AOAC RESEARCH INSTITUTE

AOAC Official Methods of Analysis℠ (OMA)

AOAC EXPERT REVIEW PANELS

POLYCYCLIC AROMATIC HYDROCARBONS
PESTICIDE RESIDUES

AOAC MIDYEAR MEETING

THURSDAY, MARCH 16, 2017
8:30 AM – 12:00PM

Gaithersburg Marriott Washingtonian Center
9751 Washingtonian Boulevard
Gaithersburg, MD 20878 USA

AOAC *OFFICIAL METHODS OF ANALYSIS*^{SM}

The *Official Methods of Analysis*^{SM} (OMA) program is AOAC INTERNATIONAL's premier methods program. The program evaluates chemistry, microbiology, and molecular biology methods. It also evaluates traditional benchtop methods, instrumental methods, and proprietary, commercial, and/or alternative methods. In 2011, AOAC augmented the *Official Methods*^{SM} program by including an approach to First Action *Official Methods*^{SM} status that relies on gathering the experts to develop voluntary consensus standards, followed by collective expert judgment of methods using the adopted standards.

The OMA program has undergone a series of transitions in support of AOAC's collaborations, evolving technology, and evolving technical requirements. Methods approved in this program have undergone rigorous scientific and systematic scrutiny such that analytical results by methods in the *Official Methods of Analysis of AOAC INTERNATIONAL* are deemed to be highly credible and defensible.

On September 7, 2012, AOAC INTERNATIONAL further clarified the AOAC *Official Methods*^{SM} program by transitioning the conformity assessment component of the *Official Methods*^{SM} program into the AOAC Research Institute. The AOAC Research Institute now administers the AOAC *Official Methods*^{SM} program for all proprietary, single and sole source methods. Methods submitted through the PTM-OMA harmonized process also will be reviewed through the AOAC Research Institute. All methods in the AOAC *Official Methods*^{SM} program are now reviewed by Expert Review Panels for First Action AOAC *Official Methods of Analysis*^{SM} status.

EXPERT REVIEW PANEL (ERP)

The AOAC Expert Review Panels (ERPs) are a key part of AOAC INTERNATIONAL's Method Approval Process. AOAC ERPs are authorized to adopt candidate methods as First Action *Official Methods* and to recommend adoption of these methods to Final Action *Official Methods* status. Scientists are recruited to serve on ERPs in a variety of ways. Normally, a call for experts is published at the same time as a call for methods is posted. Interested scientists are invited to submit their *curriculum vitae* (CV) for consideration. Advisory panel, stakeholder panel, and working group members may make recommendations to AOAC for ERP members. All CVs are reviewed and evaluated for expertise by the AOAC Chief Scientific Officer (CSO) and then to the AOAC Official Methods Board for formal review. The composition of the ERP must be fulfilled with qualified subject matter experts representing various perspectives. Please refer to our Call for Experts on the AOAC homepage for further information.

AOAC INTERNATIONAL
2275 Research Blvd, Suite 300
Rockville, Maryland 20850
Phone: (301) 924-7077

AOAC *Official Methods of Analysis*[SM] (OMA)

Expert Review Panels
Methods for AOAC Final Action Official Method Consideration

TABLE OF CONTENTS

EXPERT REVIEW PANEL (ERP) FOR POLYCYCLIC AROMATIC HYDROCARBONS

Gaithersburg Marriott Washingtonian Center
9751 Washingtonian Boulevard, Gaithersburg, MD 20878 USA

Thursday, March 16, 2017
8:30am – 10:00am

MEETING AGENDA

Expert Review Panel Co-Chairs: Tom Phillips and Jo Marie Cook

I. **Welcome and Introductions**
Expert Review Panel Co-Chairs

II. **Review of AOAC Volunteer Policies & Expert Review Panel Process Overview and Guidelines**
Deborah McKenzie, Senior Director, Standards Development and Method Approval Processes, AOAC INTERNATIONAL and AOAC Research Institute

III. **Discuss Final Action Requirements for First Action Official Methods** (if applicable)
ERP will discuss, review and track First Action methods for 2 years after adoption, review any additional information (i.e., additional collaborative study data, proficiency testing, and other feedback) and make recommendations to the Official Methods Board regarding Final Action status.

1) **AOAC OFFICIAL METHOD 2014.08: POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) IN SEAFOOD GAS CHROMATOGRAPHY-MASS SPECTROMETRY**

IV. **Next Steps and Upcoming Meetings**

V. **Adjournment**

EXPERT REVIEW PANEL (ERP) FOR PESTICIDE RESIDUES

Gaithersburg Marriott Washingtonian Center
9751 Washingtonian Boulevard, Gaithersburg, MD 20878 USA

Thursday, March 16, 2017
10:30am – 12:00pm

MEETING AGENDA

Expert Review Panel Chair: Joe Boison

I. **Welcome and Introductions**
Expert Review Panel Chair

II. **Review of AOAC Volunteer Policies & Expert Review Panel Process Overview and Guidelines**
Deborah McKenzie, Senior Director, Standards Development and Method Approval Processes, AOAC INTERNATIONAL and AOAC Research Institute

III. **Discuss Final Action Requirements for First Action Official Methods** (if applicable)
ERP will discuss, review and track First Action methods for 2 years after adoption, review any additional information (i.e., additional collaborative study data, proficiency testing, and other feedback) and make recommendations to the Official Methods Board regarding Final Action status.

1) **AOAC OFFICIAL METHOD 2014.09, DETERMINATION AND CONFIRMATION OF RESIDUES OF 653 MULTICLASS PESTICIDES AND CHEMICAL POLLUTANTS IN TEA GC/MS, GC/MS/MS, AND LC/MS/MS, FIRST ACTION 2014**

IV. **Next Steps and Upcoming Meetings**

V. **Adjournment**

**Agenda is subject to change. V1

AOAC INTERNATIONAL

POLICY AND PROCEDURES ON

VOLUNTEER CONFLICT OF INTEREST

Statement of Policy

While it is not the intention of AOAC INTERNATIONAL (AOAC) to restrict the personal, professional, or proprietary activities of AOAC members nor to preclude or restrict participation in Association affairs solely by reason of such activities, it is the sense of AOAC that conflicts of interest or even the appearance of conflicts of interest on the part of AOAC volunteers should be avoided. Where this is not possible or practical under the circumstances, there shall be written disclosure by the volunteers of actual or potential conflicts of interest in order to ensure the credibility and integrity of AOAC. Such written disclosure shall be made to any individual or group within the Association which is reviewing a recommendation which the volunteer had a part in formulating and in which the volunteer has a material interest causing an actual or potential conflict of interest.

AOAC requires disclosure of actual or potential conflicts of interest as a condition of active participation in the business of the Association. The burden of disclosure of conflicts of interest or the appearance of conflicts of interest falls upon the volunteer.

A disclosed conflict of interest will not in itself bar an AOAC member from participation in Association activities, but a three-fourths majority of the AOAC group reviewing the issue presenting the conflict must concur by secret ballot that the volunteer's continued participation is necessary and will not unreasonably jeopardize the integrity of the decision-making process.

Employees of AOAC are governed by the provision of the AOAC policy on conflict of interest by staff. If that policy is in disagreement with or mute on matters covered by this policy, the provisions of this policy shall prevail and apply to staff as well.

Illustrations of Conflicts of Interest

1. A volunteer who is serving as a committee member or referee engaged in the evaluation of a method or device; who is also an employee of or receiving a fee from the firm which is manufacturing or distributing the method or device or is an employee of or receiving a fee from a competing firm.

2. A volunteer who is requested to evaluate a proposed method or a related collaborative study in which data are presented that appear detrimental (or favorable) to a product distributed or a position supported by the volunteer's employer.

3. A referee who is conducting a study and evaluating the results of an instrument, a kit, or a piece of equipment which will be provided gratis by the manufacturer or distributor to one or more of the participating laboratories, including his or her own laboratory, at the conclusion of the study.

4. Sponsorship of a collaborative study by an interest (which may include the referee) which stands to profit from the results; such sponsorship usually involving the privilege granted by the investigator to permit the sponsor to review and comment upon the results prior to AOAC evaluation.

5. A volunteer asked to review a manuscript submitted for publication when the manuscript contains information which is critical of a proprietary or other interest of the reviewer.

The foregoing are intended as illustrative and should not be interpreted to be all-inclusive examples of conflicts of interest AOAC volunteers may find themselves involved in.

Do's and Don'ts

Do avoid the appearance as well as the fact of a conflict of interest.

Do make written disclosure of any material interest which may constitute a conflict of interest or the appearance of a conflict of interest.

Do not accept payment or gifts for services rendered as a volunteer of the Association without disclosing such payment or gifts.

Do not vote on any issue before an AOAC decision-making body where you have the appearance of or an actual conflict of interest regarding the recommendation or decision before that body.

Do not participate in an AOAC decision-making body without written disclosure of actual or potential conflicts of interest in the issues before that body.

Do not accept a position of responsibility as an AOAC volunteer, without disclosure, where the discharge of the accepted responsibility will be or may appear to be influenced by proprietary or other conflicting interests.

Procedures

Each volunteer elected or appointed to an AOAC position of responsibility shall be sent, at the time of election or appointment, a copy of this policy and shall be advised of the requirement to adhere to the provisions herein as a condition for active participation in the business of the Association. Each volunteer, at the time of his or her election or appointment, shall indicate, in writing, on a form provided for this purpose by AOAC, that he or she has read and accepts this policy.

Each year, at the spring meeting of the AOAC Board of Directors, the Executive Director shall submit a report certifying the requirements of this policy have been met; including the names and positions of any elected or appointed volunteers who have not at that time indicated in writing that they have accepted the policy.

Anyone with knowledge of specific instances in which the provisions of this policy have not been complied with shall report these instances to the Board of Directors, via the Office of the Executive Director, as soon as discovered.

* * * * * *

Adopted: March 2, 1989
Revised: March 28, 1990
Revised: October 1996

AOAC INTERNATIONAL
ANTITRUST POLICY
STATEMENT AND GUIDELINES

Introduction

It is the policy of AOAC INTERNATIONAL (AOAC) and its members to comply strictly with all laws applicable to AOAC activities. Because AOAC activities frequently involve cooperative undertakings and meetings where competitors may be present, it is important to emphasize the on_going commitment of our members and the Association to full compliance with national and other antitrust laws. This statement is a reminder of that commitment and should be used as a general guide for AOAC and related individual activities and meetings.

Responsibility for Antitrust Compliance

The Association's structure is fashioned and its programs are carried out in conformance with antitrust standards. However, an equal responsibility for antitrust compliance __ which includes avoidance of even an appearance of improper activity __ belongs to the individual. Even the appearance of improper activity must be avoided because the courts have taken the position that actual proof of misconduct is not required under the law. All that is required is whether misconduct can be inferred from the individual's activities.

Employers and AOAC depend on individual good judgment to avoid all discussions and activities which may involve improper subject matter and improper procedures. AOAC staff members work conscientiously to avoid subject matter or discussion which may have unintended implications, and counsel for the Association can provide guidance with regard to these matters. It is important for the individual to realize, however, that the competitive significance of a particular conduct or communication probably is evident only to the individual who is directly involved in such matters.

Antitrust Guidelines

In general, the U.S. antitrust laws seek to preserve a free, competitive economy and trade in the United States and in commerce with foreign countries. Laws in other countries have similar objectives. Competitors (including individuals) may not restrain competition among themselves with reference to the price, quality, or distribution of their products, and they may not act in concert to restrict the competitive capabilities or opportunities of competitors, suppliers, or customers.

Although the Justice Department and Federal Trade Commission generally enforce the U.S. antitrust laws, private parties can bring their own lawsuits.

Penalties for violating the U.S. and other antitrust laws are severe: corporations are subject to heavy fines and injunctive decrees, and may have to pay substantial damage judgments to injured competitors, suppliers, or customers. Individuals are subject to criminal prosecution, and will be punished by fines and imprisonment.
Under current U.S. federal sentencing guidelines, individuals found guilty of bid rigging, price fixing, or market allocation must be sent to jail for at least 4 to 10 months and must pay substantial minimum fines.

Since the individual has an important responsibility in ensuring antitrust compliance in AOAC activities, everyone should read and heed the following guidelines.

1. Don't make any effort to bring about or prevent the standardization of any method or product for the purpose or intent of preventing the manufacture or sale of any method or product not conforming to a specified standard.

2. Don't discuss with competitors your own or the competitors' prices, or anything that might affect prices such as costs, discounts, terms of sale, distribution, volume of production, profit margins, territories, or customers.

3. Don't make announcements or statements at AOAC functions, outside leased exhibit space, about your own prices or those of competitors.

4. Don't disclose to others at meetings or otherwise any competitively sensitive information.

5. Don't attempt to use the Association to restrict the economic activities of any firm or any individual.

6. Don't stay at a meeting where any such price or anti_competitive talk occurs.

7. Do conduct all AOAC business meetings in accordance with AOAC rules. These rules require that an AOAC staff member be present or available, the meeting be conducted by a knowledgeable chair, the agenda be followed, and minutes be kept.

8. Do confer with counsel before raising any topic or making any statement with competitive ramifications.

9. Do send copies of meeting minutes and all AOAC_related correspondence to the staff member involved in the activity.

10. Do alert the AOAC staff to any inaccuracies in proposed or existing methods and statements issued, or to be issued, by AOAC and to any conduct not in conformance with these guidelines.

Conclusion

Compliance with these guidelines involves not only avoidance of antitrust violations, but avoidance of any behavior which might be so construed. Bear in mind, however, that the above antitrust laws are stated in general terms, and that this statement is not a summary of applicable laws. It is intended only to highlight and emphasize the principal antitrust standards which are relevant to AOAC programs. You must, therefore, seek the guidance of either AOAC counsel or your own counsel if antitrust questions arise.

* * * * *

Adopted by the AOAC Board of Directors: September 24, 1989
Revised: March 11, 1991
Revised October 1996

AOAC INTERNATIONAL
POLICY ON THE USE OF THE ASSOCIATION NAME, INITIALS, IDENTIFYING INSIGNIA, LETTERHEAD, AND BUSINESS CARDS

Introduction

The following policy and guidelines for the use of the name, initials, and other identifying insignia of AOAC INTERNATIONAL have been developed in order to protect the reputation, image, legal integrity and property of the Association.

The name of the Association, as stated in its bylaws, is "AOAC INTERNATIONAL". The Association is also known by its initials, AOAC, and by its logo, illustrated below, which incorporates the Association name and a representation of a microscope, book, and flask. The AOAC logo is owned by the Association and is registered with the U.S. Patent and Trademark Office.

AOAC®
INTERNATIONAL

The full Association insignia, illustrated below, is comprised of the logo and the tagline, "The Scientific Association Dedicated to Analytical Excellence," shown below. The typeface used is Largo. The AOAC tagline is owned by the Association and is registered with the U.S. Patent and Trademark office.

AOAC®
INTERNATIONAL

The Scientific Association Dedicated to Analytical Excellence®

Policy

Policy on the use of the Association's name and logo is established by the AOAC Board of Directors as follows:

> "The Board approves and encourages reference to the Association by name, either as AOAC INTERNATIONAL or as AOAC; or reference to our registered trademark, AOAC®, in appropriate settings to describe our programs, products, etc., in scientific literature and other instances so long as the reference is fair, accurate, complete and truthful and does not indicate or imply unauthorized endorsement of any kind.
>
> The insignia (logo) of AOAC INTERNATIONAL is a registered trade and service mark and shall not be reproduced or used by any person or organization other than the Association, its elected and appointed officers, sections, or committees, without the prior written permission of the Association. Those authorized to use the AOAC INTERNATIONAL insignia shall use it only for the purposes for which permission has been specifically granted.
>
> The name and insignia of the Association shall not be used by any person or organization in any way which indicates, tends to indicate, or implies AOAC official endorsement of any product, service, program, company, organization, event or person, endorsement of which, has not been authorized by the Association, or which suggests that membership in the Association is available to any organization."

The Executive Director, in accordance with the above stated policy, is authorized to process, approve, fix rules, and make available materials containing the Association name and insignia.

It should be noted that neither the Association's name nor its insignia nor part of its insignia may be incorporated into any personal, company, organization, or any other stationery other than that of the Association; nor may any statement be included in the printed portion of such stationery which states or implies that an individual, company, or other organization is a Member of the Association.

Instructions

1. Reproduction or use of the Association name or insignia requires prior approval by the Executive Director or his designate.

2. Association insignia should not be altered in any manner without approval of the Executive Director or his designate, except to be enlarged or reduced in their entirety.

3. Artwork for reproducing the Association name or insignia, including those incorporating approved alterations, will be provided on request to those authorized to use them (make such requests to the AOAC Marketing Department). Examples of the types of alterations that would be approved are inclusion of a section name in or the addition of an officer's name and address to the letterhead insignia.

4. When the Association name is used without other text as a heading, it should, when possible, be set in the Largo typeface.

5. Although other colors may be used, AOAC blue, PMS 287, is the preferred color when printing the AOAC insignia, especially in formal and official documents. It is, of course, often necessary and acceptable to reproduce the insignia in black.

6. Do not print one part of the logo or insignia in one color and other parts in another color.

7. The letterhead of AOAC INTERNATIONAL shall not be used by any person or organization other than the Association, its elected and appointed officers, staff, sections, or committees; except by special permission.

   Correspondence of AOAC official business should be conducted using AOAC letterhead. However, those authorized to use AOAC letterhead shall use it for official AOAC business only.

   Copies of all correspondence using AOAC letterhead or conducting AOAC official business, whether on AOAC letterhead or not, must be sent to the appropriate office at AOAC headquarters.

8. AOAC INTERNATIONAL business cards shall not be used by any person or organization other than the Association, its staff, and elected officials, except by special permission.

   Those authorized to use AOAC business cards shall use them for official AOAC business only and shall not represent themselves as having authority to bind the Association beyond that authorized.

Sanctions

1. Upon learning of any violation of the above policy, the Executive Director or a designate will notify the individual or organization that they are in violation of AOAC policy and will ask them to refrain from further misuse of the AOAC name or insignia.

2. If the misuse is by an Individual Member or Sustaining Member of the Association, and the misuse continues after notification, the Board of Directors will take appropriate action.

3. If continued misuse is by a nonmember of the Association or if a member continues misuse in spite of notification and Board action, ultimately, the Association will take legal action to protect its property, legal integrity, reputation, and image.

* * * * * *

Adopted by the AOAC Board of Directors: September 24, 1989
Revised: June 13, 1991; February 26, 1992; March 21, 1995; October 1996

Official Methods of Analysis[SM] (OMA) Expert Review Panel
MEETING AND METHOD REVIEW GUIDANCE

The AOAC Research Institute administers AOAC INTERNATIONAL's premier methods program, the AOAC *Official Methods of Analysis*[SM] (OMA). The program evaluates chemistry, microbiology, and molecular biology methods. It also evaluates traditional benchtop methods, instrumental methods, and proprietary, commercial, and/or alternative methods and relies on gathering the experts to develop voluntary consensus standards, followed by collective expert judgment of methods using the adopted standards. The *Official Methods of Analysis of AOAC INTERNATIONAL* is deemed to be highly credible and defensible.

All Expert Review Panel (ERP) members are vetted by the AOAC Official Methods Board (OMB) and serve at the pleasure of the President of AOAC INTERNATIONAL. In accordance to the AOAC Expert Review Panel Member and Chair Volunteer Role Description all Expert Review Panel members are expected to 1) serve with the highest integrity, 2) perform duties and method reviews, and 3) adhere to review timelines and deadlines.

To assist the ERP Chair and its members, please note the following in preparation for Expert Review Panel meetings and method reviews.

Pre-Meeting Requirements

1. Confirm availability and plan to be present to ensure a quorum of the ERP.
   (Please refer to page 25, Quorum Guidelines, Expert Review Panel Information Packet)
2. Ensure that your laptop, CPU or mobile device can access online web documentation.
3. Be prepared for the meeting by reviewing all relevant meeting materials and method documentation.

In-Person Meeting and Teleconference Conduct

1. Arrive on time.
2. Advise the Chair and ERP members of any potential Conflicts of Interest at the beginning of the meeting.
3. Participation is required from all members of the ERP. All members have been deemed experts in the specific subject matter areas.
4. The ERP Chair will moderate the meeting to ensure that decisions can be made in a timely manner.
5. Follow Robert's Rules of Order for Motions.
6. Speak loud, clear, and concise so that all members may hear and understand your point of view.
7. Due to the openness of our meetings, it is imperative that all members communicate in a respectful manner and tone.
8. Refrain from disruptive behavior. Always allow one member to speak at a time. Please do not interrupt.
9. Please note that all methods reviewed and decisions made during the Expert Review Panel process are considered confidential and should not be discussed unless during an Expert Review Panel meeting to ensure transparency.

Reviewing Methods

Prior to the Expert Review Panel meeting, ERP members are required to conduct method reviews. All methods are reviewed under the following criteria, technical evaluation, general comments, editorial criteria, and recommendation status. These methods are being reviewed against their collaborative study protocols as provided in the supplemental documentation. *Note: The method author(s) will be present during the Expert Review Panel session to answer any questions.*

Reviewing Methods (Cont'd)

- Reviewers shall conduct in-depth review of method and any supporting information.
- In-depth reviews are completed electronically via the method review form. The method review form must be completed and submitted by the deadline date as provided.
- All reviews will be discussed during the Expert Review Panel meeting.
- Any ERP member can make the motion to adopt or not to adopt the method.
- If the method is adopted for AOAC First Action status, Expert Review Panel members must track and present feedback on assigned First Action *Official Methods*.
- Recommend additional feedback or information for Final Action consideratio**n.**

Here are some questions to consider during your review based on your scientific judgment:

1. Does the method sufficiently follow the collaborative study protocol?
2. Is the method scientifically sound and can be followed?
3. What are the strengths and weaknesses of the method?
4. How do the weaknesses weigh in your recommendation for the method?
5. Will the method serve the community that will use the method?
6. What additional information may be needed to further support the method?
7. Can this method be considered for AOAC First Action OMA status?

Reaching Consensus during Expert Review Panel Meeting

1. Make your Motion.
2. Allow another member to Second the Motion.
3. The Chair will state the motion and offer the ERP an option to discuss the motion.
4. The Chair will call a vote once deliberations are complete.
5. Methods must be adopted by unanimous decision of ERP on first ballot, if not unanimous, negative votes must delineate scientific reasons. Negative voter(s) can be overridden by 2/3 of voting ERP members after due consideration.
6. All other motions will require 2/3 majority for vote to carry.

First Action Method Updates

Expert Review Panel Tracking and Recommendations of First Action Methods

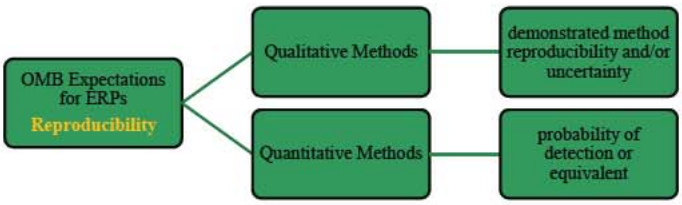
AOAC Policies & Procedures

- Policy on Antitrust
- Policy on Use of Association Name, Identifying Insignia, Letterhead, Business Cards
- Policy on Volunteer Conflict of Interest
- Expert Review Panel Policies and Procedures
- OMA Appendix G

OMA, Appendix G

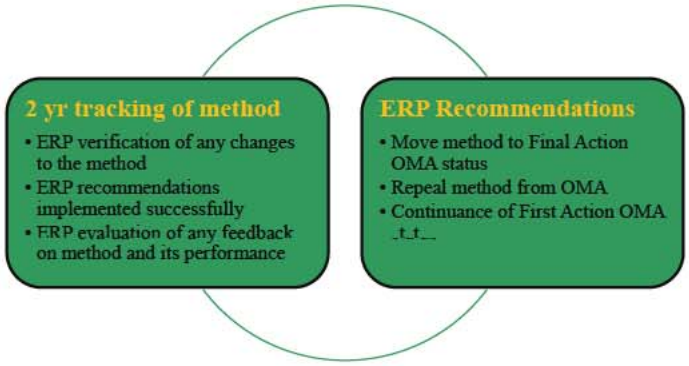
Further data indicative of adequate method reproducibility (between laboratory) performance to be collected. Data may be collected via a collaborative study or by proficiency or other testing data of similar magnitude.

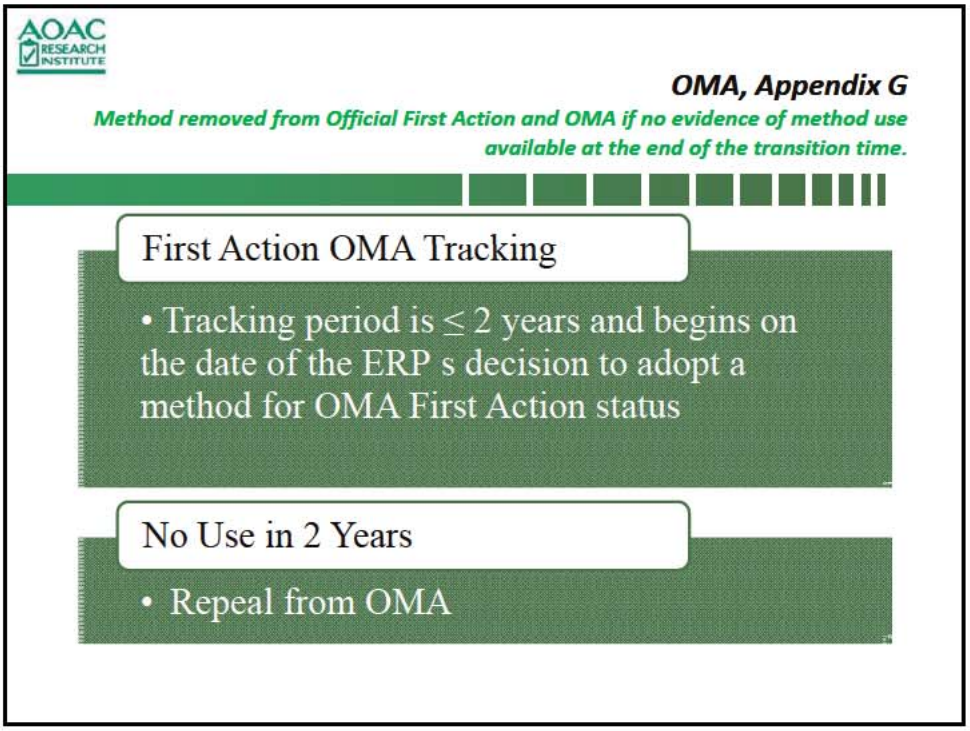
- ERP is looking to v_rif, if m_th_ _ reproducibility has been appropriately assessed and satisfactorily demonstrated

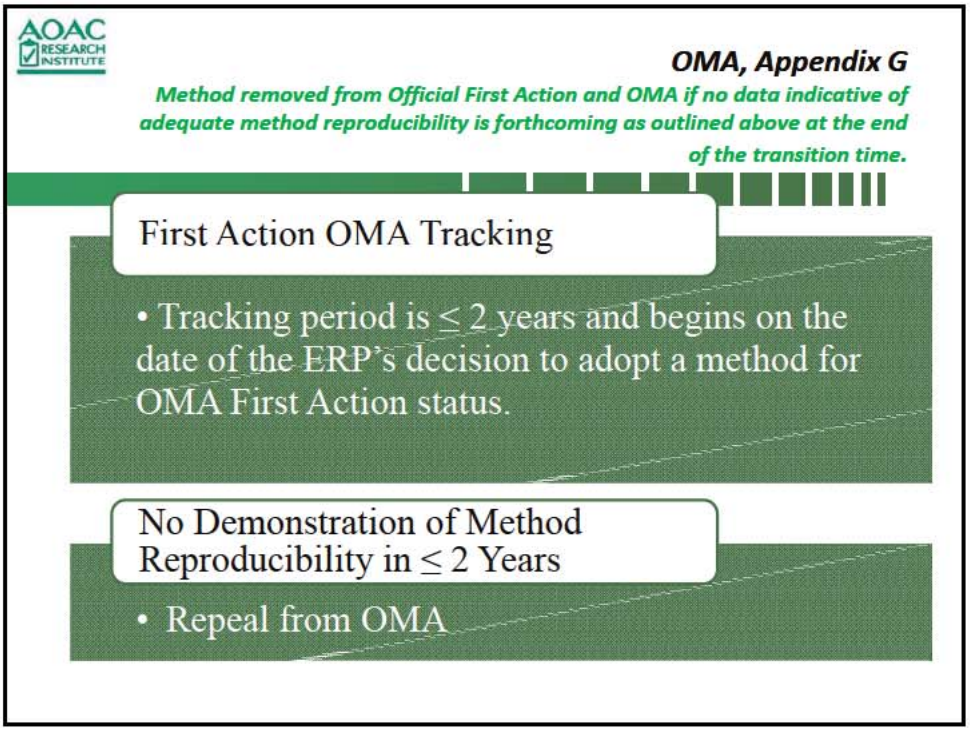


OMA, Appendix G

Two years maximum transition time (additional year(s) if ERP determines a relevant collaborative study or proficiency or other data collection is in progress).

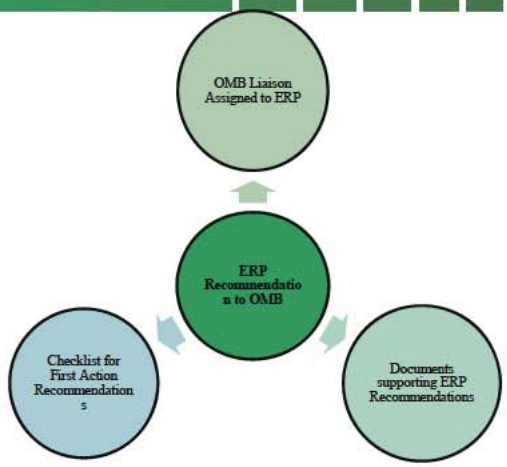



AOAC RESEARCH INSTITUTE
OMA, Appendix G
Method removed from Official First Action and OMA if no evidence of method use available at the end of the transition time.
First Action OMA Tracking
• Tracking period is ≤ 2 years and begins on the date of the ERP s decision to adopt a method for OMA First Action status
No Use in 2 Years
• Repeal from OMA


AOAC RESEARCH INSTITUTE
OMA, Appendix G
Method removed from Official First Action and OMA if no data indicative of adequate method reproducibility is forthcoming as outlined above at the end of the transition time.
First Action OMA Tracking
• Tracking period is ≤ 2 years and begins on the date of the ERP's decision to adopt a method for OMA First Action status.
No Demonstration of Method Reproducibility in ≤ 2 Years
• Repeal from OMA

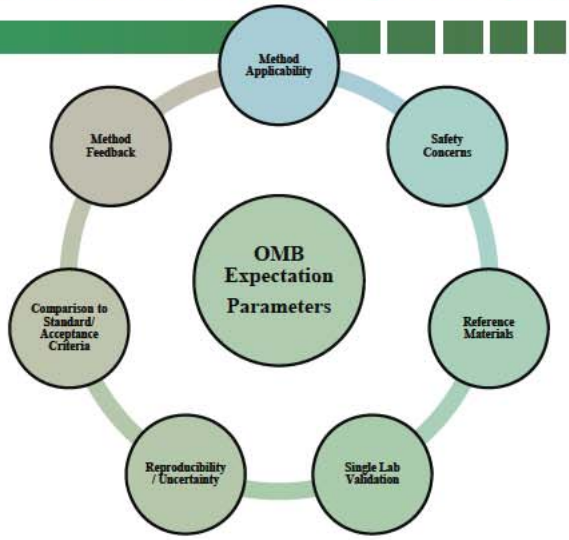
OMA, Appendix G

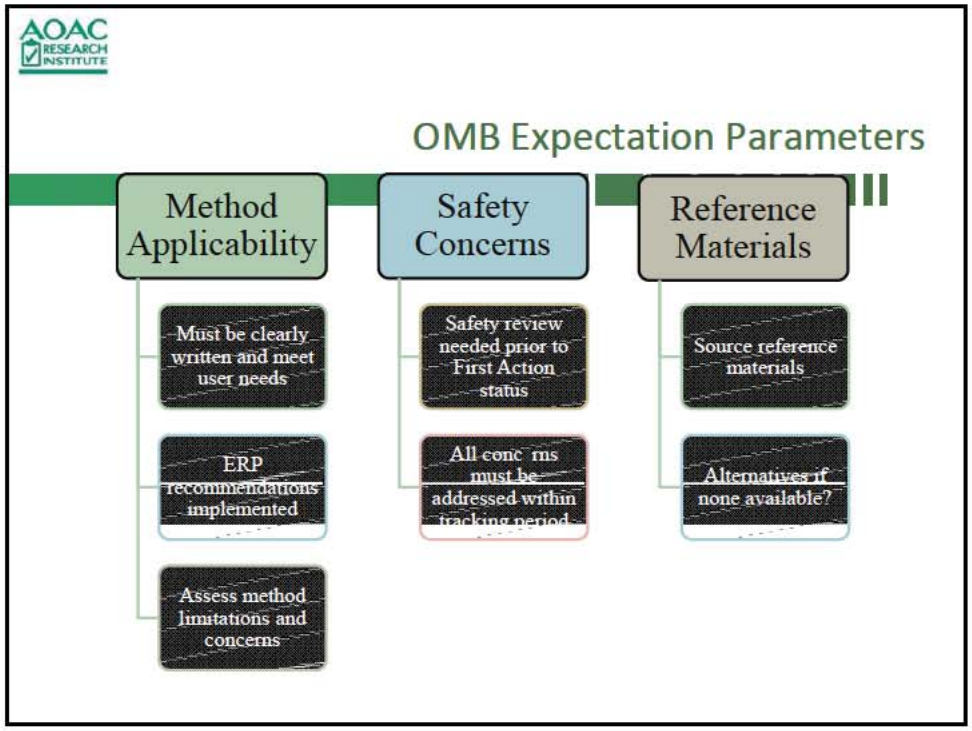
ERP to recommend Method to Official Final Action Status to the OMB.

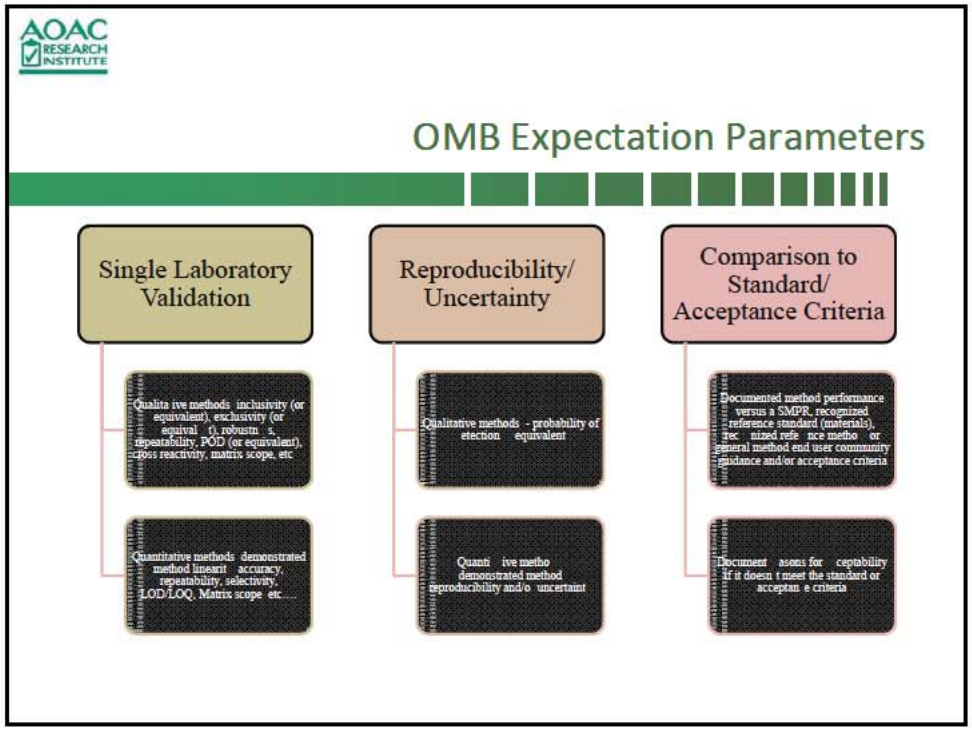


OMA, Appendix G

First Action to Final Action Methods: Guidance for AOAC Expert Review Panels




AOAC RESEARCH INSTITUTE
OMB Expectation Parameters
Method Applicability
Safety Concerns
Reference Materials
Must be clearly written and meet user needs
ERP recommendations implemented
Assess method limitations and concerns
Safety review needed prior to First Action status
All conc rns must be addressed within tracking period
Source reference materials
Alternatives if none available?


AOAC RESEARCH INSTITUTE
OMB Expectation Parameters
Single Laboratory Validation
Reproducibility/ Uncertainty
Comparison to Standard/ Acceptance Criteria
Qualita ive methods inclusivity (or equivalent), exclusivity (or equival t), robustn s, repeatability, POD (or equivalent), cross reactivity, matrix scope, etc
Quantitative methods demonstrated method linearit accuracy, repeatability, selectivity, LOD/LOQ, Matrix scope etc....
Qualitative methods - probability of etection equivalent
Quanti ive metho demonstrated method reproducibility and/o uncertaint
Documented method performance versus a SMPR, recognized reference standard (materials), rec nized refe nce metho or general method end user community guidance and/or acceptance criteria
Document asons for ceptability if it doesn t meet the standard or acceptan e criteria

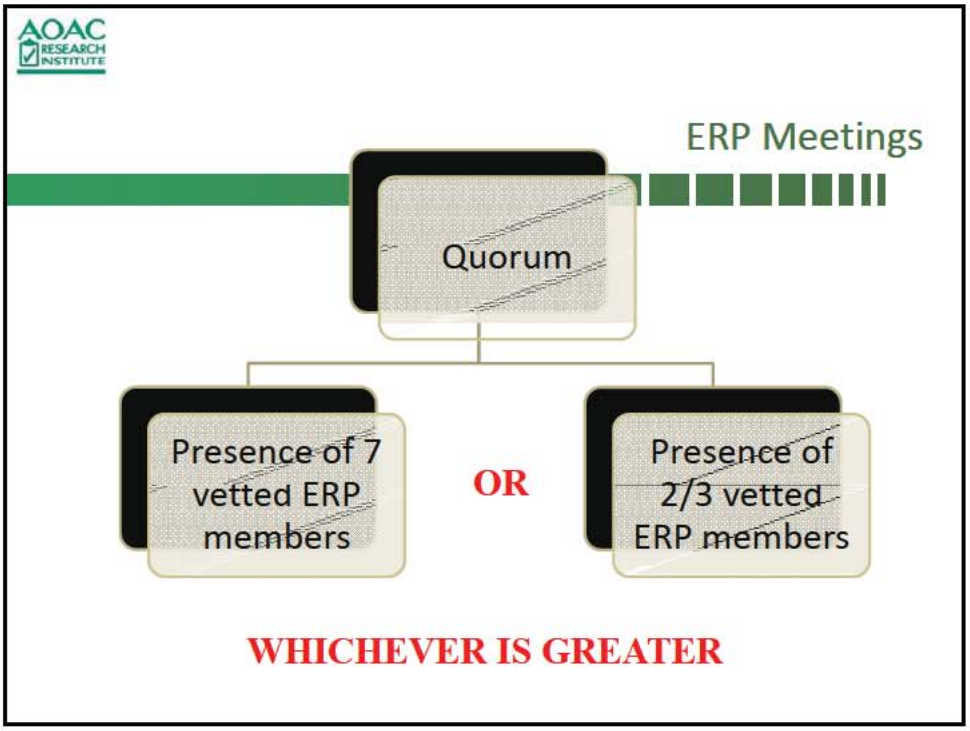
OMB Expectation Parameters

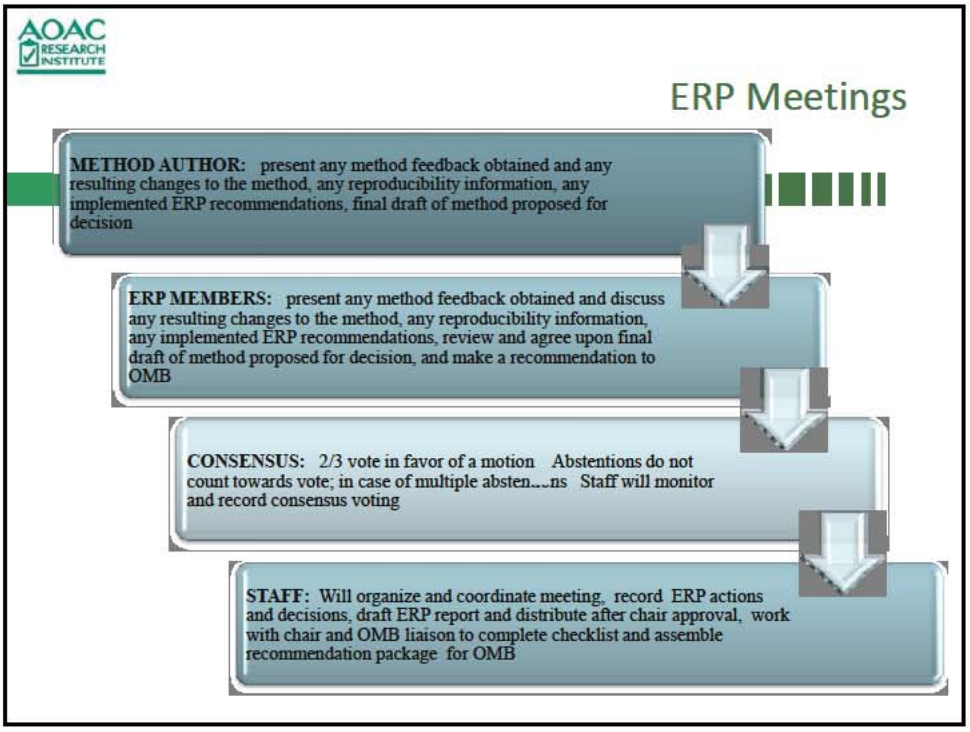
Method Feedback from End Users

Consider any positive or negative feedback on overall method performan e, applicability, availability of reference materials, matrix sc pe, method component sourcing, robustness or ruggedness parameters.

Documentation Needed

- Method Safety Evaluation
- Reference Materials
- Evidence of Single Laboratory Validation or equivalent
- Evidence of Reproducibility Assessment
- Published First Action OMA
- Method Performance versus SMPR or acceptance criteria
- Final draft of First Action OMA to be considered for status update
- Rationale or Justification for Repeal or Continuance of First Action OMA


AOAC RESEARCH INSTITUTE
ERP Meetings
Quorum
Presence of 7 vetted ERP members
OR
Presence of 2/3 vetted ERP members
WHICHEVER IS GREATER


AOAC RESEARCH INSTITUTE
ERP Meetings
METHOD AUTHOR: present any method feedback obtained and any resulting changes to the method, any reproducibility information, any implemented ERP recommendations, final draft of method proposed for decision
ERP MEMBERS: present any method feedback obtained and discuss any resulting changes to the method, any reproducibility information, any implemented ERP recommendations, review and agree upon final draft of method proposed for decision, and make a recommendation to OMB
CONSENSUS: 2/3 vote in favor of a motion Abstentions do not count towards vote; in case of multiple absten...ns Staff will monitor and record consensus voting
STAFF: Will organize and coordinate meeting, record ERP actions and decisions, draft ERP report and distribute after chair approval, work with chair and OMB liaison to complete checklist and assemble recommendation package for OMB

AOAC Official Method 2014.08
Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood

Gas Chromatography-Mass Spectrometry
First Action 2014

[Applicable for the determination of the following PAHs in mussel, oyster, and shrimp: 1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*k*]fluoranthene, chrysene, dibenz[*a,h*]anthracene, fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, naphthalene, phenanthrene, and pyrene. These were representative PAH analytes selected for the collaborative study. The method has been single-laboratory validated for 32 PAHs in fish and shrimp (1), and, therefore, is expected to be applicable to other GC-amenable PAHs and seafood matrices. The concentration ranges evaluated within the collaborative study are given in Table **2014.08A**.]

Caution: *See Appendix B: Laboratory Safety*. Use appropriate personal protective equipment such as laboratory coat, safety glasses or goggles, appropriate chemical-resistant gloves, and a fume hood. Dispose of solvents and solutions according to federal, state, and local regulations. Always handle open containers of solvents inside the fume hood, including the pouring, mixing, evaporating, and preparing standard solution. Keep containers covered or closed when not in use.

Hexane and isooctane.—Highly flammable, liquid irritants. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation.

Ethyl acetate.—Highly flammable, liquid irritants. Harmful if swallowed in quantity. Vapors may cause drowsiness.

Toluene.—Highly flammable, liquid irritant. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation. May cause drowsiness. Possible teratogen.

Dichloromethane.—Noncombustible, liquid irritant. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation. Asphyxiant. Causes central nervous system (CNS) depression. Possible carcinogen and mutagen.

PAHs.—Carcinogens, respiratory sensitizers, teratogens, reproductive hazard, mutagens. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation.

See Tables **2014.08B–D** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Homogenized seafood samples (10 g sample with a 5 µg/kg addition of ^{13}C-PAH surrogate mixture) are mixed with 5 mL water (or 10 mL water in the case of shrimp and other more viscous samples) and shaken vigorously by hand with 10 mL ethyl acetate in a 50 mL polypropylene centrifuge tube for 1 min. Subsequently, 4 g anhydrous magnesium sulfate and 2 g sodium chloride are added to the mixture to induce phase separation and force the analytes into the ethyl acetate layer. The tube is again shaken by hand for 1 min and then centrifuged for 10 min at >1500 rcf. A 5 mL aliquot of the ethyl acetate layer is evaporated, reconstituted in 1 mL hexane, and cleaned on an SPE column with 1 g silica gel and approximately 0.2 g anhydrous sodium sulfate on the top. The column is conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane, followed by application of the 1 mL extract in hexane. The analytes are eluted with hexane–dichloromethane (3 + 1, v/v) using volume determined for the given silica gel SPE cartridges from the elution profiles of target analytes and fat, which are dependent on the silica deactivation. The clean extract is carefully evaporated, reconstituted in 0.5 mL isooctane, and analyzed by GC/MS. *See* Figure **2014.08A** for the method flow chart.

B. Apparatus

(**a**) *Homogenizer*.—WARING blender Model 38BL40 (Conair Corp., Stamford, CT) or equivalent.

(**b**) *Solvent evaporator*.—Any suitable solvent evaporator, such as a rotary vacuum evaporator, Kuderna-Danish evaporator,

Table 2014.08A. PAH and ^{13}C-PAH concentrations in the calibration standard solutions

	Concentration, µg/L				Equivalent concentration, µg/kg			
Calibration level	BaP and others[a]	Chr and others[b]	Naph[c]	^{13}C-PAHs	BaP and others	Chr and others	Naph	^{13}C-PAHs
1	5	12.5	25	50	0.5	1.25	2.5	5
2	10	25	50	50	1	2.5	5	5
3	20	50	100	50	2	5	10	5
4	50	125	250	50	5	12.5	25	5
5	100	250	500	50	10	25	50	5
6	200	500	1000	50	20	50	100	5
7	500	1250	2500	50	50	125	250	5
8	1000	2500	5000	50	100	250	500	5

[a] Analytes at 10 µg/mL in the mixed stock standard solution.

[b] Analytes at 25 µg/mL in the mixed stock standard solution.

[c] Analytes at 50 µg/mL in the mixed stock standard solution.

Table 2014.08B. Statistical results for the studied PAHs at three different concentration levels in shrimp after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	9	18	21.7	108.6	2.6	4.1	11.8	18.9	0.66
	9	18	22.7	113.7	1.8	4.2	8.0	18.7	0.66
	9	18	21.7	108.3	1.9	4.4	8.8	20.4	0.72
1-MN	9	18	23.1	115.4	6.2	6.8	26.9	29.4	1.04
	9	18	81.5	108.6	6.7	15.6	8.3	19.1	0.82
	9	18	203.2	101.6	17.8	55.4	8.8	27.3	1.34
1-MP	9	18	10.0	99.9	1.0	1.4	9.8	14.0	0.44
	9	18	25.0	99.9	1.9	3.7	7.6	14.9	0.53
	9	18	119.4	95.5	6.7	15.7	5.6	13.1	0.60
2,6-DMN	8	16	15.6	103.9	1.1	2.6	6.9	16.4	0.55
	8	16	37.8	94.6	4.8	7.4	12.6	19.5	0.74
	7	14	146.7	83.8	16.6	20.3	11.3	13.9	0.65
6-MC	9	18	11.1	110.5	0.6	1.5	5.8	13.1	0.42
	9	18	32.2	107.2	1.6	3.6	5.1	11.1	0.42
	9	18	145.2	100.1	8.7	13.7	6.0	9.4	0.44
Ant	9	18	4.9	98.5	0.3	0.5	6.7	10.3	0.29
	9	18	10.6	105.7	0.8	1.7	7.3	16.2	0.51
	9	18	38.9	97.4	2.4	4.6	6.2	11.7	0.45
BaA	9	18	4.8	95.9	0.3	0.5	7.0	9.7	0.27
	9	18	15.0	99.9	0.6	1.3	4.3	8.4	0.28
	9	18	56.6	94.4	2.5	5.2	4.5	9.2	0.37
BaP	9	18	1.9	96.2	0.1	0.2	6.5	12.1	0.29
	9	18	4.9	98.7	0.4	0.5	7.3	9.6	0.27
	8	16	23.1	92.3	1.1	1.6	4.6	7.0	0.25
BbF	9	18	4.8	96.7	0.3	0.5	6.6	10.1	0.28
	9	18	9.8	98.2	0.3	0.7	2.6	7.0	0.22
	9	18	71.6	95.5	3.9	6.3	5.5	8.8	0.37
BghiP	8	16	1.9	94.7	0.1	0.2	7.0	11.7	0.28
	8	16	4.9	98.5	0.2	0.5	4.3	9.9	0.28
	8	16	18.0	90.1	1.0	1.4	5.7	7.9	0.27
BkF	9	18	2.0	99.5	0.1	0.3	6.2	13.7	0.34
	9	18	8.1	101.7	0.4	0.7	4.9	8.7	0.26
	9	18	38.3	95.8	1.8	2.8	4.7	7.2	0.28
Chr	8	16	15.2	101.5	0.5	1.4	3.1	9.4	0.31
	9	18	50.7	101.4	2.2	4.1	4.4	8.1	0.32
	9	18	167.4	95.6	9.0	14.5	5.3	8.7	0.41
DBahA	9	18	1.9	95.9	0.2	0.3	10.9	13.5	0.33
	9	18	5.0	100.4	0.3	0.6	6.6	11.2	0.32
	9	18	13.8	91.8	0.9	1.2	6.4	8.4	0.28
Fln	8	16	5.2	103.0	0.1	0.5	1.4	10.0	0.28
	9	18	15.4	102.3	0.6	1.1	4.2	7.5	0.25
	9	18	47.3	94.7	2.1	4.1	4.5	8.7	0.34
Flt	9	18	9.7	97.2	0.6	1.0	6.0	10.4	0.32
	9	18	25.1	100.3	1.4	2.4	5.5	9.7	0.35
	9	18	93.9	93.9	4.9	8.7	5.2	9.3	0.41
IcdP	9	18	2.0	98.2	0.1	0.3	5.3	13.3	0.32
	9	18	5.1	102.2	0.5	0.6	9.1	11.0	0.31

Table 2014.08B. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	9	18	18.4	92.1	1.1	1.9	5.7	10.5	0.36
Naph	8	16	27.7	110.7	2.8	2.8	10.3	10.3	0.37
	9	18	84.1	105.1	5.6	8.8	6.7	10.5	0.45
	8	16	158.7	99.2	9.7	34.2	6.1	21.6	1.02
Phe	8	16	15.1	100.5	0.5	1.2	3.3	7.8	0.26
	9	18	49.7	99.4	1.5	3.0	3.1	6.0	0.24
	9	18	168.0	96.0	8.6	16.6	5.1	9.9	0.47
Pyr	9	18	14.8	98.5	0.9	1.3	6.1	8.8	0.29
	9	18	40.3	100.8	1.6	3.3	3.8	8.2	0.32
	8	16	118.7	95.0	2.9	6.4	2.5	5.4	0.25

or a nitrogen blow-down system, may be used as long as it provides results meeting the laboratory qualification/method set-up requirements (absolute analyte recoveries >70% in both evaporation steps).

(**c**) *Centrifuge*.—Capable of centrifugation of 50 mL tubes at >1500 rcf for 10 min.

(**d**) *Furnace/oven*.—Capable of 600°C operation.

(**e**) *Balance(s)*.—Analytical, capable of accurately measuring weights from 1 mg to 10 g.

(**f**) *Gas chromatograph-mass spectrometer*.—Any GC/MS instrument [single quadrupole, triple quadrupole, time-of-flight (TOF), or ion trap] with electron ionization (EI) may be used as long as it provides results meeting the laboratory qualification requirements (to provide reliable results for the calibration range specified in Table **2014.08A**).

(**g**) *GC column*.—Capillary column BPX-50 (30 m, 0.25 mm id, 250 µm film thickness; Trajan Scientific, Austin, TX, USA) or equivalent (USP specification G3), such as Rxi-17Sil MS (Restek Corp., Bellefonte, PA, USA); DB-17MS, DB-17, or HP-50 (Agilent Technologies, Santa Clara, CA, USA); or any other column that enables adequate separation of PAHs as specified in the laboratory qualification requirements (*see* **G**).

C. Reagents and Materials

(**a**) *Hexane.*—>98.5%, mixture of isomers.

(**b**) *Isooctane.*—ACS or better grade.

(**c**) *Ethyl acetate.*—>99.5%, for GC residue analysis.

(**d**) *Dichloromethane.*—≥99.9%, for GC residue analysis.

(**e**) *Toluene.*—≥99.9%, for GC residue analysis.

(**f**) *Water.*—Purified, free of interfering compounds.

(**g**) *Anhydrous sodium sulfate (Na_2SO_4).*—≥99.0%, powder, heated at 600°C for 7 h and then stored in a desiccator before use (Na_2SO_4 prepared and stored as indicated can be used for 1 month from preparation).

(**h**) *Silica gel SPE column.*—Containing 1 g silica gel. Any commercially available silica gel SPE column can be used as long as it provides adequate fat cleanup and meets requirements for low background contamination specified in the laboratory qualification requirements: the concentrations of all analytes in the reagent blanks had to be below the concentrations in the lowest calibration level standard; for naphthalene, levels below the second lowest calibration standard (equivalent to 5 ng/g naphthalene in the sample) are still acceptable if the source of contamination could not be eliminated.

Silica gel SPE columns can be prepared in-house using the following procedure: Activate the silica gel by heating at 180°C for 5 h, and then deactivate it by adding 5% deionized water, shaking for 3 h. Store in a desiccator for 16 h before use (silica gel prepared and stored as indicated can be used for 14 days). Place a piece of deactivated glass wool in a Pasteur pipet (5 mL), add 1 g activated silica gel (Silica gel 60, 0.063–0.2 mm, 70–230 mesh or equivalent) and top it with approximately 0.2 g muffled anhydrous Na_2SO_4.

(**i**) *Anhydrous magnesium sulfate ($MgSO_4$).*—≥99.0%, powder, heated (muffled) at 600°C for 7 h, and then store in a desiccator before use ($MgSO_4$ prepared and stored as indicated can be used for 1 month from preparation). *Note*: A preweighed (commercially available) mixture of 2 g sodium chloride and 4 g anhydrous magnesium sulfate (muffled) in pouches or tubes can be used.

(**j**) *Sodium chloride (NaCl).*—≥99.0%.

(**k**) *Helium 5.0 or better, nitrogen 4.0 or better.*

(**l**) *Polypropylene centrifuge tubes.*—50 mL.

(**m**) *Glass Pasteur pipet.*—5 mL (for solvent transfers and/or in-house preparation of silica gel minicolumns).

(**n**) *Syringes/pipets.*—Capable of accurate measurement and transfer of appropriate volumes for standard solution preparation and sample fortification (50–1000 µL).

(**o**) *Volumetric flasks*.—5–100 mL.

(**p**) *Glassware for evaporation steps*.—Depending on the evaporation technique (e.g., small round-bottom flasks, suitable tubes, or glassware for Kuderna-Danish evaporation). It is recommended to heat the glassware for at least 2 h at 250°C to remove potential contamination.

D. Reference Standards

(**a**) *PAH standards.*—High-purity reference standards of the PAH analytes (1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*k*]fluoranthene, chrysene, dibenz[*a,h*]anthracene, fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, naphthalene, phenanthrene, and pyrene).

(**b**) *U.S. Environmental Protection Agency (EPA) 16 PAH cocktail.*—(^{13}C, 99%), Product No. ES-4087 (5 µg/mL, 1.2 mL in nonane), Cambridge Isotope Labs (Tewksbury, MA, USA) or equivalent.

Table 2014.08C. Statistical results for the studied PAHs at three different concentration levels in mussel after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	10	20	38.1	95.3	5.7	8.8	14.9	23.2	0.89
	9	18	39.4	98.6	4.6	8.3	11.7	21.1	0.81
	10	20	38.9	97.3	4.6	8.9	11.9	22.8	0.87
1-MN	10	20	19.3	96.5	3.1	4.5	16.2	23.2	0.80
	10	20	96.9	96.9	9.0	19.2	9.2	19.8	0.87
	8	16	208.7	104.4	26.6	26.6	12.7	12.7	0.63
1-MP	9	18	9.8	98.1	0.7	1.8	7.2	18.0	0.56
	9	18	45.8	91.6	5.3	9.6	11.6	21.0	0.82
	9	18	117.2	93.7	10.2	24.4	8.7	20.8	0.94
2,6-DMN	10	20	13.8	91.9	1.5	2.9	11.2	20.7	0.68
	8	16	65.4	87.2	3.1	13.6	4.8	20.7	0.86
	10	20	153.8	87.9	10.7	36.8	6.9	23.9	1.13
3-MC	10	20	9.9	98.6	0.7	1.7	7.6	16.7	0.52
	10	20	87.2	96.8	4.1	10.9	4.7	12.5	0.54
	10	20	138.0	95.2	3.8	16.7	2.8	12.1	0.56
Ant	10	20	3.9	77.9	0.3	1.3	6.7	32.5	0.88
	9	18	13.0	86.8	1.7	3.2	12.8	24.8	0.81
	9	18	31.5	78.8	1.4	7.9	4.6	25.0	0.93
BaA	10	20	4.3	85.1	0.3	0.7	6.9	15.9	0.44
	10	20	21.5	85.9	1.1	2.3	5.1	10.9	0.38
	8	16	52.6	87.7	1.3	2.2	2.5	4.2	0.17
BaP	9	18	1.6	79.2	0.1	0.3	5.6	20.5	0.49
	9	18	8.1	80.5	0.6	1.4	6.9	17.7	0.54
	9	18	19.3	77.3	0.6	2.6	3.0	13.4	0.46
BbF	10	20	4.7	94.4	0.3	0.5	7.2	10.6	0.30
	10	20	27.1	90.2	1.9	2.8	6.9	10.2	0.37
	10	20	69.1	92.1	2.1	5.8	3.0	8.4	0.35
BghiP	9	18	2.0	98.1	0.1	0.2	4.9	10.6	0.26
	10	20	9.3	92.7	0.4	1.1	4.4	12.1	0.37
	10	20	17.9	89.6	0.6	1.5	3.2	8.6	0.29
BkF	9	18	1.9	97.4	0.1	0.2	7.2	10.3	0.25
	10	20	18.5	92.7	1.1	2.4	6.2	12.8	0.44
	10	20	36.6	91.5	1.0	3.8	2.7	10.4	0.40
Chr	10	20	14.2	94.4	0.8	1.3	5.7	9.5	0.31
	10	20	91.6	91.6	4.1	8.6	4.5	9.4	0.41
	10	20	159.8	91.3	4.9	11.8	3.1	7.4	0.35
DBahA	10	20	1.9	93.1	0.2	0.2	9.1	11.2	0.27
	10	20	9.1	90.5	0.5	1.2	5.4	13.5	0.41
	10	20	13.6	90.8	0.5	1.2	4.0	8.5	0.28
Fln	10	20	5.4	107.7	0.2	0.6	3.5	10.6	0.30
	10	20	25.5	101.8	1.0	1.9	3.7	7.6	0.27
	9	18	48.1	96.2	1.7	2.4	3.5	4.9	0.20
Flt	9	18	10.2	102.4	1.1	1.4	10.7	13.2	0.41
	10	20	48.9	97.7	2.7	4.3	5.5	8.8	0.35
	9	18	93.3	93.3	4.9	6.6	5.3	7.1	0.31
IcdP	10	20	2.0	97.7	0.1	0.2	7.3	11.3	0.28
	10	20	9.4	93.7	0.5	1.1	5.6	11.9	0.37

Table 2014.08C. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	10	20	17.9	89.3	0.9	1.6	4.9	8.8	0.30
Naph	9	18	23.7	94.6	1.9	4.0	8.1	17.1	0.61
	10	20	105.9	84.7	10.1	26.7	9.5	25.2	1.12
	8	16	146.7	91.7	7.2	19.2	4.9	13.1	0.61
Phe	8	16	14.5	96.8	0.8	0.9	5.3	6.1	0.20
	8	16	93.1	93.1	3.9	8.5	4.1	9.2	0.40
	8	16	160.8	91.9	5.8	13.0	3.6	8.1	0.38
Pyr	10	20	14.2	94.6	0.7	1.3	5.0	9.3	0.31
	10	20	71.5	95.4	2.9	7.0	4.0	9.8	0.41
	10	20	116.5	93.2	4.5	9.6	3.8	8.3	0.37

Containing: Acenaphthene ($^{13}C_6$, 99%), acenaphthylene ($^{13}C_6$, 99%), anthracene ($^{13}C_6$, 99%), benz[*a*]anthracene ($^{13}C_6$, 99%), benzo[*b*]fluoranthene ($^{13}C_6$, 99%), benzo[*k*]fluoranthene ($^{13}C_6$, 99%), benzo[*g,h,i*]perylene ($^{13}C_{12}$, 99%), benzo[*a*]pyrene ($^{13}C_4$, 99%), chrysene ($^{13}C_6$, 99%), dibenz[*a,h*]anthracene ($^{13}C_6$, 99%), fluoranthene ($^{13}C_6$, 99%), fluorene ($^{13}C_6$, 99%), indeno[1,2,3-*cd*]pyrene ($^{13}C_6$, 99%), naphthalene ($^{13}C_6$, 99%), phenanthrene ($^{13}C_6$, 99%), and pyrene ($^{13}C_6$, 99%).

E. Preparation of Standard Solutions

(**a**) *Individual stock solutions.*—Prepare individual PAH stock solutions at approximately 1000 or 2500 µg/mL in toluene.

(**b**) *Mixed stock standard solution.*—Use analyte individual stock solutions to obtain a mixed solution of each PAH at 10 µg/mL (for benzo[*a*]pyrene and other low-level PAHs) or 25 µg/mL (for chrysene and other higher-level PAHs) or 50 µg/mL (for naphthalene) in isooctane. *See* Table **2014.08E** for analyte concentrations in the mixed stock standard solution.

(**c**) *Working PAH Solution A.*—Accurately transfer 0.5 mL of the mixed stock standard solution into a 5 mL volumetric flask and dilute to volume with isooctane.

(**d**) *Working PAH Solution B.*—Accurately transfer 0.5 mL of the Working PAH Solution A into a 5 mL volumetric flask and dilute to volume with isooctane.

(**e**) *Internal standard solution.*—Prepare 1 µg/mL solution of ^{13}C-PAHs in isooctane by 5-fold dilution of the 5 µg/mL EPA 16 ^{13}C-PAHs cocktail with isooctane.

(**f**) *Calibration standard solutions.*—Prepare eight levels of calibration standard solutions (1 mL each) in 2 mL amber screw-cap vials. It is recommended to distribute small portions (enough for a single injection) of the calibration standard solutions into multiple crimp-top vials with 100 µL deactivated glass inserts. *See* Table **2014.08A** for analyte concentrations in the calibration standards and Table **2014.08F** for the dilution scheme.

(*1*) *For level 1 calibration standard.*—Accurately transfer 50 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 900 µL isooctane. Cap the vial and vortex mix briefly.

(*2*) *For level 2 calibration standard.*—Accurately transfer 100 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

(*3*) *For level 3 calibration standard.*—Accurately transfer 200 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 750 µL isooctane. Cap the vial and vortex mix briefly.

(*4*) *For level 4 calibration standard.*—Accurately transfer 500 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 450 µL isooctane. Cap the vial and vortex mix briefly.

(*5*) *For level 5 calibration standard.*—Accurately transfer 100 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

(*6*) *For level 6 calibration standard.*—Accurately transfer 200 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 750 µL isooctane. Cap the vial and vortex mix briefly.

(*7*) *For level 7 calibration standard.*—Accurately transfer 500 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 450 µL isooctane. Cap the vial and vortex mix briefly.

(*8*) *For level 8 calibration standard.*—Accurately transfer 100 µL of the mixed stock standard solution into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

F. Extraction and Cleanup Procedure

(*1*) Add 50 µL of the 1 µg/mL ^{13}C-PAHs solution to 10 ± 0.1 g of thoroughly homogenized seafood sample in a 50 mL polypropylene centrifuge tube.

(*2*) Vortex sample for 15 s and let equilibrate for 15 min.

(*3*) Add 5 mL (10 mL in the case of shrimp) of purified water and 10 mL ethyl acetate.

(*4*) Shake tube vigorously by hand for 1 min.

(*5*) Add 4 g of muffled anhydrous magnesium sulfate and 2 g sodium chloride, and seal the tube well (ensure that powder does not get into the screw threads or rim of the tube).

(*6*) Shake tube vigorously by hand for 1 min, ensuring that crystalline agglomerates are broken up sufficiently during shaking.

(*7*) Centrifuge tube at >1500 rcf for 10 min.

(*8*) Take a 5 mL aliquot of the upper ethyl acetate layer, add 50 µL isooctane as a keeper, and gently evaporate all ethyl acetate until only isooctane and co-extracted sample fat are left.

(*9*) Reconstitute in 1 mL hexane.

Table 2014.08D. Statistical results for the studied PAHs at three different concentration levels in oyster after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	8	16	72.3	90.4	4.1	11.7	5.7	16.2	0.68
	8	16	69.0	86.2	4.9	12.6	7.1	18.3	0.76
	8	16	65.6	82.0	5.5	13.2	8.4	20.1	0.83
1-MN	8	16	67.9	90.5	4.3	14.8	6.4	21.8	0.91
	9	18	90.8	90.8	20.6	28.9	22.7	31.9	1.39
	9	18	236.6	94.6	26.4	55.3	11.2	23.4	1.18
1-MP	7	14	20.2	80.8	1.8	4.8	8.8	23.9	0.83
	8	16	39.9	79.8	2.6	7.7	6.4	19.3	0.74
	8	16	154.7	77.3	16.9	34.7	10.9	22.4	1.06
2,6-DMN	7	14	30.5	76.2	3.0	5.3	9.8	17.5	0.65
	7	14	57.9	77.2	4.9	10.8	8.5	18.6	0.76
	7	14	161.3	71.7	12.7	20.9	7.9	13.0	0.62
3-MC	9	18	27.8	92.5	1.7	3.6	6.0	13.0	0.47
	9	18	79.8	88.6	5.1	10.3	6.4	13.0	0.55
	9	18	196.8	87.5	10.1	23.5	5.1	12.0	0.59
Ant	7	14	5.3	53.2	0.5	2.9	8.8	55.0	1.56
	7	14	7.5	50.3	0.8	4.9	10.0	64.7	1.94
	6	12	34.0	56.6	3.0	15.1	8.8	44.5	1.67
BaA	9	18	10.9	72.6	0.8	2.2	7.7	19.7	0.62
	9	18	17.2	68.6	1.0	3.6	5.8	21.1	0.71
	9	18	71.6	71.6	3.9	12.5	5.4	17.5	0.73
BaP	9	18	2.5	49.7	0.3	1.1	11.9	43.5	1.10
	9	18	4.8	48.2	0.4	2.0	9.0	42.2	1.18
	9	18	24.6	49.3	1.6	10.0	6.4	40.5	1.45
BbF	9	18	8.6	85.9	0.6	1.0	6.6	11.6	0.35
	9	18	24.6	81.8	1.7	2.7	7.1	11.2	0.40
	9	18	82.8	82.8	3.4	9.8	4.1	11.9	0.51
BghiP	9	18	4.1	82.4	0.2	0.5	5.9	12.3	0.34
	9	18	8.2	81.9	0.7	1.1	8.6	13.6	0.41
	9	18	19.6	78.4	1.0	2.3	4.9	11.7	0.41
BkF	9	18	6.9	85.9	0.5	1.1	7.7	16.3	0.48
	9	18	16.9	84.3	1.1	2.6	6.5	15.4	0.52
	9	18	62.9	83.8	3.8	8.2	6.1	13.1	0.54
Chr	9	18	43.0	85.9	2.8	4.3	6.5	9.9	0.39
	9	18	81.6	81.6	5.0	8.6	6.2	10.6	0.45
	9	18	204.1	81.6	8.7	19.0	4.3	9.3	0.46
DBahA	9	18	4.1	82.7	0.4	0.5	9.0	13.0	0.35
	8	16	8.2	82.2	0.6	1.1	7.5	13.4	0.41
	9	18	16.0	80.0	0.7	2.0	4.4	12.7	0.43
Fln	9	18	12.5	83.3	1.0	1.9	8.2	15.4	0.50
	9	18	20.3	81.2	1.4	3.0	6.8	14.6	0.51
	9	18	57.0	76.0	2.3	11.3	4.0	19.9	0.81
Flt	9	18	22.0	88.2	2.1	3.6	9.5	16.2	0.57
	9	18	42.0	83.9	5.1	7.5	12.2	17.9	0.69
	9	18	120.7	80.5	7.0	17.1	5.8	14.2	0.65
IcdP	9	18	4.3	86.8	0.4	0.6	9.6	13.1	0.36
	9	18	8.3	83.3	0.8	1.1	9.0	13.7	0.42

Table 2014.08D. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	9	18	20.0	80.1	1.0	2.2	4.8	10.7	0.37
Naph	9	18	71.0	88.7	5.3	9.4	7.5	13.2	0.55
	9	18	106.2	84.9	7.3	14.7	6.9	13.9	0.62
	8	16	193.9	86.2	6.0	29.8	3.1	15.4	0.75
Phe	9	18	41.6	83.2	3.0	5.5	7.2	13.2	0.51
	9	18	80.3	80.3	6.1	10.7	7.6	13.3	0.57
	8	16	203.9	81.6	9.5	22.5	4.7	11.0	0.54
Pyr	9	18	34.0	85.1	2.2	3.3	6.4	9.8	0.37
	8	16	63.2	84.3	2.2	5.3	3.5	8.4	0.35
	9	18	163.4	81.7	8.0	16.6	4.9	10.2	0.48

(*10*) Condition a silica SPE column (1 g silica gel with approximately 0.2 g of muffled anhydrous sodium sulfate on the top) with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane.

(*11*) Apply the extract in hexane onto the silica SPE cartridge.

(*12*) Elute with hexane–dichloromethane (3 + 1, v/v) using volume determined for the given silica gel SPE cartridges from the elution profiles of target analytes and fat, which are dependent on the silica deactivation, *see Note* (*4*) below. Collect the eluent.

(*13*) Add 0.5 mL isooctane (and 1–2 mL ethyl acetate) to the eluent as a keeper and gently evaporate down to 0.5 mL to remove hexane and dichloromethane from the final extract.

(*14*) Transfer the final extract into an autosampler vial for the GC/MS analysis.

Notes: (*1*) The fat capacity of the 1 g silica gel SPE column is approximately 0.1 g. If the 5 mL ethyl acetate extract aliquot contains more than 0.1 g fat, it is necessary to use a smaller aliquot volume to avoid sample breakthrough during the cleanup step.

(*2*) Ethyl acetate should not be present in the extract applied to the silica cartridge because it can affect the extract polarity, thus potentially retention of fat and analytes on the silica gel. The coextracted fat and 50 µL isooctane act as keepers during the first evaporation step (step *8*), thus the evaporation should be conducted gently until there is no significant change in the volume, i.e., until only the isooctane and coextracted fat are left in the evaporation tube or flask.

(*3*) Addition of 1–2 mL ethyl acetate to the eluent in step *13* is recommended for a better control of the evaporation process and higher absolute recoveries of volatile PAHs.

(*4*) The deactivation and storage of silica gel SPE cartridges can vary, potentially resulting in different amounts of water in the silica, thus its potentially different retention characteristics. Therefore, it is important to test the elution profiles of PAHs and fat and determine the optimum volume of the elution solvent to ensure adequate analyte recoveries and fat cleanup. The following procedure is recommended:

(**a**) Prepare a PAH solution in hexane by combining 50 µL of the Working PAH Solution A and 1 mL hexane in a vial. Mix well and apply onto a silica SPE column (1 g silica gel with approximately 0.2 g of muffled anhydrous sodium sulfate on the top), which was conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane.

(**b**) Elute with 10 mL hexane–dichloromethane (3 + 1, v/v), collecting 0.5 mL elution fractions in 20 evaporation tubes or flasks. Add 0.5 mL isooctane to each elution fraction and evaporate down to 0.5 mL using the optimized evaporation conditions. Analyze each fraction by GC/MS.

(**c**) Determine PAH elution profile by plotting analyte response (peak area or height) in a given fraction normalized to the sum of analyte responses in all tested fractions vs the elution volume. *See* Figure **2014.08B** for an example of a PAH elution profile. It is recommended to add an additional 0.5 mL on top of the determined elution fraction (corresponding to 100% recovery) as a safety margin ensuring good analyte recoveries in routine practice. This would result in the optimum elution volume of 7 mL for the silica cartridge tested in Figure **2014.08B**.

(**d**) To check the effectiveness of fat removal, dissolve 100 mg pure fish oil (or any suitable fat) in 1 mL hexane and apply it onto the silica gel cartridge, which was conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane. Elute with the optimum elution volume of hexane–dichloromethane (3 + 1, v/v), which was determined in the previous step (e.g., 7 mL for the example in Figure **2014.08B**). Collect this fraction in an evaporation tube or flask, which empty weight (after heating in an oven to remove moisture) was recorded to four decimal places

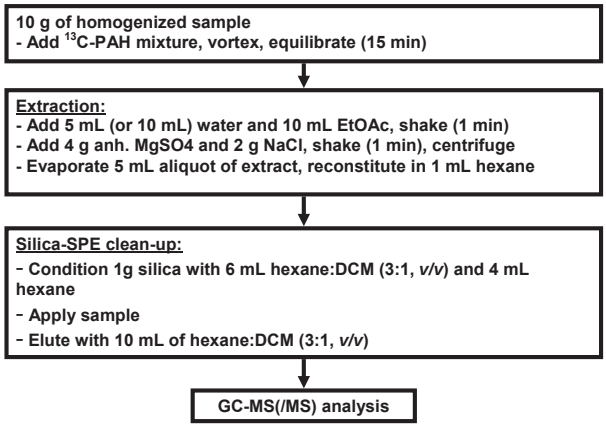


Figure 2014.08A. Flow chart of the method for determination of PAHs in seafood using GC/MS.

Table 2014.08E. Analyte concentrations in the mixed stock standard solution

Analyte	Concentration, µg/mL
Anthracene	10
Benz[*a*]anthracene	10
Benzo[*a*]pyrene	10
Benzo[*b*]fluoranthene	10
Benzo[*g,h,i*]perylene	10
Benzo[*k*]fluoranthene	10
Chrysene	25
Dibenz[*a,h*]anthracene	10
Fluoranthene	25
Fluorene	10
Indeno[1,2,3-*cd*]pyrene	10
Naphthalene	50
Phenanthrene	25
Pyrene	25
1-Methylnaphthalene	25
2,6-Dimethylnaphthalene	25
1-Methylphenanthrene	25
1,7-Dimethylphenanthrene	10
3-Methylchrysene	25

using an analytical balance. Elute the cartridge with additional 3 × 1 mL hexane–dichloromethane (3 + 1, v/v) and collect in three evaporation tubes/flasks of known empty weight.

(**e**) Evaporate the four elution fractions to dryness and gravimetrically determine the amount of fat eluting in each fraction by subtracting the empty weights from newly recorded weights after solvent evaporation. There should be no fat eluting in the optimum elution fraction for PAHs (this can also be observed visually in the tubes).

(**f**) If there is fat coeluting with PAHs, then the PAH and fat elution profiles have to be reexamined to determine optimum elution volume for PAH and fat separation (potentially sacrificing up to 5% of late-eluting PAH amounts if necessary) or a different silica gel cartridge has to be used.

Table 2014.08F. Dilution scheme for preparation of the calibration standard solutions

Calibration level	Vol. of mixed stock standard solution, µL	Vol. of working PAH solution[a], µL	Vol. of working PAH solution B, µL	Vol. of ^{13}C-PAH 1 µg/mL solution, µL	Final vol.[a], µL
1	—	—	50	50	1000
2	—	—	100	50	1000
3	—	—	200	50	1000
4	—	—	500	50	1000
5	—	100	—	50	1000
6	—	200	—	50	1000
7	—	500	—	50	1000
8	100	—	—	50	1000

[a] Bring to volume using isooctane.

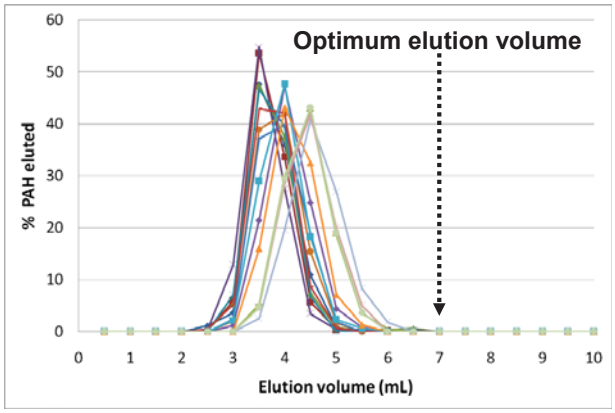


Figure 2014.08B. An example of elution profiles of PAHs on a silica gel SPE cartridge and determination of the optimum elution volume.

G. GC/MS Analysis

(**a**) *GC conditions.*—Table **2014.08G** provides GC conditions that were used by the collaborative study participants. Other conditions (e.g., column, temperature and flow program, and injection technique and volume) can be used as long as the laboratory qualification criteria for separation, sensitivity, and linearity are met. The injection temperature or program needs to be optimized to enable quantitative transfer of less volatile PAHs. If programmable temperature vaporizer (PTV) solvent vent mode is used, solvent venting parameters (temperature, time, flow, pressure) need to be carefully optimized to prevent losses of the volatile PAHs, especially naphthalene. The separation criteria (demonstrated in Figure **2014.08C**) include (*1*) a baseline separation of benzo[*a*]pyrene and benzo[*e*]pyrene (concentration ratio of 1:5), (*2*) at least 50% valley separation of anthracene and phenanthrene (concentration ratio 1:2.5; evaluated for the anthracene peak), and (*3*) at least 50% valley separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*]fluoranthene (concentration ratio of 1:1:1). *Note*: Criteria for separation of chrysene and triphenylene (another PAH critical pair) were not set for the collaborative study. For accurate quantitation of chrysene, at least 50% valley separation is recommended, which can be achieved using selective stationary phases.

The maximum oven temperature program may not exceed the maximum temperature limit for a given column. Backbone-modified columns, such as Rxi-17Sil MS or DB-17MS, are recommended for their better temperature stability and also good selectivity for critical PAH pairs or groups, including the anthracene/phenanthrene pair or benzofluoranthenes. Conduct proper inlet and column maintenance to ensure adequate operation of the GC instrument. Perform system checks.

(**b**) *MS conditions.*—Any GC/MS instrument (single quadrupole, triple quadrupole, TOF, or ion trap) with EI may be used as long as it provides results meeting the laboratory qualification requirements. The 10 study participants used the following instruments: single quadrupole (Agilent 5973–Laboratory 4; Agilent 5975B XL Inert–Laboratories 3 and 8–10; Agilent 5975C–Laboratories 6 and 7), triple quadrupole (Agilent 7000B–Laboratory 5; Thermo TSQ–Laboratory 1), and time-of-flight (Leco Pegasus 4D–Laboratory 2). Pay special attention to the optimization of the MS transfer line and MS source temperature. Higher MS source temperatures

Table 2014.08G. GC conditions used by collaborative study participants

Laboratory No.	1	2	3	4	5
GC Instrument	Thermo Trace GC Ultra	Agilent 6890N	Agilent 6890N	Agilent 6890	Agilent 7890A
Inlet type	PTV (Thermo 816, RP - 2004)	PTV (Gerstel CIS 4)	S/SL (Agilent)	PTV (Gerstel CIS 4)	Multi-mode (Agilent)
Column type	Thermo TR-50MS	Restek Rxi-17Sil MS	Agilent J&W DB-17MS	Restek Rxi-17Sil MS	Restek Rxi-17Sil MS
Column dimension	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm
Retention gap dimension	NA	NA	NA	2 m × 0.25 mm id, 0.25 µm	NA
Run time, min	40	49.97	47.17	46	39.33
Oven temperature program	60°C (1 min), 12°C/min to 210°C, 8°C/min to 340°C (10 min)	50°C (4.3 min), 30°C/min to 220°C, 2°C/min to 240°C, 5°C/min to 360°C (6 min)	80°C (1 min), 30°C/min to 220°C, 2°C/min to 270°C (1.5 min), 5°C/min to 320°C	65°C (4 min), 30°C/min to 220°C (0.5 min), 3°C/min to 320°C (5 min)	65°C (0.5 min), 15°C/min to 220°C, 4°C/min to 330°C (1 min)
He flow/pressure program	1.2 mL/min (constant flow)	1.3 mL/min (constant flow)	1.3 mL/min (constant flow)	1.3 mL/min (15 min), 50 mL/min^2 to 2 mL/min	2 mL/min (constant flow)
Injection mode	PTV solvent split	PTV solvent vent	Pulsed splitless	PTV solvent vent (stop flow)	PTV solvent vent
Injection volume, µL	8	8	1	8	5
Inlet temperature (program)	50°C (2.3 min), 6.7°C/s to 300°C (1 min), 10°C/s to 350°C (10 min)	50°C (2.3 min), 12°C/s to 340°C	320°C	15°C (0.5 min), 12°C/min to 275°C	70°C (0.15 min), 600°C/min to 325°C (5 min)
Liner	Thermo, LVI Silcosteel, 2 mm id	Gerstel, CIS4 deactivated baffled liner, 2 mm id	Restek, single-gooseneck splitless, 4 mm id	Gerstel, CIS4 deactivated baffled liner, 2 mm id	Agilent, dimpled deactivated, 2 mm id
PTV solvent vent time	0.1 min	138 s	NA	0.5 min	0.1 min
PTV solvent vent flow, mL/min	50	50	NA	100	50
PTV solvent vent pressure, psi	NA	50	NA	1.80	5
Pressure pulse and duration	NA	NA	50 psi for 0.2 min	NA	NA
Split vent purge flow and start	50 mL/min, 1 min	20 mL/min	50 mL/min, 1 min	50 mL/min, 2.5 min	60 mL/min, 2.6 min
Gas saver flow and start time	15 mL/min, 3 min	NA	15 mL/min, 6 min	30 mL/min, 3 min	NA
MS transfer line temperature, °C	250	280	280	280	300

	6	7	8	9	10
GC instrument	Agilent 7890	Agilent 7890	Agilent 6890N	Agilent 6890N	Agilent 6890N
Inlet type	S/SL (Agilent)	S/SL (Agilent)	S/SL (Agilent)	PTV (Apex, ProSep 800 Plus)	PTV (Gerstel CIS 4)
Column type	Restek Rxi-17Sil MS	Restek Rxi-17Sil MS	Agilent J&W DB-EUPAH	Phenomenex ZB-50	Agilent J&W DB-EUPAH
Column dimension	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	20 m × 0.18 mm id, 0.14 µm	30 m × 0.25 mm id, 0.25 µm	20 m × 0.18 mm id, 0.14 µm
Retention gap dimension	1 m × 0.25 mm	NA	NA	NA	NA
GC run time, min	46.33	41.63	31.83	48.27	36.17
Oven temperature program	65°C (0.5 min), 15°C/min to 220°C, 4°C/min to 330°C (8 min)	80°C (4.3 min), 30°C/min to 220°C, 2°C/min to 240°C, 5°C/min to 300°C (1 min), 30°C/min to 350°C (8 min)	80°C (1 min), 30°C/min to 130°C, 12°C/min to 240°C, 3.5°C/min to 310°C	80°C (4.6 min), 30°C/min to 130°C (5 min), 10°C/min to 260°C (15 min), 10°C/min to 320°C (3 min); Post run: 80°C (1 min)	55°C (3 min), 30°C/min to 220°C, 2°C/min to 240°C, 3°C/min to 285°C; 15°C/min to 310°C (1 min); Post run: 330°C (2 min)

Table 2014.08G. (*continued*)

Laboratory No.	6	7	8	9	10
He flow/pressure program	1.5 mL/min (13 min), 10 mL/min² to 2 mL/min	1.5 mL/min (constant flow)	1 mL/min (constant flow)	1.3 mL/min (19.5 min), 50 mL/min² to 2 mL/min	2 mL/min (constant flow)
Injection mode	Hot splitless	Hot splitless	Pulsed splitless	Splitless	PTV solvent vent
Injection volume, µL	1	1	1	10	5
Inlet temperature (program)	300°C	280°C	320°C	60°C (2.25 min), 300°C/min to 335°C (45 min)	50°C (0.5 min), 600°C/min 400°C (15 min), 30°C/min 70°C
Liner	SGE, Focus liner	Agilent, single taper, glass wool-packed, 4 mm id	Agilent, double taper, 4 mm id	Apex, ProSep taper liner, 2 mm id	Agilent, PTV multi-baffle, 2 mm id
PTV solvent vent time	NA	NA	NA	NA	0.5 min
PTV solvent vent flow	NA	NA	NA	NA	100 mL/min
PTV solvent vent pressure	NA	NA	NA	NA	50 kPa
Pressure pulse and duration	NA	NA	50 psi for 0.2 min	NA	NA
Split vent purge flow and start	50 mL/min, 1 min	50 mL/min, 1 min	50 mL/min, 1 min	50.0 mL/min, 2.30 min	100 mL/min, 3 min
Gas saver flow and start time	20 mL/min, 3 min	20 mL/min, 2 min	15 mL/min, 6 min	NA	15 mL/min, 6 min
MS transfer line temperature, °C	300	280	320	300	325

Figure 2014.08C. GC separation criteria: (1) A baseline separation of benzo[*a*]pyrene and benzo[*e*]pyrene (concentration ratio of 1:5), (2) at least 50% valley separation of phenanthrene and anthracene (concentration ratio 2.5:1; evaluated for the anthracene peak, which is the second peak in the figure), and (3) at least 50% valley separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*] fluoranthene (concentration ratio of 1:1:1).

are recommended (e.g., ≥280°C for Agilent sources) to provide optimum analysis (quantitative transfers, minimum peak tailing) for less volatile PAHs.

Table **2014.08H** provides MS ions (*m/z*) and MS/MS transitions used by the study participants for quantification and identification of target PAHs and ^{13}C-PAHs using single-stage MS (single quadrupole and TOF) and tandem MS/MS (triple quadrupole) instruments, respectively.

Use adequate data acquisition rate (dwell times in scanning instruments) and solvent delay time. Perform air/water checks and autotune to verify and obtain adequate operation of the instrument. Verify identification of the analyte peaks by comparing the ion

Table 2014.08H. MS ions (*m*/*z*) and MS/MS precursor to product ion transitions used by study participants for quantification (quant) and identification (qual) of target PAHs and ^{13}C-PAHs using single-stage MS (single quadrupole and TOF) and tandem MS/MS (triple quadrupole) instruments, respectively

	Single quad/TOF MS		Triple quad MS/MS	
Compound	Quant	Qual	Quant	Qual
1,7-DMP	206	191	206>190	206>205, 206>165
1-MN	142	115	142>115	142>141, 142>116
1-MP	192	189	192>191	192>165
2,6-DMN	156	141, 144	156>115	156>141
3-MC	242	241	242>239	242>226
Ant	178	177	178>176	178>177, 178>151
BaA	228	226	228>226	228>224, 228>202
BaP	252	253	252>250	250>248, 252>224
BbF	252	253	252>250	250>248, 252>224
BghiP	276	277	276>274	274>272, 276>275
BkF	252	253	252>250	250>248, 252>224
Chr	228	226	228>226	228>224, 228>202
DBahA	278	276	278>276	276>274, 278>274
Fln	166	165	166>165	166>164, 166>163
Flt	202	200	202>200	202>201
IcdP	276	277	276>274	274>272, 276>248
Naph	128	127	128>102	128>127
Phe	178	177	178>176	178>177, 178>151
Pyr	202	200	202>200	202>201
13C-Ant	184	183	184>183	184>182, 184>156
13C-BaA	234	232	234>232	234>206
13C-BaP	256	257	256>254	256>228
13C-BbF	258	259	258>256	258>255
13C-BghiP	288	289	288>286	288>287
13C-BkF	258	259	258>256	258>255
13C-Chr	234	232	234>232	234>206
13C-DBahA	284	282	284>282	284>280
13C-Fln	172	171	172>171	172>170
13C-Flt	208	205	208>206	208>207
13C-IcdP	282	283	282>280	282>281
13C-Naph	134	133	134>133	134>105
13C-Phe	184	183	184>183	184>156
13C-Pyr	205	203, 206, 208	205>203	205>204

ratios of contemporaneously analyzed calibration standards, which have been analyzed under the same conditions.

(**c**) *Injection sequence.*—Bracket the seven test samples with two sets of calibration standards. Inject solvent blanks after the calibration level 8 (highest) standard and after the samples. In addition, analyze a reagent blank with each set of samples. Inject only once from each vial, thus preventing potential losses of volatile PAHs and/or contamination.

H. Calculations

Quantification is based on linear least-squares calibration of analyte signals (S_{PAH}) divided by signals ($S_{13C\text{-}PAH}$) of corresponding ^{13}C-labeled internal standards (*see* Table **2014.08I**) plotted versus analyte concentrations. Peak areas are generally preferred as signals used for the quantification, but peak heights should be used for peaks that are not well resolved, such as in the case of anthracene and phenanthrene. The analyte concentrations in the final extract (c_{PAH}, µg/L) are determined from the equation:

$$c_{PAH} = [(S_{PAH}/S_{13C\text{-}PAH}) - b]/a$$

where a is the slope of the calibration curve and b is the y-intercept.

The concentration of PAHs in the sample (C, µg/kg) is then calculated:

$$C = (c_{PAH}/c_{13C\text{-}PAH}) \times (X_{13C\text{-}PAH}/m)$$

Table 2014.08I. PAH analytes and corresponding ^{13}C-PAHs used for PAH signal normalization

Analyte	^{13}C-PAH used for signal normalization
Anthracene	Anthracene ($^{13}C_6$)
Benz[*a*]anthracene	Benz[*a*]anthracene ($^{13}C_6$)
Benzo[*a*]pyrene	Benzo[*a*]pyrene ($^{13}C_4$)
Benzo[*b*]fluoranthene	Benzo[*b*]fluoranthene ($^{13}C_6$)
Benzo[*g,h,i*]perylene	Benzo[*g,h,i*]perylene ($^{13}C_{12}$)
Benzo[*k*]fluoranthene	Benzo[*k*]fluoranthene ($^{13}C_6$)
Chrysene	Chrysene ($^{13}C_6$)
Dibenz[*a,h*]anthracene	Dibenz[*a,h*]anthracene ($^{13}C_6$)
Fluoranthene	Fluoranthene ($^{13}C_6$)
Fluorene	Fluorene ($^{13}C_6$)
Indeno[1,2,3-*cd*]pyrene	Indeno[1,2,3-*cd*]pyrene ($^{13}C_6$)
Naphthalene	Naphthalene ($^{13}C_6$)
Phenanthrene	Phenanthrene ($^{13}C_6$)
Pyrene	Pyrene ($^{13}C_6$)
1-Methylnaphthalene	Naphthalene ($^{13}C_6$)
2,6-Dimethylnaphthalene	Phenanthrene ($^{13}C_6$)
1-Methylphenanthrene	Phenanthrene ($^{13}C_6$)
1,7-Dimethylphenanthrene	Phenanthrene ($^{13}C_6$)
3-Methylchrysene	Chrysene ($^{13}C_6$)

where $c_{13C\text{-}PAH}$ is the concentration of the corresponding ^{13}C-PAH in the calibration standard solutions (in µg/L); $X_{13C\text{-}PAH}$ is the amount of the corresponding ^{13}C-PAH added to the sample (in ng); and m is the sample weight (in g). Based on the method procedure and preparation of the calibration standard solutions, $c_{13C\text{-}PAH}$ is 50 µg/L, $X_{13C\text{-}PAH}$ is 50 ng, and m for the test samples is 10 g.

In the collaborative study, eight concentration levels were used for the calibration, corresponding to 5, 10, 20, 50, 100, 200, 500, and 1000 µg/L for benzo[*a*]pyrene and other lower-level PAHs, to 12.5, 25, 50, 125, 250, 500, 1250, and 2500 µg/L for higher-level PAHs, except for naphthalene that was present at 25, 50, 100, 250, 500, 1000, 2500, and 5000 µg/L. Coefficients of determination (r^2) should be 0.990 or greater and back-calculated concentrations of the calibration standards should not exceed ±20% of theoretical. For lower concentration levels, a limited calibration curve (without the higher-end concentration points) may be used for better accuracy. If a well-characterized quadratic relationship occurs, then a best-fitted quadratic curve may be used for calibration. Otherwise, if the back-calculated concentrations exceed ±20% of theoretical, normalized signals of the nearest two calibration standards that enclose the analyte signal in the sample can be used to interpolate the analyte concentration in the final extract.

References: (1) Kalachova, K., Pulkrabova, J., Drabova, L., Cajka, T., Kocourek, V., & Hajslova, J. (2011) *Anal. Chim. Acta* **707**, 84–91. http://dx.doi.org/10.1016/j.aca.2011.09.016

J. AOAC Int. **98**, 477 (2105)
DOI: 10.5740/jaoacint.15-032

Posted: April 30, 2015

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-07-06 15:33:49
Please indicate your perspective	Expert Review Panel Member
AOAC Official Methods Number (XXXX.XX)	2014.08
Method Name or Manuscript Title	Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No
Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!

Name	Cheryl L. Lassitter
Organization	DOC, NOAA, NMFS, NSIL
E-mail Address	cheryl.lassitter@noaa.gov

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-09-14 05:04:54
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	2014.08
Method Name or Manuscript Title	Determination of Polycyclic Aromatic Hydrocarbons PPAHs) in Seafood Using Gas Chromatography-Mass Spectrometry: Collaborative Study
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Mastovska K., Sorenson W.R., Hajslova J., Perez R., Betz J., Perez S., Keide K., Misunis M., Wang J., Jabusch J., Schmitz J., Stepp J., Hammack W., Cook J.M., Lopez-Sanchez P., Lizak R., Wenzl T., Drabova L., Pulkrabova J., Binkley J., Staples D., Pugh S., Taffe B., Rosmus J., Bousova K., Mittendorf K.: Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in seafood using gas chromatography-mass spectrometry: Collaborative study. Journal of AOAC International (2015) 98: 477–505. (doi: 10.5740/jaoacint.15-032).
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
Upload documentation to support the Method Applicability Information	PAHs method-applicability.pdf
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
Upload documentation to support the Reference Materials/ Reference Method	SRM 2977_mussletissue.pdf SRM_1974c_musseltissue.pdf
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
Upload documentation to support the Single Laboratory Validation Information	PAHs_Validation data.pdf

5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No
Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Jana Hajslova
Organization	University of Chemistry and Technology Prague, Czech Republic
E-mail Address	jana.hajslova@vscht.cz

The analytical procedure previously developed within the European project Conffidence and subsequently validated within the AOAC interlaboratory study is applicable not only for analysis of PAHs in seafood, but in our lab it is successfully applied for analysis of other groups of organic pollutants such as PCBs, OCPs, PBDEs and other brominated flame retardants. In our laboratory, the analytical method is accredited according to ISO 17025 for the whole spectrum of above mentioned analytes.

It has been applied in the studies summarized below:

- Analysis of oil/fish oil samples on halogenated POPs and PAHs with the aim of documentation of contamination of products in the common market.
- Participation in the interlaboratory test FAPAS no. 665 – PAHs in olive oil and nbr. 659 – smoked fish.
- Homogeneity testing during the preparation of CRM for PAHs analysis in smoked fish within the Conffidence project at IRMM.
- Analysis of fish and seafood samples on PAHs, PCBs, PBDEs and OCPs within the Conffidence project.
- Analysis of total diet samples and human breast milk samples on PAHs with the national project - „Impact of air pollution to genome of newborns”.
- Analysis of organohalogenated pollutants in human breast milk samples in the human biomonitoring surveys conducted in the Czech Republic within the years 2012 – 2016.
- Analysis of fish/seafood samples on halogenated POPs and PAHs within various commercial analysis in our laboratory with the aim of documentation of contamination of products in the common market, as well as within the monitoring of environmental contamination.

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2977

Mussel Tissue (Organic Contaminants and Trace Elements)

This Standard Reference Material (SRM) 2977 is intended for use in evaluating analytical methods for the determination of selected polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCB) congeners, chlorinated pesticides, polybromoinated diphenyl ether (BDE) congeners, methylmercury, and inorganic constituents in marine bivalve mollusk tissue and similar matrices. All of the constituents for which certified, reference, and information values are provided are naturally present in the freeze-dried mussel tissue. A unit of SRM 2977 consists of one bottle containing approximately 10 g of freeze-dried mussel tissue.

The development of this material was in response to the recommendations of the Group of Experts on Standards and Reference Materials (GESREM) established by the Intergovernmental Oceanographic Commission (IOC), United Nations Environment Program (UNEP), and the International Atomic Energy Agency (IAEA) [1]. The collection, preparation, and value assignment of SRM 2977 was a collaboration between the National Institute of Standards and Technology (NIST) and National Research Council of Canada (NRCC).

Certified Values: Certified values for concentrations, expressed as mass fractions, for 13 PAHs, 19 PCB congeners, 6 chlorinated pesticides, 5 BDE congeners (some in combination), 6 trace elements, and methylmercury are provided in Tables 1 to 6. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or accounted for by NIST. The certified values for the PAHs, PCB congeners, and chlorinated pesticides are based on the agreement of results obtained at NIST from two independent analytical techniques and from an interlaboratory comparison study. The certified values for the BDE congeners are based on the agreement of results obtained at NIST, from a collaborating laboratory, and from an interlaboratory comparison study. The certified values for the trace elements and methylmercury are based on NIST measurements by one technique and additional results from several collaborating laboratories.

Reference Values: Reference concentration values, expressed as mass fractions, are provided in Table 7 for 10 additional PAHs, 3 additional PCB congeners, 2 additional chlorinated pesticides, and five additional BDE congeners. Reference concentration values are provided in Table 8 for nine additional inorganic constituents. Reference values are noncertified values that represent best estimates of the true value; however, the values do not meet the NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

Information Values: Information values for concentrations, expressed as mass fractions, are provided in Table 9 for 23 additional trace elements. An information value is considered to be a value that will be of use to the SRM user, but insufficient information is available to assess the uncertainty associated with the value or only a limited number of analyses were performed.

Expiration of Certification: The certification of SRM 2977 is valid, within the measurement uncertainty specified, until **31 December 2017,** provided the SRM is handled in accordance with the instructions given in this certificate (see "Instructions for Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

The coordination of the technical measurements leading to certification was under the direction of M.M. Schantz and S.A. Wise of the NIST Analytical Chemistry Division.

Consultation on the statistical design of the experimental work and evaluation of the data were provided by S. D. Leigh, M.G Vangel and M.S. Levenson of the NIST Statistical Engineering Division.

Stephen A. Wise, Chief
Analytical Chemistry Division

Gaithersburg, MD 20899
Certificate Issue Date: 12 September 2008
See Certificate Revision History on Last Page

Robert L. Watters, Jr., Chief
Measurement Services Division

The mussels were collected under the supervision of A. Wagener from the Pontificia Unversidade Catolica, Do Rio De Janeiro, Brazil. The mussel tissue was freeze-dried at the Natural Products Support Group at the Frederick Cancer Research and Development Center (Frederick, MD) under the direction of T. McCloud. Preparation of the freeze-dried material was performed by M.P. Cronise and C.N. Fales of the NIST Measurement Services Division.

Analytical measurements at NIST were performed by W.C. Davis, J.M. Keller, J.R. Kucklick, M.J. Lopez de Alda, B.J. Porter, M.M. Schantz, S. Tutschku, and L. Yu of the NIST Analytical Chemistry Division.

Analytical measurements for selected PCB congeners were also performed at the Institute for National Measurement Standards, NRCC (Ottawa, Canada) by G. Gardner and C. Frasier. Results for selected PAHs, PCB congeners, chlorinated pesticides, and BDE congeners were also used from 12 laboratories that participated in an intercomparison exercise coordinated by M. M. Schantz of the NIST Analytical Chemistry Division. Analytical measurements for selected trace elements and methylmercury were also performed at the Institute of Applied Physical Chemistry, Research Centre Jülich (Jülich, Germany) by H. Emons and at the Department of Environmental Sciences, Jožef Stefan Institute (Ljubljana, Slovenia) by M. Horvat. Analytical measurements for selected BDE congeners were also performed at Indiana University (Bloomington, IN) by Y.L. Zhu and R.A. Hites. Results for selected trace elements were also used from six laboratories that participated in an intercomparison exercise coordinated by S. Willie of the Institute for National Measurement Standards, NRCC.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

INSTRUCTIONS FOR USE

Prior to removal of subsamples for analysis, the contents of the bottle should be mixed. The concentrations of constituents in SRM 2977 are reported on a dry-mass basis. The freeze-dried mussel tissue homogenate is hygroscopic, and as received, contains greater than 3 % (mass fraction expressed as percent) residual moisture. The mussel tissue sample should be dried to a constant mass before weighing for analysis, or if the constituents of interest are volatile, a separate subsample of the mussel tissue should be removed from the bottle at the time of analysis and dried to determine the concentration on a dry-mass basis.

NOTICE AND WARNING TO USERS

Storage: SRM 2977 is provided as a freeze-dried tissue homogenate in amber glass bottles. The tissue material should be stored at room temperature or below.

Handling: Normal biohazard safety precautions for the handling of biological tissues should be exercised.

PREPARATION AND ANALYSIS[1]

Sample Collection and Preparation: The mussels (*Perna perna*, edible brown mussel) used for the preparation of SRM 2977 were collected in Guanabara Bay, Brazil. The mussels were shucked, and the tissue was shipped to NIST on dry ice in two batches, each containing approximately 35 kg. For processing, the tissue was allowed to partially thaw and was transferred into a Robot Coupe Vertical Cutter Mixer until it was half full. The mussel tissue was blended for 5 min into a puree form and then poured into metal trays and frozen. The material was then freeze-dried with a starting temperature of -10 °C and slowly warmed to a temperature of 10 °C. The dry material was broken into smaller chunks and then jet milled to produce a fine powder. The powder was blended for homogeneity by processing through the jet mill twice. The material was radiation sterilized (^{60}Co) and then aliquoted into jars (~ 10 g each).

[1]Certain commercial equipment, instruments or materials are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

PAHs, PCBs , and Chlorinated Pesticides: The general approach used for the value assignment of the PAHs, PCBs, and chlorinated pesticides in SRM 2977 was similar to that reported for the recent certification of several environmental matrix SRMs [2] and consisted of combining results from analyses using various combinations of different extraction techniques, cleanup/isolation procedures, and chromatographic separation and detection techniques.

Two sets of gas chromatography/mass spectrometry (GC/MS) results, designated as GC/MS (I) and GC/MS (II), were obtained at NIST. For GC/MS (I) analyses, single subsamples of 3 g from three bottles of SRM 2977 were extracted using PFE with DCM as described by Schantz et al. [3]. Size exclusion chromatography (SEC) on a preparative-scale divinylbenzene-polystyrene column (10 μm particle size, 10 nm (100 Å pore size, 2.5 cm i.d. × 60 cm, PL-Gel, Polymer Labs, Inc., Amherst, MA) was used to remove the majority of the lipid and biogenic material. The extract was further fractionated using a silica solid phase extraction (SPE) column to isolate the fraction of interest. The processed extract was then analyzed by GC/MS using a 0.25 mm i.d. × 30 m fused silica capillary column with a 5 % (mole fraction) phenyl methylpolysiloxane phase (0.25 μm film thickness) (HP-5 MS, Agilent Technologies, Wilmington, DE) and a 0.25 mm i.d. × 60 m fused silica capillary column with a 50 % (mole fraction) phenyl methylpolysiloxane phase (0.25 μm film thickness) (DB-17 MS, Agilent Technologies). For the GC/MS (II) analyses, one sample (2 g) from each of three bottles was extracted using PFE with DCM. The fraction of interest was isolated using an alumina column (5% deactivated) followed by an amiopropylsilane SPE column. The isolated fraction was then analyzed by GC/MS using a 0.18 mm i.d. × 30 m fused silica capillary column with a proprietary non-polar phase (0.18 μm film thickness) (DB-XLB, Agilent Technologies). For both methods described above, selected perdeuterated PAHs, carbon-13 labeled PCBs, and perdeuterated pesticides were added to the mussel tissue prior to solvent extraction for use as internal standards for quantification purposes.

In addition to the analyses performed at NIST, SRM 2977 was used in 2005 as part of the NIST Intercomparison Exercise Program for Organic Contaminants in the Marine Environment [4]. Results from 12 laboratories that participated in this exercise were used as the third data set in the determination of the assigned values for PAHs, PCBs, and chlorinated pesticides in SRM 2977. The laboratories participating in this exercise used the analytical procedures routinely used in their laboratories to measure the analytes of interest.

Homogeneity Assessment for PAHs, PCBs, and Chlorinated pesticides: The homogeneity of SRM 2977 was assessed by analyzing duplicate 3 g samples from eight bottles selected by stratified random sampling. Samples were extracted, processed, and analyzed as described above for GC/MS (I). No statistically significant differences among bottles were observed for the PAHs at the 3 g sample size.

BDEs: Value assignment of concentrations for BDE congeners was based on three sets of data (one set from NIST, one set from a collaborating laboratory, and one set from an interlaboratory comparison study) using a variety of different extraction, cleanup, and quantification methods. All measurements were performed by using GC/MS operated in either electron impact (GC/EI-MS) or negative chemical ionization (GC/NCI-MS) mode.

For the NIST data set (GC/MS III), 3 g to 4 g subsamples of tissue from each of three bottles were extracted using PFE with DCM. The extracts were processed as above using SEC followed by a second cleanup step using a 5 % deactivated alumina SPE column. The extracts were analyzed by using GC/EI-MS on a 0.25 mm × 60 m fused silica capillary column with a 5 % phenyl methylpolysiloxane phase (0.25 μm film thickness) (DB-5MS, Agilent Technologies). ^{13}C-Labeled 2,2,4,4′5-pentabromodiphenyl ether (BDE 99) was added to the tissue samples prior to extraction for use as an internal standard for quantification of the BDEs.

For the measurements from the collaborating laboratory (Indiana University, Bloomington, IN) (GC/MS IV), five subsamples of SRM 2977 were Soxhlet extracted using hexane:acetone (1:1, volume fraction) after spiking with two internal standards, ^{13}C-labeled 2,3,3′,4,4′,5-hexachlorodiphenyl ether (CDE 156) and ^{13}C-labeled 2,2′,3,3′,4,4′,5,5′-octachlorodiphenyl ether (CDE 194). Lipids were removed by adding concentrated H_2SO_4 and shaking; the organic phase was collected and the extracts were further cleaned using a 3 % deactivated silica column and an alumina column in series. The extracts were analyzed by using GC/NCI-MS on a 0.25 mm × 60 m fused silica capillary column with a 5 % phenyl methylpolysiloxane phase (0.25 μm film thickness) (DB-5, Agilent Technologies). Details of the analyses by the collaborating laboratory are presented by Zhu and Hites [5].

SRM 2977 was used in 2005 as part of the NIST Intercomparison Exercise Program for Organic Contaminants in the Marine Environment [4]. Results from 12 laboratories that participated in this exercise were used as the third data set in the determination of the assigned values for BDEs in SRM 2977. The laboratories participating in this exercise used the analytical procedures routinely used in their laboratories to measure the analytes of interest.

Analytical Approach for Inorganic Constituents: Value assignment of the concentrations of selected trace elements was accomplished by combining results of the analyses of SRM 2977 at NIST, NRCC, Research Centre Jülich, Jožef Stefan Institute, and six selected laboratories that participated in an interlaboratory comparison exercise coordinated by the NRCC [6].

For the certified concentration values listed in Table 5, results were combined from analyses at NIST using inductively coupled plasma mass spectrometry (ICP-MS), analyses at NRCC using isotope dilution (ID) ICP-MS and graphite furnace atomic absorption spectrometry (GFAAS), analyses at Research Centre Jülich using one to four techniques, analyses at Jožef Stefan Institute using one or two techniques, and the mean of the results from six laboratories that participated in the NRCC interlaboratory comparison exercise. For the reference values provided in Table 8, results were combined from NIST, NRCC, Jožef Stefan Institute, Research Centre Jülich, and the NRCC interlaboratory comparison exercise. The information values in Table 9 are based on results of analyses at NRCC, Jožef Stefan Institute, and/or Research Centre Jülich. The analytical techniques used for the analysis of SRM 2977 for inorganic constituents are summarized in Table 10.

NIST Trace Element Analyses: The elements cadmium, cobalt, nickel, lead, copper, manganese, and strontium were determined using ICP-MS, quantified by the method of standard addition. Five mL of concentrated HNO_3 was added to 0.5 g subsamples from each of five bottles of SRM 2977. These samples were digested in closed vessels using programmed heating in a microwave oven. The resulting tissue digests were quantitatively diluted into two concentration ranges; rhodium was added to each as an internal standard. The elements copper, manganese, and strontium were determined in the more dilute solution; cadmium, copper, nickel, and lead were determined in the more concentrated solution. Two spike solutions containing each of these groups of elements were prepared and added to a split portion of each digest solution for the purpose of quantification by the method of standard addition. Prior to the quantitative determination of the analyte elements, an ICP-MS semi-quantitative analysis was performed to assess possible isobaric interferences. A correction was made for a molybdenum oxide interference on cadmium, the only interference observed.

NRCC Trace Element Analyses: Subsamples (0.25 g) from each of six bottles were placed in polytetrafluoroethylene (PTFE) digestion vessels with nitric acid; the vessels were sealed and heated in a microwave oven. (For the samples intended for ICP-MS analyses, a suitable amount of each enriched isotope solution was added to each sample prior to digestion). The digestion vessels were opened, (H_2O_2 was added to the samples for GFAAS and H_2O_2 and HF were added to the samples for ICP-Atomic Emission Spectrometry (AES), and the contents were evaporated to dryness. The residues were dissolved in nitric acid and double distilled water. The samples were analyzed by ID-ICP-MS for the determination of silver, cadmium, copper, nickel, lead, tin, and zinc. GFAAS was used for determination of silver, arsenic, cadmium, chromium, copper, nickel, and selenium, and ICP-AES was used for the determination of aluminum, iron, and zinc.

Research Centre Jülich Trace Element Analyses: The elements manganese, strontium, iron, zinc, calcium, magnesium, phosphorus, potassium, sodium, sulfur, and barium were determined by ICP-AES after pressure digestion (0.2 g of sample + 2 mL of HNO_3) in PTFE vessels. Aqueous acid-matched standard solutions containing scandium as an internal standard were used for calibration. The elements phosphorus and sulfur were determined without an internal standard. The elements cadmium, lead, and copper were determined in aliquots of corresponding digestion solutions by GFAAS using the method of standard addition [7,8]. ID-TIMS was used for the determination of cadmium, lead, copper, zinc, and thallium in solutions from pressure digestion (0.2 g of sample + 2 mL of HNO_3 + 0.2 mL HF) [9].

For mercury determination by cold vapor atomic absorption spectrometry (CVAAS), a subsample of 0.3 g to 0.5 g of material was digested with 10 mL concentrated nitric acid in heated quartz vessels closed with a cap [10]. The measuring system was calibrated using mercury (II) standard solutions in nitric acid. After high-pressure digestion (HPA) in quartz vessels (0.2 g of sample + 2 mL of HNO_3), cadmium, lead, copper, nickel, and thallium were determined by ICP-MS using aqueous standard solutions for calibration. In aliquots of HPA digestion solutions, electrochemical techniques were used for the determination of lead (differential pulse anodic stripping voltammetry (DPASV)), nickel (adsorptive stripping voltammetry (ADSV)), and selenium (cathodic stripping voltammetry (CSV)) at the hanging mercury drop electrode by standard addition method [11]. Selenium was quantified in HPA digestion and arsenic after open wet digestion (0.2 g of sample + 3 mL of HNO_3) by HG-AAS using aqueous standard solutions for calibration.

Jožef Stefan Institute Trace Element Analyses: Subsamples from each of six bottles of SRM 2977 were analyzed by electrothermal atomic absorption spectrometry (ETAAS), flame atomic absorption spectrometry (FAAS), instrumental neutron activation analysis (INAA), and radiochemical neutron activation analysis (RNAA). For the determination of trace elements by FAAS (iron, manganese, zinc, and copper) and ETAAS (cadmium, lead, and vanadium), subsamples of 300 mg were placed in PTFE Parr bombs with nitric acid and heated at 105 °C for 12 h. After digestion, the samples were equilibrated to room temperature and diluted with double distilled water.

For INAA, subsamples of 150 mg to 200 mg were sealed in plastic containers and irradiated for 20 h at a fluence rate of 1.0×10^{12} $cm^{-2} \cdot s^{-1}$. For the short-lived radionuclides, samples were irradiated for 1 min. The irradiated samples were transferred to clean polyethylene containers and counted after 2, 8, and 30 days. For the short-lived radionuclides, samples

were counted at 2 min after irradiation for 5 min and at 3 h for 30 min. Samples were counted with a germanium detector. For the determination of mercury and selenium by RNAA, subsamples were sealed in quartz ampoules and irradiated for 16 h to 20 h at the fluence rate above. The samples were pyrolyzed resulting in volatilization of the mercury and selenium; selenium was trapped on soda lime and mercury was trapped on selenium-impregnated paper. The gamma activity of the isolated radionuclides was counted with a NaI(Tl) detector.

Methylmercury: The certified value for methylmercury is based on results of analyses of SRM 2977 at NIST and two other laboratories: Institute of Applied Physical Chemistry, Research Centre Jülich, (Jülich, Germany) and the Jožef Stefan Institute (Ljubljana, Slovenia). For the determination of methylmercury, SRM 2977 was analyzed at NIST using solid phase microextraction (SPME) with speciated isotope dilution GC/inductively coupled plasma mass spectrometry (GC/ICPMS). For the speciated isotope dilution GC/ICPMS analyses, approximately 1.0 g to 2.0 g subsamples were spiked with an appropriately diluted sample of IRMM-670 ^{202}Hg enriched methylmercury isotopic CRM and subjected to an alkaline microwave digestion (using 25 % volume fraction tetraammoniumhydroxide in water). Sodium tetraethylborate was used for ethylation. The derivatized methylmercury was back-extracted into isooctane and injected into a GC/ICPMS. The GC analysis used a 30 m × 0.32 mm column with a 100 % dimethylpolysiloxane phase (0.17 µm film thickness) (HP-1, Agilent Technolgies) [12]. At the Research Centre Jülich, the analytical procedure for methylmercury consisted of water steam distillation under acid conditions, anion exchange chromatographic separation of inorganic mercury and methylmercury, followed by CVAAS detection before and after ultraviolet radiation [13-15]. Triplicate subsamples (~ 300 mg) from each of three bottles were analyzed. Three methods were used for the determination of methylmercury at the Jožef Stefan Institute: (1) HCl extraction for 12 h anion exchange chromatographic separation of inorganic mercury and organomercury followed by cold vapor atomic absorption spectrometric detection before and after ultraviolet radiation (IEC-CVAAS) [13,14,16]; (2) H_2SO_4 extraction followed by ethylation at room temperature precollection, GC-pyrolysis with cold vapor atomic fluorescence spectrometric detection (GC-CVAFS) [17-20]; and (3) solid-liquid extraction into toluene followed by GC-ECD [16,19,21]. Six subsamples (200 mg to 500 mg) from one bottle of SRM 2977 were analyzed for each of the three analytical techniques and a subsample (500 mg) from each of six bottles of SRM 2977 was analyzed by one technique (GC-ECD).

Table 1. Certified Concentrations for Selected PAHs in SRM 2977

	Mass Fraction µg/kg (dry mass basis)		
Fluorene[b,c,d]	10.30	±	0.13[a]
Phenanthrene[b,c,d]	36.2	±	2.5[a]
1-Methylphenanthrene[b,c,d]	39.0	±	1.9[e]
Fluoranthene[b,c,d]	38.90	±	0.63[e]
Pyrene[b,c,d]	77.4	±	2.1[a]
Benz[*a*]anthracene[b,c,d]	20.19	±	0.87[a]
Benzo[*b*]fluoranthene[b,c,d]	11.10	±	0.50[e]
Benzo[*j*]fluoranthene[b,c,d]	4.48	±	0.15[a]
Benzo[*e*]pyrene[b,c,d]	13.29	±	0.43[a]
Benzo[*a*]pyrene[b,c,d]	5.30	±	0.61[a]
Perylene[b,c,d]	3.69	±	0.38[e]
Benzo[*ghi*]perylene[b,c,d]	9.45	±	0.37[e]
Indeno[1,2,3-*cd*]pyrene[b,c,d]	4.76	±	0.15[a]

[a] The certified value is a weighted mean of the results from three analytical methods [22]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence) calculated by combining a between-method variance incorporating inter-method bias with a pooled, within-method variance following the ISO and NIST Guides [23].

[b] GC/MS I

[c] GC/MS II

[d] Results from up to 12 laboratories participating in an interlaboratory comparison exercise.

[e] The certified value is an unweighted mean of the results from three analytical methods. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence), calculated by combining a between-method variance [24] with a pooled, within-method variance following the ISO and NIST Guides [23].

Table 2. Certified Concentrations for Selected PCB Congeners[a] in SRM 2977

			Mass Fraction µg/kg (dry mass basis)		
PCB	8	(2,4′-Dichlorobiphenyl)[c,d,e]	1.99	±	0.14[b]
PCB	28	(2,4,4′-Trichlorobiphenyl)[c,d,e]	5.17	±	0.36[f]
PCB	31	(2,4′,5-Trichlorobiphenyl)[c,d,e]	3.86	±	0.29[f]
PCB	44	(2,2′3,5′-Tetrachlorobiphenyl)[c,d,e]	3.22	±	0.21[f]
PCB	49	(2,2′,4,5′-Tetrachlorobiphenyl)[c,d,e]	2.44	±	0.27[b]
PCB	52	(2,2′,5,5′-Tetrachlorobiphenyl)[c,d,e]	8.02	±	0.56[b]
PCB	66	(2,3′,4,4′-Tetrachlorobiphenyl)[c,d,e]	3.55	±	0.18[f]
PCB	95	(2,2′,3,5′,6-Pentachlorobiphenyl)[c,d,e]	5.17	±	0.53[b]
PCB	101	(2,2′,4,5,5′-Pentachlorobiphenyl)[c,d,e]	10.6	±	0.9[b]
PCB	118	(2,3′,4,4′,5-Pentachlorobiphenyl)[c,d,e]	10.0	±	0.41[b]
PCB	128	(2,2′,3,3′,4,4′-Hexachlorobiphenyl)[c,d,e]	2.38	±	0.28[b]
PCB	138	(2,2′,3,4,4′,5′-Hexachlorobiphenyl)[c,d,e]	7.94	±	0.63[f]
PCB	149	(2,2′,3,4′,5′,6-Hexachlorobiphenyl)[c,d,e]	8.95	±	0.67[b]
PCB	153	(2,2′,4,4′,5,5′-Hexachlorobiphenyl)[c,d,e]	14.1	±	1.3[b]
PCB	156	(2,3,3′,4,4′,5-Hexachlorobiphenyl)[c,d,e]	0.959	±	0.036[f]
PCB	170	(2,2′,3,3′,4,4′,5-Heptachlorobiphenyl)[c,d,e]	2.74	±	0.25[b]
PCB	180	(2,2′,3,4,4′,5,5′-Heptachlorobiphenyl)[c,d,e]	6.32	±	0.72[b]
PCB	187	(2,2′,3,4′,5,5′6-Heptachlorobiphenyl)[c,d,e]	4.47	±	0.32[b]
PCB	194	(2,2′,3,3′,4,4′,5,5′-Octachlorobiphenyl)[c,d,e,f]	0.881	±	0.032[f]

[a] PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell [25] and later revised by Schulte and Malisch [26] to conform with IUPAC rules; for the specific congeners mentioned in this SRM, the Ballschmiter-Zell numbers correspond to those of Schulte and Malisch. When two or more congeners are known to coelute under the GC analysis conditions used, the PCB congener listed first is the major component and the additional congeners may be present as minor components. The quantitative results are based on the response of the congener listed first.

[b] The certified value is a weighted mean of the results from three analytical methods [22]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence) calculated by combining a between-method variance incorporating inter-method bias with a pooled, within-method variance following the ISO and NIST Guides [23].

[c] GC/MS I

[d] GC/MS II

[e] Results from up to 12 laboratories participating in an interlaboratory comparison exercise.

[f] The certified value is an unweighted mean of the results from three analytical methods. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence), calculated by combining a between-method variance [24] with a pooled, within-method variance following the ISO and NIST Guides [23].

Table 3. Certified Concentrations for Selected Chlorinated Pesticides in SRM 2977

	Mass Fraction µg/kg (dry mass basis)
trans-Nonachlor[b,c,d]	1.25 ± 0.17[a]
Dieldrin[b,c,d]	5.55 ± 0.61[a]
4,4′-DDE[b,c,d]	11.8 ± 1.2[e]
2,4′-DDD[b,c,d]	3.15 ± 0.25[e]
4,4′-DDD[b,c,d]	3.92 ± 0.56[a]
4,4′-DDT[b,c,d]	1.32 ± 0.16[a]

[a] The certified value is a weighted mean of the results from three analytical methods [22]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence) calculated by combining a between-method variance incorporating inter-method bias with a pooled, within-method variance following the ISO and NIST Guides [23].
[b] GC/MS I
[c] GC/MS II
[d] Results from up to 12 laboratories participating in an interlaboratory comparison exercise.
[e] The certified value is an unweighted mean of the results from three analytical methods. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence), calculated by combining a between-method variance [24] with a pooled, within-method variance following the ISO and NIST Guides [23].

Table 4. Certified Concentrations for Selected BDE Congeners[a] in SRM 2977

			Mass Fraction µg/kg (dry mass basis)
BDE	28	(2,4,4′-Tribromodiphenyl ether)[c,d,e]	2.54 ± 0.40[b]
	33	(2′,3,4-Tribromodiphenyl ether)	
BDE	47	(2,2′,4,4′-Tetrabromodiphenyl ether)[c,d,e]	36.5 ± 4.0[b]
BDE	49	(2,2′,4,5′-Tetrabromodiphenyl ether)[c,d,e]	1.20 ± 0.19[b]
BDE	66	(2,3′,4,4′-Tetrabromodiphenyl ether)[c,d,e]	0.453 ± 0.046[b]

[a] BDE congeners are numbered according to IUPAC rules. When two or more congeners are known to coelute under the GC analysis conditions used, the BDE congener listed first is the major component and the additional congeners may be present as minor components. The quantitative results are based on the response of the congener listed first.
[b] The certified value is a weighted mean of the results from three analytical methods [22]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence) calculated by combining a between-method variance incorporating inter-method bias with a pooled, within-method variance following the ISO and NIST Guides [23].
[c] GC/MS III
[d] GC/MS IV [5]
[e] Results from up to 12 laboratories participating in an interlaboratory comparison exercise.

Table 5. Certified Concentrations for Selected Inorganic Constituents in SRM 2977

Element	Degrees of Freedom	Mass Fraction mg/kg (dry mass basis)[a]
Cadmium[b,c,d,e,f,g,h]	4	0.179 ± 0.003
Copper[b,c,d,e,f,g,h,j]	5	9.42 ± 0.52
Lead[b,c,d,e,f,g,h,k,l]	4	2.27 ± 0.13
Manganese[b,i,m]	2	23.93 ± 0.29
Nickel[b,c,d,e,g,n]	4	6.06 ± 0.24
Strontium[b,i,m]	2	69.3 ± 4.2

[a] The results are expressed as the certified value ± the expanded uncertainty. The certified value is the mean of three to six results from the following: (1) the mean of ICP-MS analyses performed at NIST; (2) the mean of ID-ICP-MS analyses performed at NRCC; (3) the mean of GFAAS analyses performed at NRCC; (4) the mean of results from six selected laboratories participating in the NRCC intercomparison exercise; (5) the mean of results from analyses by HGAAS, GFAAS, ICP-MS, ID-TIMS, ADSV, and/or DPASV performed at Research Centre Jülich; and (6) the mean of results from analyses by INAA, GFAAS, and FAAS performed at Jožef Stefan Institute. The expanded uncertainty in the certified value is equal to $U = ku_c$, where u_c is the combined standard uncertainty calculated according to the ISO and NIST Guides [23] and k is the coverage factor. The value of u_c is intended to represent, at the level of one standard deviation, the combined effect of all the uncertainties in the certified value. Here u_c is given by the standard error of the mean of the available values.
[b] Measured at NIST using ICP-MS.
[c] Measured at NRCC using ID-ICP-MS.
[d] Measured at NRCC using GFAAS.
[e] Measured by six laboratories as part of the NRCC interlaboratory comparison exercise.
[f] Measured at Research Centre Jülich using GFAAS
[g] Measured at Research Centre Jülich using ICP-MS.
[h] Measured at Research Centre Jülich using ID-TIMS.
[i] Measured at Jožef Stefan Institute using INAA.
[j] Measured at Jožef Stefan Institute using FAAS.
[k] Measured at Jožef Stefan Institute using GFAAS.
[l] Measured at Research Centre Jülich using DPASV.
[m] Measured at Research Centre Jülich using ICP-AES.
[n] Measured at Research Centre Jülich using ADSV.

Table 6. Certified Concentration of Methylmercury in SRM 2977

	Mass Fraction μg/kg (dry mass basis)
Methylmercury[a,b]	36.6 ± 1.0

[a] Results for methylmercury are reported as μg/kg mercury.
[b] The certified value is a weighted mean of the results from three analytical methods [22]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence) calculated by combining a between-method variance incorporating inter-method bias with a pooled, within-method variance following the ISO and NIST Guides [23].

Table 7. Reference Concentrations for Selected PAHs, PCB Congeners, Chlorinated Pesticides, and BDE Congeners in SRM 2977

	Mass Fraction μg/kg (dry mass basis)
Naphthalene[b,c,d]	21.1 ± 1.4[a]
1-Methylnaphthalene[b,c,d]	15.6 ± 1.5[e]
2-Methylnaphthalene[b,c,d]	17.3 ± 1.7[e]
Biphenyl[b,c,d]	6.0 ± 1.3[a]
Acenaphthene[b,c,d]	4.9 ± 1.2[a]
Anthracene[b,c,d]	6.2 ± 1.4[a]
Chrysene[b,c,d]	42.2 ± 5.5[a]
Triphenylene[b,c,d]	36.1 ± 2.4[a]
Benzo[*k*]fluoranthene[b,c,d]	4.02 ± 0.75[a]
Dibenz[*a*,*h*]anthracene[b,c,d]	1.47 ± 0.33[a]
PCB 18[f] (2,2′,5-Trichlorobiphenyl) [b,c,d]	2.24 ± 0.74[a]
PCB 99[f] (2,2′,4,4′,5-Pentachlorobiphenyl) [b,c,d]	3.0 ± 1.2[a]
PCB 105[f] (2,3,3′,4,4′-Pentachlorobiphenyl) [b,c,d]	2.93 ± 0.46[a]
cis-Chlordane (α-Chlordane) [b,c,d]	1.14 ± 0.39[a]
trans-Chlordane (γ-Chlordane) [b,c,d]	2.01 ± 0.39[a]
BDE 17[f] (2,2′,4-Tribromodiphenyl ether) [d,g,h]	1.04 ± 0.19[a]
BDE 99[f] (2,2′,4,4′,5-Pentabromodiphenyl ether) [d,g,h]	4.68 ± 0.92[e]
BDE 100[f] (2,2′,4,4′,6-Pentabromodiphenyl ether) [d,g,h]	1.82 ± 0.64[a]
BDE 153[f] (2,2′,4,4′,5,5′-Hexabromodiphenyl ether) [d,g,h]	0.16 ± 0.04[e]
BDE 154[f] (2,2′,4,4′,5,6′-Hexabromodiphenyl ether) [d,g,h]	0.20 ± 0.09[e]

[a] The reference value is a weighted mean of the results from three analytical methods [22]. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence) calculated by combining a between-method variance incorporating inter-method bias with a pooled, within-method variance following the ISO and NIST Guides [23].

[b] GC/MS I

[c] GC/MS II

[d] Results from up to 12 laboratories participating in an interlaboratory comparison exercise.

[e] The reference value is an unweighted mean of the results from three analytical methods. The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (approximately 95 % confidence), calculated by combining a between-method variance [24] with a pooled, within-method variance following the ISO and NIST Guides [23].

[f] PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell [25] and later revised by Schulte and Malisch [26] to conform with IUPAC rules; for the specific congeners mentioned in this SRM, the Ballschmiter-Zell numbers correspond to those of Schulte and Malisch. BDE congeners are numbered according to IUPAC rules.

[g] GC/MS III

[h] GC/MS IV [5]

Table 8. Reference Concentrations for Selected Inorganic Constituents in SRM 2977 as Determined by Multiple Laboratories

Element	Degrees of Freedom	Mass Fraction mg/kg (dry mass basis)[a]
Arsenic[b,c,d,e]	3	8.83 ± 0.91
Chromium[b,c,d]	2	3.91 ± 0.47
Cobalt[c,f,g]	2	0.48 ± 0.13
Iron[b,c,d,h]	4	274 ± 18
Mercury[d,i,j,k,l]	3	0.101 ± 0.004
Selenium[b,c,d,e,k,m]	3	1.78 ± 0.16
Silver[b,c,d,n]	3	4.58 ± 0.33
Tin[d,n]	5	1.47 ± 0.27
Zinc[b,c,d,h,n,p]	4	135 ± 5

[a] The results are expressed as the reference value ± the expanded uncertainty. The reference value is the mean of three to five values from the following values: (1) the mean of ID-ICP-MS analyses performed at NIST; (2) the mean of ID-ICP-MS analyses performed at NRCC; (3) the mean of GFAAS or ICP-AES analyses performed at NRCC; (4) the mean of results from five or six selected laboratories participating in the NRCC intercomparison exercise; (5) the mean of results from analyses by CSV, HGAAS, ICP-AES, and/or ID-TIMS performed at Research Centre Jülich; and (6) the mean of results from analyses by CVAAS, INAA, and/or RNAA performed at Ljubljana. The expanded uncertainty in the certified value is equal to $U = ku_c$ where u_c is the combined standard uncertainty calculated according to the ISO and NIST Guides [23] and k is the coverage factor. The value of u_c is intended to represent at the level of one standard deviation the combined effect of all the uncertainties in the certified value. Here u_c is given by the standard error of the mean of the available values. The coverage factor, k, is the Student's t-value for a 95 % confidence interval with four degrees of freedom.

[b] Measured at NRCC using GFAAS or ICP-AES.

[c] Measured at Jožef Stefan Institute using INAA.

[d] Measured by five or six laboratories as part of the NRCC interlaboratory comparison exercise.

[e] Measured at Research Centre Jülich using HGAAS.

[f] Measured at NIST using ICP-MS.

[g] Measured at Research Centre Jülich using ICP-MS.

[h] Measured at Research Centre Jülich using ICP-AES.

[i] Measured at NRCC using CVAAS.

[j] Measured at Research Centre Jülich using CVAAS.

[k] Measured at Jožef Stefan Institute using RNAA.

[l] Measured at Jožef Stefan Institute using CVAAS.

[m] Measured at Research Centre Jülich using CSV.

[n] Measured at NRCC using ID-ICP-MS.

[o] The reference value for tin is the mean of the results from NRCC using ID-ICP-MS and the individual results from five laboratories participating in the NRCC interlaboratory exercise (n = 6).

[p] Measured at Research Centre Jülich using ID-TIMS.

Table 9. Information Values for the Concentrations for Selected Inorganic Constituents in SRM 2977

Element	Mass Fraction
	% (dry mass basis)
Calcium[a,b]	0.83
Chlorine[b]	4.3
Magnesium[a,b]	3.9
Phosphorus[a]	1.1
Potassium[a,b]	1.2
Sodium[a,b]	2.4
Sulfur[a,b]	2.9
	mg/kg (dry mass basis)
Aluminum[b,c]	400
Antimony[b]	0.048
Barium[a]	4.7
Bromine[b]	215
Cerium[b]	0.93
Cesium[b]	0.039
Gold[b]	0.013
Iodine[b]	26
Lanthanum[b]	0.44
Rubidium[b]	6.7
Samarium[b]	0.064
Scandium[b]	0.055
Thorium[b]	0.19
Uranium[b]	0.083
Vanadium[b]	1.1
	µg/kg (dry-mass basis)
Thallium[d,e]	10.2

[a] Measured at Research Centre Jülich using ICP-AES.
[b] Measured at Jožef Stefan Institute using INAA.
[c] Measured at NRCC using ICP-AES.
[d] Measured at Research Centre Jülich using ICP-MS.
[e] Measured at Research Centre Jülich using ID-TIMS.

Table 10. Analytical Methods Used for the Analysis of SRM 2977 for Inorganic Constituents

Elements	Analytical Methods
Aluminum	ICP-AES, INAA
Antimony	INAA
Arsenic	CSV, GFAAS, HGAAS, ICP-AES, ICP-MS, INAA
Barium	ICP-AES
Bromine	INAA
Cadmium	FAAS, GFAAS, ICP-MS, ICP-AES, ID-ICP-MS, ID-TIMS
Calcium	INAA, ICP-AES
Cerium	INAA
Cesium	INAA
Chlorine	INAA
Chromium	GFAAS, ICP-MS, INAA
Cobalt	ICP-MS, INAA, RNAA
Copper	FAAS, GFAAS, ICP-AES, ICP-MS, ID-ICP-MS, ID-TIMS
Iodine	INAA
Iron	FAAS, ICP-AES, ICP-MS, INAA
Lanthanum	INAA
Lead	DPASV, GFAAS, ICP-MS, ID-TIMS, XRF
Magnesium	ICP-AES, INAA
Manganese	FAAS, ICP-AES, ICP-MS, INAA
Mercury	CVAAS, ICP-MS INAA, RNAA
Nickel	ADSV GFAAS, ICP-AES, ICP-MS
Potassium	ICP-AES, INAA
Rubidium	INAA
Samarium	INAA
Scandium	INAA
Selenium	CSV, GFAAS, HGAAS, ICP-MS, INAA, RNAA
Silver	GFAAS, ID-ICP-MS, ICP-MS, INAA
Sodium	ICP-AES, INAA
Strontium	ICP-MS, HG-AAS, INAA
Sulfur	ICP-AES, INAA
Thallium	ICP-MS, ID-TIMS, INAA
Tin	GFAAS, ICP-AES, ICP-MS, ID-ICP-MS
Thorium	INAA
Uranium	INAA
Vanadium	INAA
Zinc	FAAS, ICP-AES, ICP-MS, ID-ICP-MS, ID-TIMS, XRF, INAA

Methods	
ADSV	Adsorptive stripping voltammetry
CSV	Cathodic stripping voltammetry
CVAAS	Cold vapor atomic absorption spectrometry
DPASV	Differential pulse anodic stripping voltammetry
ETAAS	Electrothermal atomic absorption spectrometry
FAAS	Flame atomic absorption spectrometry
GFAAS	Graphite furnace atomic absorption spectrometry
HGAAS	Hydride generation atomic absorption spectrometry
ICP-AES	Inductively coupled plasma atomic emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
ID-ICP-MS	Isotope dilution inductively coupled plasma mass spectrometry
ID-TIMS	Isotope dilution thermal ionization mass spectrometry
INAA	Instrumental neutron activation analysis
RNAA	Radiochemical neutron activation analysis
XRF	X-ray fluorescence spectrometry

REFERENCES

[1] Calder, J.A.; Jamieson, W.D.; *The Purpose and Program of the IOC/UNEP/IAEA Group of Experts on Standards and Reference Materials*; Fresenius J. Anal. Chem., Vol. 338, pp. 378-379 (1990).

[2] Wise, S.A.; Poster, D.L.; Kucklick, J.R.; Keller, J.M.; VanderPol, S.S.; Sander, L.C.; Schantz, M.M.; *Standard Reference Materials (SRMs) for Determination of Organic Contaminants in Environmental Samples*; Anal. Bioanal. Chem., Vol. 386, pp. 1153-1190 (2006).

[3] Schantz, M.M.; Nichols, J.J.; Wise, S.A.; *Evaluation of Pressurized Fluid Extraction for the Extraction of Environmental Matrix Reference Materials*; Anal. Chem., Vol 69, pp. 4210-4219 (1997).

[4] Schantz, M.M.; Kucklick, J.R.; Parris, R.M.; Poster, D.L.; Wise, S.A.; *NIST Intercomparison Exercise Program for Organic Contaminants in the Marine Environment: Description and Results of 2005 Organic Intercomparison Exercises*; NISTIR 7340; National Institute of Standards and Technology, U.S. Department of Commerce: Gaithersburg, MD (2006).

[5] Zhu, L.Y.; Hites, R.A.; *Determination of Polybrominated Diphenyl Ethers in Environmental Standard Reference Materials*; Anal. Chem., Vol. 75, pp. 6696-6700 (2003).

[6] Willie, S.; *NOAA National Status and Trends Program Twelfth Round Intercomparison Exercise Results for Trace Metals in Marine Sediments and Biological Tissues*; NOAA Technical Memorandum NOS ORCA 137, p. 51, Silver Spring, MD (1998).

[7] Emons, H.; *Environmental Specimen Banking - Aspects of Metal Determination and Distribution*; Fresenius J. Anal. Chem., Vol 354, pp. 507-510 (1996).

[8] Rossbach, M.; Emons, H.; Hoppstock, K.; Ostapczuk, P.; Schladot, J.D.; *Intermethod Comparison for Quality Control at the Environmental Specimen Bank (ESB) of the Federal Republic of Germany*; Acta Chim. Slov., Vol. 44, pp. 213-223 (1997).

[9] Waidmann, E.; Emons, H.; Dürbeck, H.W.; *Trace Determination of Tl, Cu, Pb, Cd and Zn in Specimens of the Limnic Environment using Isotope Dilution Mass Spectrometry with Thermal Ionization*; Fresenius J. Anal. Chem., Vol. 350, pp. 293-297 (1994).

[10] May, K.; Stoeppler, M.; *Pretreatment Studies with Biological and Environmental Materials, IV. Complete Wet Digestion in Partly and Completely Closed Quartz Vessels for Subsequent Trace and Ultratrace Mercury Determinations*; Fresenius Z. Anal. Chem., Vol. 317, pp. 248-251 (1984).

[11] Emons, H.; Ostapzcuk, P.; *Electroanalysis for the Purpose of Environmental Monitoring and Specimen Banking - Is There a Future?*; Analyst, Vol. 121, pp. 1917-1921 (1996).

[12] Davis, W.C.; Vander Pol, S.S.; Schantz, M.M.; Long, S.E.; Christopher, S.J.; *An Accurate and Sensitive Method for the Determination of Methyl Mercury in Biological Specimens Using GC-ICPMS with Solid Phase Microextraction*; J. Anal. At. Spectrom., Vol. 19, pp. 1546-1551 (2004).

[13] May, K.; Stoeppler, M.; Reisinger, K.; *Studies of the Ratio of Total Mercury/Methylmercury in the Aquatic Food Chain*; Toxicol. Environ. Chem., Vol. 13, pp. 153-159 (1987).

[14] Ahmed, R.; May, K.; Stoeppler, M.; *Ultratrace Analysis of Mercury and Methylmercury (MM) in Rain Water using Cold Vapour Atomic Absorption Spectrometry*; Fresenius Z. Anal. Chem., Vol. 326, pp. 510-516 (1987).

[15] Padberg, S.; Burow, M.; Stoeppler, M.; *Methylmercury Determination in Environmental and Biological Reference and Other Materials by Quality Control with Certified Reference Materials*; Fresenius J. Anal. Chem., Vol. 346, pp. 686-688 (1993).

[16] Horvat, M.; May, K.; Stoeppler, M.; Byrne, A.R.; *Comparative Studies of Methylmercury Determination in Biological and Environmental Samples*; Appl. Organomet. Chem., Vol. 2, pp. 515-524 (1988).

[17] Horvat, M.; Liang, L.; Bloom, N.S.; *Comparison of Distillation with Other Current Isolation Methods for the Determination of Methylmercury Compounds in Low Level Environmental Samples, Part 1: Sediments*; Anal. Chim. Acta., Vol. 281, pp. 135-152 (1993).

[18] Horvat, M.; Liang, L.; Bloom, N.S.; *Comparison of Distillation with Other Current Isolation Methods for the Determination of Methylmercury Compounds in Low Level Environmental Samples, Part 2: Water*; Anal. Chim. Acta., Vol.282, pp. 153-168 (1993).

[19] Horvat, M.; Byrne, A.R.; May, K.; *Rapid Quantitative Separation and Determination of Methylmercury by Gas Chromatography*; Talanta, Vol. 37, pp. 207-212 (1989).

[20] Liang, L.; Horvat, M.; Bloom, N.S.; *An Improved Method for Speciation of Mercury by Aqueous Phase Ethylation, Room Temperature Precollection, GC Separation and CV AFS Detection*; Talanta, Vol. 41, pp. 371-379 (1994).

[21] Akagi, H.; Nishimura, H.; *Speciation of Mercury in the Environment*; In: Advances in Mercury Toxicology, Suzuki, T.; Imura, N.; Clarkson, T.W., Eds., pp. 53-76, Plenum Press, New York (1992).

[22] Ruhkin, A.L.; Vangel, M.G.; *Estimation of a Common Mean and Weighted Means Statistics*; J Am Statist Assoc, Vol. 93, pp. 303–308 (1998).

[23] ISO; G*uide to the Expression of Uncertainty in Measurement*; ISBN 92-67-10188-9, 1st ed.; International Organization for Standardization: Geneva, Switzerland (1993); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at http://physics.nist.gov/Pubs/.

[24] Levenson, M.S.; Banks, D.L.; Eberhardt, K.R.; Gill, L.M.; Guthrie, W.F.; Liu, H.K.; Vangel, M.G.; Yen, J.H.; Zhang, N.F.; *An Approach to Combining Results From Multiple Methods Motivated by the ISO GUM*; J. Res. Natl. Inst. Stand. Technol., Vol. 105, pp. 571–579 (2000).

[25] Ballschmiter, K.; Zell, M.; *Analysis of Polychlorinated Biphenyls (PCB) by Glass Capillary Gas Chromatography - Composition of Technical Aroclor- and Clophen-PCB Mixtures*; Fresenius Z. Anal. Chem., Vol. 302, pp. 20-31 (1980).

[26] Schulte, E.; Malisch, R.; *Calculation of the Real PCB Content in Environmental Samples. I. Investigation of the Composition of Two Technical PCB Mixtures*; Fresenius Z. Anal. Chem., Vol. 314, pp. 545-551 (1983).

Certificate Revision History: 12 September 2008 (Updated existing certified and reference values for PAHs, PCBs, pesticides and methylmercury; added certified and reference values for PBDEs and for additional PAHs and PCBs; extension of certification period); 01 May 2000 (Original certificate date).

Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 1974c

Organics in Mussel Tissue (*Mytilus edulis*)

This Standard Reference Material (SRM) is a frozen mussel tissue homogenate intended for use in evaluating analytical methods for the determination of selected polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCB) congeners, chlorinated pesticides, and polybrominated diphenyl ether (PBDE) congeners in marine bivalve mollusk tissue and similar matrices. All of the constituents for which certified and reference values are provided in SRM 1974c were naturally present in the tissue material before processing. A unit of SRM 1974c consists of five jars each containing approximately 10 g (wet basis) of frozen tissue homogenate.

Certified Mass Fraction Values: Certified mass fraction values for 22 PAHs, 38 PCB congeners, 11 chlorinated pesticides, and 5 PBDE congeners are provided in Tables 1 to 4. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [1]. The certified values are based on the agreement of results obtained at NIST using multiple analytical techniques. The measurand is the total mass fraction of each analyte listed in Tables 1 to 4. Metrological traceability is to the SI unit for mass (expressed as micrograms per kilogram).

Reference Mass Fraction Values: Reference mass fraction values are provided in Tables 5 to 7 for an additional 18 PAHs, 14 PCB congeners, and 2 chlorinated pesticides, respectively. A NIST reference value is a non-certified value that is the best estimate of the true value; however, the value does not meet the NIST criteria for certification and is provided with an associated uncertainty that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods [1]. The measurand is the mass fraction of each analyte listed in Tables 5 to 7 as determined by the methods used. Metrological traceability is to the SI unit for mass (expressed as micrograms per kilogram).

Expiration of Certification: The certification of **SRM 1974c** is valid, within the measurement uncertainty specified, until **30 September 2022**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Handling, Storage, and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Overall direction and coordination of technical measurements leading to certification were performed by M.M. Schantz and L.C. Sander of the NIST Chemical Sciences Division.

Preparation of the material was performed by G. Ballihaut, P.R. Becker, W.C. Davis, M.B. Ellisor, J. Hoguet, A.J. Moors, B.J. Porter, R.S. Pugh, L.B. Rust, and J.M. Yordy of the NIST Chemical Sciences Division.

Analytical measurements were performed by M.M. Schantz and S.S. Van der Pol of the NIST Chemical Sciences Division.

Statistical analyses of the certification data were performed by N.A. Heckert and A.L. Pintar of the NIST Statistical Engineering Division.

Carlos A. Gonzalez, Chief
Chemical Sciences Division

Gaithersburg, MD 20899
Certificate Issue Date: 05 November 2015
Certificate Revision History on Last Page

Robert L. Watters, Jr., Director
Office of Reference Materials

Support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

NOTICE TO USERS: SRM 1974c IS INTENDED FOR LABORATORY USE ONLY, NOT FOR HUMAN CONSUMPTION.

INSTRUCTIONS FOR HANDLING, STORAGE, AND USE

Storage: The SRM should be stored at –80 °C (or lower). Extended storage at temperatures of –25 °C or higher, or if allowed to warm, the tissue homogenate will lose its powder-like form.

Handling: For the handling of this material during sample preparation, the following procedures and precautions are recommended:

- If weighing relatively large quantities (≥3 g), remove a portion from the jar and reweigh the jar to determine the weight of the subsample. (Avoid heavy frost buildup by handling the jars rapidly and wiping them prior to weighing.)
- For weighing smaller quantities, transfer subsamples to a pre-cooled thick-walled glass container rather than a thin-walled plastic container to minimize heat transfer to the sample.
- If possible, use a cold work space, e.g., an insulated container with dry ice or liquid nitrogen coolant on the bottom and pre-cooled implements, such as Teflon-coated spatulas, for transferring the powder.
- If the material has been previously thawed and is no longer powder-like, allow the sample to completely thaw, stir well, and use the contents of the entire jar for analysis.

Use: Subsamples of this SRM for analysis (minimum of 3 g) should be withdrawn from the jar immediately after opening and used without delay for the certified values listed in Tables 1 to 4 to be valid within the stated uncertainties. The mass fractions of constituents in SRM 1974c are reported on both a wet-mass and a dry-mass basis for user convenience. The SRM tissue homogenate, as received, contains approximately 90 % moisture. A separate subsample of the SRM should be removed from the jar at the time of analysis and dried to determine the concentration on a dry-mass basis (see "Conversion to Dry-Mass Basis").

PREPARATION AND ANALYSIS[1]

Sample Collection and Preparation: The mussels (*Mytilus edulis*) used for the preparation of SRM 1974c were collected in Dorchester Bay, MA in 2004 by TDI-Brooks International, College Station, TX. The mussels were frozen and delivered to NIST (Hollings Marine Laboratory, Charleston, SC) where they were stored in a liquid nitrogen (LN_2) vapor-phase freezer at –150 °C. For processing, the mussels were allowed to warm to approximately 0 °C, shells were opened, and the tissue removed using titanium knives. Approximately 70 kg of mussel tissue was stored in Teflon bags in an LN_2 vapor-phase freezer (–150 °C) until homogenization. For homogenization, the frozen mussel material was removed from the Teflon bags, placed in a pre-frozen Teflon smasher, and crushed into smaller pieces using a manual smashing device and/or a compressed-air smashing device. The frozen, crushed mussel material was then immediately placed back in an LN_2 vapor-phase freezer (–150 °C) and divided among four stainless steel buckets within the freezer. The Palla VM-KT Vibrating Cryomill (KHD Humboldt Wedag GmbH, Cologne, Germany) was cooled allowing LN_2 to flow through the mill until a temperature of –180 °C was reached. The LN_2 was shut off and the crushed mussel tissue from all 4 buckets was processed through the cryomill until a fresh, frozen powder was created. This procedure was repeated four times prior to bottling to ensure the mussel material was completely blended. Subsamples (approximately 10 g) of the frozen mussel powder homogenate were aliquoted into cleaned, pre-cooled glass jars within an LN_2 vapor-phase freezer (–150 °C) and the glass jars were then stored in –80 °C upright mechanical freezers.

Conversion to Dry-Mass Basis: Sixteen samples were analyzed for moisture using an automated moisture/solids microwave analysis system (CEM, Matthews, NC). Each sample was approximately 1 g of material; the automated moisture determination temperature maximum was set to 105 °C and the power was set to 100 %. A sample was determined to have reached dry mass when the mass of the sample had not changed more than 0.1 mg in 10 s. The moisture content at the time of the certification analyses was 89.75 % ± 0.08 % (expanded uncertainty at a 95 % confidence level for 16 samples with a standard deviation of 0.0015). Analytical results for the constituents were determined on a wet-mass basis and then converted to a dry-mass basis by dividing by the conversion factor of 0.1025 (grams dry mass per gram wet mass). The uncertainty component for the conversion factor obtained from the

[1] Certain commercial equipment, instrumentation, or materials are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

moisture measurements is incorporated in the uncertainties of the certified and reference values using the methods of reference 6, reported on a dry-mass basis, that are provided in this certificate.

PAHs, PCBs, Chlorinated Pesticides, and PBDEs: Value assignments of the PAHs, PCBs, chlorinated pesticides, and PBDEs in SRM 1974c consisted of combining results from analyses using various combinations of different extraction techniques, cleanup/isolation procedures, and chromatographic separation and detection techniques. Two sets of gas chromatography/mass spectrometry (GC/MS) analysis methods, designated as GC/MS (I) and GC/MS (II), were used at NIST.

For GC/MS (I) analyses, duplicate test portions of approximately 3 g from each of 10 jars of SRM 1974c were mixed with diatomaceous earth (Hydromatrix, Restek, Bellefonte, PA) in glass extraction thimbles. The mixtures were extracted using Soxhlet extraction with hexane:acetone (1:1 volume fraction) for 20 h. The extract was fractionated using two aminopropyl solid-phase extraction (SPE) columns to isolate the fraction of interest. The processed extract was then analyzed by GC/MS using a 0.25 mm i.d. × 60 m fused silica capillary column with a 50 % (mole fraction) phenyl methylpolysiloxane phase (0.25 μm film thickness; DB-17MS, Agilent Technologies, Wilmington, DE). The PAHs, PCBs, and pesticides were analyzed on the DB-17MS column using electron impact MS (EI-MS), method GC/MS (Ia). The PBDEs were analyzed on a 0.25 mm × 15 m fused silica capillary column containing a 5 % phenyl methylsubstituted polysiloxane phase (Restek), 0.25 μm film thickness using negative chemical ionization MS (NCI-MS), method GC/MS (Ib).

For the GC/MS (II) analyses, a 9 g sample from each of six jars was extracted using pressurized-fluid extraction (PFE) with dichloromethane (DCM). The fraction of interest was first isolated using an alumina (5 % deactivated) SPE column. Size exclusion chromatography (SEC) on a divinylbenzene-polystyrene column (10 μm particle size, 10 nm (100 Å) pore size, 7.5 mm × 300 mm i.d. PLGel column, Polymer Labs, Inc., Amherst, MA) was used to remove the majority of the remaining lipid and biogenic material. The processed extract was then analyzed by GC/MS using a 0.18 mm i.d. × 30 m fused silica capillary column with a low-bleed, low-polarity phase (0.18 μm film thickness; DB-XLB, Agilent Technologies, Wilmington, DE). The PAHs, PCBs, PBDEs, and certain pesticides were analyzed on the DB-XLB column using EI-MS, method GC/MS (IIa). The remaining pesticides were analyzed on the same capillary column using NCI-MS, method GC/MS (IIb). For the methods described above, selected perdeuterated PAHs, carbon-13 labeled PCB congeners, chlorinated pesticides, and PBDE congeners, and fluorinated PBDE congeners were added to the mussel tissue prior to extraction for use as internal standards for quantification purposes.

Homogeneity Assessment for PAHs, PCBs, Chlorinated Pesticides, and PBDEs: The homogeneity of SRM 1974c was assessed by analyzing duplicate test portions of 3 g from 10 jars selected by stratified random sampling. Test portions were processed and analyzed as described above for GC/MS (I). No differences among jars were observed for the PAHs, PCBs, chlorinated pesticides, or PBDEs for a 3 g test portion size.

Table 1. Certified Mass Fraction Values for Selected PAHs in SRM 1974c

	Mass Fraction[a,b,c] (μg/kg)						*k*
	Wet-Mass Basis			Dry-Mass Basis			
Fluorene	2.31	±	0.04	22.6	±	0.4	1.97
Dibenzothiophene	1.53	±	0.02	15.0	±	0.2	1.99
Phenanthrene	19.6	±	0.4	191	±	4	1.96
Anthracene	1.17	±	0.08	11.4	±	0.8	1.97
1-Methylphenanthrene	3.07	±	0.11	30.0	±	1.1	1.97
2-Methylphenanthrene	4.56	±	0.04	44.5	±	0.5	1.97
3-Methylphenanthrene	4.09	±	0.03	39.9	±	0.4	1.97
9-Methylphenanthrene	2.46	±	0.02	24.0	±	0.3	1.97
2-Methylanthracene	0.951	±	0.007	9.3	±	0.1	1.97
Fluoranthene	45.3	±	0.8	442	±	9	1.97
Pyrene	23.9	±	1.6	233	±	15	1.97
Benzo[*ghi*]fluoranthene	3.03	±	0.09	29.5	±	0.9	1.96
Benzo[c]phenanthrene	1.99	±	0.04	19.4	±	0.4	1.97
Benz[*a*]anthracene	5.69	±	0.11	55.5	±	1.1	1.96
Benzo[*k*]fluoranthene	2.75	±	0.02	26.8	±	0.3	2.04
Benzo[*a*]fluoranthene	0.543	±	0.006	5.30	±	0.07	1.97
Benzo[*e*]pyrene	7.33	±	0.05	71.6	±	0.7	1.98
Benzo[*a*]pyrene	2.32	±	0.03	22.6	±	0.3	1.96
Perylene	0.560	±	0.022	5.46	±	0.22	1.97
Benzo[*ghi*]perylene	2.82	±	0.05	27.6	±	0.5	1.97
Benzo[*b*]chrysene	0.694	±	0.013	6.77	±	0.13	1.97
Picene	1.36	±	0.08	13.2	±	0.8	1.97

[a] Mass fractions are reported on both wet- and dry-mass basis; material as received contains 89.75 % ± 0.08 % (95 % confidence level) water.

[b] The certified value reported on a wet-mass basis is a weighted mean of average mass fractions, with one average each from two analytical methods [3,4]. The expanded uncertainty is the half width of a symmetric 95 % parametric bootstrap confidence interval [5], which is consistent with the ISO Guide [6,7]. The effective coverage factor *k* is included in the table for each PAH.

[c] GC/MS (Ia) using SPE clean-up followed by analysis on a DB-17MS column using EI-MS and GC/MS (IIa) using SPE and SEC clean-up followed by analysis on a DB-XLB column using EI-MS.

Table 2. Certified Mass Fraction Values for Selected PCB Congeners[a] in SRM 1974c

			Mass Fraction[b,c,d] (µg/kg)				*k*
			Wet-Mass Basis		Dry-Mass Basis		
PCB	8	(2,4'-Dichlorobiphenyl)	0.191 ±	0.003	1.86 ±	0.03	1.97
PCB	18	(2,2',5-Trichlorobiphenyl)	0.589 ±	0.007	5.75 ±	0.08	1.97
PCB	28	(2,4,4'-Trichlorobiphenyl)	1.47 ±	0.02	14.4 ±	0.2	1.97
PCB	31	(2,4',5-Trichlorobiphenyl)	1.16 ±	0.06	11.3 ±	0.6	1.97
PCB	44	(2,2'3,5'-Tetrachlorobiphenyl)	1.54 ±	0.08	15.1 ±	0.8	1.97
PCB	45	(2,2',3,6-Tetrachlorobiphenyl)	0.214 ±	0.019	2.09 ±	0.18	1.96
PCB	49	(2,2',4,5'-Tetrachlorobiphenyl)	1.76 ±	0.02	17.1 ±	0.2	1.97
PCB	52	(2,2',5,5'-Tetrachlorobiphenyl)	2.49 ±	0.06	24.3 ±	0.6	1.97
PCB	56	(2,3,3',4'-Tetrachlorobiphenyl)	0.663 ±	0.008	6.46 ±	0.09	1.98
PCB	63	(2,3,4',5-Tetrachlorobiphenyl)	0.137 ±	0.013	1.34 ±	0.13	1.97
PCB	66	(2,3',4,4'-Tetrachlorobiphenyl)	1.65 ±	0.02	16.1 ±	0.2	2.05
PCB	70	(2,3',4',5-Tetrachlorobiphenyl)	1.57 ±	0.05	15.3 ±	0.5	1.97
PCB	74	(2,4,4',5-Tetrachlorobiphenyl)	0.850 ±	0.011	8.29 ±	0.12	1.96
PCB	82	(2,2',3,3',4-Pentachlorobiphenyl)	0.507 ±	0.008	4.95 ±	0.09	1.97
PCB	87	(2,2',3,4,5'-Pentachlorobiphenyl)	2.08 ±	0.02	20.3 ±	0.2	2.01
PCB	92	(2,2',3,5,5'-Pentachlorobiphenyl)	1.06 ±	0.02	10.4 ±	0.2	1.97
PCB	95	(2,2',3,5',6-Pentachlorobiphenyl)	1.82 ±	0.02	17.8 ±	0.2	2.15
PCB	99	(2,2',4,4'5-Pentachlorobiphenyl)	3.55 ±	0.05	34.7 ±	0.6	1.97
PCB	101	(2,2',4,5,5'-Pentachlorobiphenyl)	6.67 ±	0.05	65.1 ±	0.7	1.97
PCB	105	(2,3,3',4,4'-Pentachlorobiphenyl)	1.57 ±	0.03	15.3 ±	0.3	1.97
PCB	110	(2,3,3',4',6-Pentachlorobiphenyl)	5.47 ±	0.06	53.4 ±	0.7	1.97
PCB	118	(2,3',4,4',5-Pentachlorobiphenyl)	4.08 ±	0.09	39.8 ±	0.9	1.97
PCB	128	(2,2',3,3',4,4'-Hexachlorobiphenyl)	0.801 ±	0.011	7.81 ±	0.11	1.97
PCB	138	(2,2',3,4,4',5'-Hexachlorobiphenyl)	4.39 ±	0.04	42.9 ±	0.5	1.97
PCB	146	(2,2',3,4',5,5'-Hexachlorobiphenyl)	0.904 ±	0.005	8.82 ±	0.09	1.97
PCB	149	(2,2',3,4',5',6-Hexachlorobiphenyl)	3.97 ±	0.04	38.8 ±	0.5	1.97
PCB	151	(2,2',3,5,5',6-Hexachlorobiphenyl)	1.13 ±	0.03	11.0 ±	0.3	1.96
PCB	153	(2,2',4,4',5,5'-Hexachlorobiphenyl)[a]	6.76 ±	0.12	66.0 ±	1.3	1.97
	132	(2,2',3,3',4,6'-Hexachlorobiphenyl)					
PCB	156	(2,3,3',4,4',5-Hexachlorobiphenyl)	0.253 ±	0.005	2.47 ±	0.05	1.96
PCB	158	(2,3,3',4,4',6-Hexachlorobiphenyl)	0.443 ±	0.003	4.33 ±	0.04	1.98
PCB	163	(2,3,3',4',5,6-Hexachlorobiphenyl)	1.10 ±	0.09	10.8 ±	0.9	1.96
PCB	170	(2,2',3,3',4,4',5-Heptachlorobiphenyl)	0.105 ±	0.009	1.03 ±	0.09	1.97
PCB	177	(2,2'3,3',4',5,6-Heptachlorobiphenyl)	0.696 ±	0.011	6.79 ±	0.12	1.97
PCB	178	(2,2',3,3',5,5',6-Heptachlorobiphenyl)	0.350 ±	0.011	3.42 ±	0.11	1.97
PCB	180	(2,2',3,4,4',5,5'-Heptachlorobiphenyl)	0.594 ±	0.008	5.79 ±	0.09	1.97
PCB	183	(2,2',3,4,4'5',6-Heptachlorobiphenyl)	0.848 ±	0.006	8.27 ±	0.09	1.98
PCB	187	(2,2',3,4',5,5',6-Heptachlorobiphenyl)	2.09 ±	0.05	20.4 ±	0.5	1.97

[a] PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell [8] and later revised by Schulte and Malisch [9] to conform with IUPAC rules; for the specific congeners mentioned in this table, the Ballschmiter-Zell numbers correspond to those of Schulte and Malisch. When two or more congeners are known to coelute under the GC analysis conditions used, the PCB congener listed first is the major component and the additional congeners may be present as minor components. The quantitative results are based on the response of the congener listed first.

[b] Mass fractions are reported on both wet- and dry-mass basis; material as received contains 89.75 % ± 0.08 % (95 % confidence level) water.

[c] The certified value reported on a wet-mass basis is a weighted mean of average mass fractions, with one average each from two analytical methods [3,4]. The expanded uncertainty is the half width of a symmetric 95 % parametric bootstrap confidence interval [5], which is consistent with the ISO Guide [6,7]. The effective coverage factor *k* is included in the table for each PCB congener.

[d] GC/MS (Ia) using SPE clean-up followed by analysis on a DB-17MS column using EI-MS and GC/MS (IIa) using SPE and SEC clean-up followed by analysis on a DB-XLB column using EI-MS.

Table 3. Certified Mass Fraction Values for Selected Chlorinated Pesticides in SRM 1974c

	Mass Fraction[a,b] (µg/kg)		*k*
	Wet-Mass Basis	Dry-Mass Basis	
Heptachlor[c]	0.132 ± 0.006	1.29 ± 0.06	1.97
cis-Chlordane[d]	1.20 ± 0.05	11.7 ± 0.5	1.97
trans-Chlordane[d]	0.741 ± 0.013	7.23 ± 0.14	1.97
cis-Nonachlor[d]	0.286 ± 0.006	2.79 ± 0.06	1.98
trans-Nonachlor[d]	0.742 ± 0.005	7.24 ± 0.07	1.97
Dieldrin[d]	0.285 ± 0.021	2.78 ± 0.20	1.97
2,4'-DDE[c]	0.346 ± 0.002	3.38 ± 0.04	1.98
4,4'-DDE[c]	1.85 ± 0.02	18.1 ± 0.2	1.99
2,4'-DDD[c]	0.398 ± 0.004	3.88 ± 0.05	1.96
4,4'-DDD[c]	1.30 ± 0.09	12.7 ± 0.8	1.97
2,4'-DDT[c]	0.942 ± 0.027	9.19 ± 0.27	1.97

[a] Mass fractions are reported on both wet- and dry-mass basis; material as received contains 89.75 % ± 0.08 % (95 % confidence level) water.

[b] The certified value reported on a wet-mass basis is a weighted mean of average mass fractions, with one average each from two analytical methods [3,4]. The expanded uncertainty is the half width of a symmetric 95 % parametric bootstrap confidence interval [5], which is consistent with the ISO Guide [6,7]. The effective coverage factor *k* is included in the table for each chlorinated pesticide.

[c] GC/MS (Ia) using SPE clean-up followed by analysis on a DB-17MS column using EI-MS and GC/MS (IIa) using SPE and SEC clean-up followed by analysis on a DB-XLB column using EI-MS.

[d] GC/MS (Ia) using SPE clean-up followed by analysis on a DB-17MS column using EI-MS and GC/MS (IIb) using SPE and SEC clean-up followed by analysis on a DB-XLB column using NCI-MS.

Table 4. Certified Mass Fraction Values for Selected PBDE Congeners[a] in SRM 1974c

			Mass Fraction[b,c,d] (µg/kg)		*k*
			Wet-Mass Basis	Dry-Mass Basis	
PBDE	17	(2,2',4-Tribromodiphenyl ether)	0.078 ± 0.003	0.761 ± 0.032	1.97
PBDE	25	(2,3',4-Tribromodiphenyl ether)	0.103 ± 0.005	1.00 ± 0.05	1.97
PBDE	47	(2,2',4,4'-Tetrabromodiphenyl ether)	0.939 ± 0.017	9.16 ± 0.18	1.96
PBDE	49	(2,2',4,5'-Tetrabromodiphenyl ether)	0.140 ± 0.005	1.37 ± 0.05	1.97
PBDE	99	(2,2',4,4',5-Pentabromodiphenyl ether)	0.375 ± 0.004	3.66 ± 0.05	1.97

[a] PBDE congeners are numbered according to IUPAC rules.

[b] Mass fractions are reported on both wet- and dry-mass basis; material as received contains 89.75 % ± 0.08 % (95 % confidence level) water.

[c] The certified value reported on a wet-mass basis is a weighted mean of average mass fractions, with one average each from two analytical methods [3,4]. The expanded uncertainty is the half width of a symmetric 95 % parametric bootstrap confidence interval [5], which is consistent with the ISO Guide [6,7]. The effective coverage factor *k* is included in the table for each PBDE congener.

[d] GC/MS (Ib) using SPE clean-up followed by analysis on a DB-17MS column using NCI-MS and GC/MS (IIa) using SPE and SEC clean-up followed by analysis on a DB-XLB column using EI-MS.

Table 5. Reference Mass Fraction Values for Selected PAHs in SRM 1974c

	Mass Fraction[a] (µg/kg)						*k*
	Wet-Mass Basis			Dry-Mass Basis			
Naphthalene[b,c]	0.990	±	0.039	9.66	±	0.39	1.96
1-Methylnaphthalene[b,c]	1.41	±	0.03	13.7	±	0.3	1.97
2-Methylnaphthalene[b,c]	1.50	±	0.06	14.6	±	0.6	1.97
1,2-Dimethylnaphthalene[b,c]	0.913	±	0.007	8.91	±	0.10	2.09
1,6-Dimethylnaphthalene[b,c]	1.19	±	0.02	11.6	±	0.2	1.97
2,6-Dimethylnaphthalene[b,c]	0.206	±	0.006	2.01	±	0.06	1.97
Biphenyl[b,c]	0.860	±	0.008	8.39	±	0.10	1.97
Acenaphthylene[b,c]	0.523	±	0.007	5.11	±	0.08	1.96
Acenaphthene[b,c]	0.343	±	0.019	3.35	±	0.18	1.97
1-Methylfluoranthene[d,e]	0.451	±	0.014	4.40	±	0.14	2.57
3-Methylfluoranthene[d,e]	1.32	±	0.02	12.9	±	0.2	2.57
Chrysene[d,e]	19.2	±	0.5	187	±	5	2.09
Triphenylene[d,e]	10.1	±	0.1	98.5	±	1.6	2.09
4-H-Cyclopenta[*def*]phenanthrene[d,e]	2.02	±	0.04	19.7	±	0.4	2.57
Benzo[*b*]fluoranthene[d,e]	5.95	±	0.05	58.0	±	0.7	2.09
Benzo[*j*]fluoranthene[d,e]	2.07	±	0.01	20.2	±	0.2	2.09
Dibenz[*a,c*+*a,h*]anthracene[d,e]	0.100	±	0.001	0.976	±	0.016	2.57
Dibenzo[*b,k*]fluoranthene[d,e]	0.490	±	0.010	4.78	±	0.11	2.57

[a] Mass fractions are reported on both wet- and dry-mass basis; material as received contains 89.75 % ± 0.08 % (95 % confidence level) water.

[b] GC/MS (Ia) using SPE clean-up followed by analysis on a DB-17MS column using EI-MS and GC/MS (IIa) using SPE and SEC clean-up followed by analysis on a DB-XLB column using EI-MS.

[c] The reference value reported on a wet-mass basis is a weighted mean of average mass fractions, with one average each from two analytical methods [3,4]. The expanded uncertainty is the half width of a symmetric 95 % parametric bootstrap confidence interval [5], which is consistent with the ISO Guide [6,7]. The effective coverage factor *k* is included in the table for each PAH.

[d] GC/MS (IIa) using SPE and SEC clean-up followed by analysis on a DB-XLB column using EI-MS.

[e] The reference value reported on a wet-mass basis is the mean of results obtained using one analytical technique. The expanded uncertainty, U, is calculated as $U = ku_c$, where u_c is one standard deviation of the analyte mean, and the coverage factor, k, is determined from the Student's t-distribution corresponding to the associated degrees of freedom and a 95 % confidence level for each analyte.

Table 6. Reference Mass Fraction Values for Selected PCB Congeners[a] in SRM 1974c

			Mass Fraction[b,c,d] (µg/kg)		k
			Wet-Mass Basis	Dry-Mass Basis	
PCB	29	(2,4,5-Trichlorobiphenyl)	0.131 ± 0.003	1.28 ± 0.03	2.57
PCB	109	(2,3,3',4,6-Pentachlorobiphenyl)	0.451 ± 0.004	4.40 ± 0.06	2.57
PCB	114	(2,3,4,4',5-Pentachlorobiphenyl)	0.155 ± 0.005	1.51 ± 0.05	2.57
PCB	119	(2,3',4,4',6-Pentachlorobiphenyl)	0.341 ± 0.004	3.33 ± 0.05	2.57
PCB	130	(2,2',3,3',4,5'-Hexachlorobiphenyl)	0.356 ± 0.008	3.47 ± 0.08	2.57
PCB	137	(2,2',3,4,4',5-Hexachlorobiphenyl)	0.095 ± 0.001	0.924 ± 0.014	2.57
PCB	154	(2,2',4,4',5,6'-Hexachlorobiphenyl)	0.990 ± 0.020	9.66 ± 0.22	2.57
PCB	157	(2,3,3',4,4',5'-Hexachlorobiphenyl)	0.086 ± 0.003	0.840 ± 0.026	2.57
PCB	165	(2,3,3',5,5',6-Hexachlorobiphenyl)	1.56 ± 0.02	15.2 ± 0.3	2.57
PCB	166	(2,3,4,4',5,6-Hexachlorobiphenyl)	0.020 ± 0.001	0.192 ± 0.010	2.57
PCB	167	(2,3',4,4',5,5'-Hexachlorobiphenyl)	0.305 ± 0.004	2.98 ± 0.05	2.57
PCB	175	(2,2',3,3',4,5',6-Heptachlorobiphenyl)	0.139 ± 0.002	1.36 ± 0.03	2.57
PCB	176	(2,2',3,3',4,6,6'-Heptachlorobiphenyl)	0.165 ± 0.004	1.61 ± 0.04	2.57
PCB	202	(2,2',3,3',5,5',6,6'-Octachlorobiphenyl)	0.214 ± 0.003	2.09 ± 0.04	2.57

[a] PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell [8] and later revised by Schulte and Malisch [9] to conform with IUPAC rules; IUPAC PCB 109 is BZ#107.

[b] Mass fractions are reported on both wet- and dry-mass basis; material as received contains 89.75 % ± 0.08 % (95 % confidence level) water.

[c] The reference value reported on a wet-mass basis is the mean of results obtained using one analytical technique. The expanded uncertainty, U, is calculated as $U = ku_c$, where u_c is one standard deviation of the analyte mean, and the coverage factor, k, is determined from the Student's t-distribution corresponding to the associated degrees of freedom and a 95 % confidence level for each analyte.

[d] GC/MS (IIa) using SPE and SEC clean-up followed by analysis on a DB-XLB column using EI-MS.

Table 7. Reference Mass Fraction Values for Selected Chlorinated Pesticides in SRM 1974c

	Mass Fraction[a,b,c] (µg/kg)		k
	Wet-Mass Basis	Dry-Mass Basis	
Hexachlorobenzene	0.021 ± 0.001	0.205 ± 0.014	2.57
Mirex	0.164 ± 0.005	1.60 ± 0.05	2.57

[a] Mass fractions are reported on both wet- and dry-mass basis; material as received contains 89.75 % ± 0.08 % (95 % confidence level) water.

[b] The reference value reported on a wet-mass basis is the mean of results obtained using one analytical technique. The expanded uncertainty, U, is calculated as $U = ku_c$, where u_c is one standard deviation of the analyte mean, and the coverage factor, k, is determined from the Student's t-distribution corresponding to the associated degrees of freedom and a 95 % confidence level for each analyte.

[c] GC/MS (IIb) using SPE and SEC clean-up followed by analysis on a DB-XLB column using NCI-MS.

REFERENCES

[1] May, W.; Parris, R.; Beck, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G..; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*; NIST Special Publication 260–136; U.S. Government Printing Office: Washington, DC (2000); available at http://www.nist.gov/srm/upload/SP260-136.PDF (accessed Nov 2015).

[2] Wise, S.A.; Poster, D.L.; Kucklick, J.R.; Keller, J.M.; VanderPol, S.S.; Sander, L.C.; Schantz, M.M.; *Standard Reference Materials (SRMs) for Determination of Organic Contaminants in Environmental Samples*; Anal. Bioanal. Chem., Vol. 386, pp. 1153–1190 (2006).

[3] Dersimonian, R.; Laird, N.; *Meta-Analysis in Clinical Trials*; Control Clin. Trials, Vol. 7, pp. 177–188 (1986).

[4] Rukhin, A.L.; *Weighted Means Statistics in Interlaboratory Studies*; Metrologia, Vol. 46, pp. 323–331 (2009).

[5] Efron, B.; Tibshirani, R.J.; *An Introduction to the Bootstrap*; Chapman & Hall (1993).

[6] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (ISO GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (JCGM) (2008); available at http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Nov 2015); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at http://www.nist.gov/pml/pubs/tn1297/index.cfm (accessed Nov 2015).

[7] JCGM 101:2008; *Evaluation of Measurement Data — Supplement 1 to the "Guide to the Expression of Uncertainty in Measurement" — Propagation of Distributions Using a Monte Carlo Method*; JCGM (2008); available at www.bipm.org/utils/common/documents/jcgm/JCGM_101_2008_E.pdf (accessed Nov 2015).

[8] Ballschmiter, K.; Zell, M.; *Analysis of Polychlorinated Biphenyls (PCB) by Glass Capillary Gas Chromatography - Composition of Technical Aroclor- and Clophen-PCB Mixtures*; Fresenius Z. Anal. Chem., Vol. 302, pp. 20–31 (1980).

[9] Schulte, E.; Malisch, R.; *Calculation of the Real PCB Content in Environmental Samples. I. Investigation of the Composition of Two Technical PCB Mixtures*; Fresenius Z. Anal. Chem., Vol. 314, pp. 545-551 (1983).

Certificate Revision History: 05 November 2015 (Corrected PBDE 49 and PCB 109 names; editorial changes); 18 September 2012 (Original certificate date)

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

Validation data – method performance characteristics

Smoked salmon – spiked concentrations – 1 and 5 µg/kg

Concentration level 1 µg/kg

Analyte	Spike 1	Spike 2	Spike 3	Spike 4	Spike 5	Spike 6	Recovery (%)	Repeatability, RSD (%)	LOQ (µg/kg)	Linearity range (µg/kg)
BcFln	106	103	108	107	98	99	**104**	4	0.05	0.05 - 100
BaA	103	97	100	97	94	94	**97**	4	0.03	0.03 - 100
CPcdP	82	82	82	90	91	91	**86**	5	0.03	0.03 - 100
Chr	109	101	106	101	101	107	**104**	3	0.03	0.03 - 100
5 MeCHr	85	88	86	86	83	87	**86**	2	0.05	0.03 - 100
BbF	84	82	88	86	88	87	**86**	3	0.03	0.03 - 100
BkF	94	98	102	98	97	91	**97**	5	0.03	0.03 - 100
BjF	100	101	101	94	93	93	**97**	4	0.03	0.03 - 100
BaP	93	90	95	96	91	90	**93**	3	0.03	0.03 - 100
IcdP	94	100	93	70	83	84	**91**	4	0.03	0.03 - 100
DbahA	86	83	81	83	80	82	**83**	3	0.03	0.03 - 100
BghiP	100	102	101	101	102	103	**101**	1	0.03	0.03 - 100
DBalP	98	91	97	93	88	93	**93**	4	0.10	0.10 - 100
DBaeP	91	95	92	97	90	91	**93**	3	0.10	0.10 - 100
DBaiP	95	98	98	99	95	93	**98**	2	0.10	0.10 - 100
DBahP	79	60	95	82	82	93	**88**	8	0.10	0.10 - 100

Concentration level 5 µg/kg

Analyte	Spike 1	Spike 2	Spike 3	Spike 4	Spike 5	Spike 6	Recovery (%)	Repeatability, RSD (%)	LOQ (µg/kg)	Linearity range (µg/kg)
BcFln	89	80	86	84	93	85	**86**	5	0.05	0.05 - 100
BaA	86	83	85	82	88	84	**85**	3	0.03	0.03 - 100
CPcdP	98	88	98	89	92	95	**93**	5	0.03	0.03 - 100
Chr	92	89	89	87	90	88	**89**	2	0.03	0.03 - 100
5 MeCHr	95	91	92	89	93	93	**92**	2	0.05	0.03 - 100
BbF	84	83	84	82	88	84	**84**	3	0.03	0.03 - 100
BkF	87	85	85	86	89	87	**87**	2	0.03	0.03 - 100
BjF	96	94	92	97	97	99	**96**	3	0.03	0.03 - 100
BaP	89	89	88	88	91	86	**89**	2	0.03	0.03 - 100
IcdP	85	83	81	84	84	81	**83**	2	0.03	0.03 - 100
DbahA	94	90	91	87	92	89	**91**	3	0.03	0.03 - 100
BghiP	91	88	89	85	92	87	**89**	3	0.03	0.03 - 100
DBalP	92	97	89	84	91	82	**89**	6	0.10	0.10 - 100
DBaeP	91	80	93	94	81	92	**88**	7	0.10	0.10 - 100
DBaiP	89	85	88	90	85	84	**87**	3	0.10	0.10 - 100
DBahP	84	84	81	84	85	86	**84**	2	0.10	0.10 - 100

Validation data – method performance characteristics

Shrimps – spiked concentrations – 1 and 5 µg/kg

Concentration level 1 µg/kg

Analyte	Spike 1	Spike 2	Spike 3	Spike 4	Spike 5	Spike 6	Recovery (%)	Repeatability, RSD (%)	LOQ (µg/kg)	Linearity range (µg/kg)
Naph	77	80	81	77	86	67	**78**	9	0.1	0.10 - 100
Ace	72	79	82	84	75	84	**79**	6	0.1	0.10 - 100
Fln	80	79	80	93	79	87	**83**	7	0.05	0.05 - 100
Phe	80	72	87	86	68	80	**79**	9	0.05	0.05 - 100
Ant	75	73	76	85	74	85	**78**	7	0.05	0.05 - 100
Flt	100	87	101	97	96	103	**97**	6	0.05	0.05 - 100
Pyr	89	78	82	93	80	89	**85**	7	0.05	0.05 - 100
BaA	82	88	83	92	82	89	**86**	5	0.05	0.05 - 100
Chr	84	79	84	96	83	91	**86**	7	0.05	0.05 - 100
BbF	84	82	87	98	81	93	**88**	8	0.05	0.05 - 100
BkF	87	84	90	100	85	97	**91**	7	0.05	0.05 - 100
BaP	87	84	89	99	84	95	**90**	7	0.05	0.05 - 100
DBahA	89	85	91	100	87	97	**91**	6	0.05	0.05 - 100
BghiP	88	85	91	99	87	97	**91**	6	0.05	0.05 - 100
IcdP	89	86	92	100	86	100	**92**	7	0.05	0.05 - 100

Concentration level 5 µg/kg

Analyte	Spike 1	Spike 2	Spike 3	Spike 4	Spike 5	Spike 6	Recovery (%)	Repeatability, RSD (%)	LOQ (µg/kg)	Linearity range (µg/kg)
Naph	70	80	55	70	80	84	**77**	8	0.10	0.10 - 100
Ace	84	89	78	86	90	90	**86**	6	0.10	0.10 - 100
Fln	77	83	75	80	85	84	**81**	5	0.05	0.05 - 100
Phe	80	85	81	84	90	86	**84**	4	0.05	0.05 - 100
Ant	77	81	79	79	78	79	**79**	2	0.05	0.05 - 100
Flt	108	115	51	99	102	116	**108**	7	0.05	0.05 - 100
Pyr	76	83	84	85	85	82	**82**	4	0.05	0.05 - 100
BaA	85	89	86	87	93	89	**88**	3	0.05	0.05 - 100
Chr	86	90	86	89	93	90	**89**	3	0.05	0.05 - 100
BbF	84	90	89	93	93	89	**90**	4	0.05	0.05 - 100
BkF	89	93	91	94	96	92	**93**	3	0.05	0.05 - 100
BaP	87	92	90	93	96	92	**92**	3	0.05	0.05 - 100
DBahA	88	93	90	93	94	92	**92**	3	0.05	0.05 - 100
BghiP	89	95	92	96	98	93	**94**	3	0.05	0.05 - 100
IcdP	91	96	93	98	100	95	**95**	4	0.05	0.05 - 100

RESIDUES AND TRACE ELEMENTS

Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood Using Gas Chromatography-Mass Spectrometry: Collaborative Study

KATERINA MASTOVSKA and WENDY R. SORENSON
Covance Laboratories Inc., Nutritional Chemistry and Food Safety, 3301 Kinsman Blvd, Madison, WI 53704
JANA HAJSLOVA
Institute of Chemical Technology, Faculty of Food and Biochemical Technology, Department of Food Chemistry and Analysis, Technická 3, 166 28 Prague 6, Czech Republic

Collaborators: J. Betzand; J. Binkley; K. Bousova; J.M. Cook; L. Drabova; W. Hammack; J. Jabusch; K. Keide; R. Lizak; P. Lopez-Sanchez; M. Misunis; K. Mittendorf; R. Perez; S. Perez; S. Pugh; J. Pulkrabova; J. Rosmus; J. Schmitz; D. Staples; J. Stepp; B. Taffe; J. Wang; T, Wenzl

A collaborative study was conducted to determine selected polycyclic aromatic hydrocarbons (PAHs) and their relevant alkyl homologs in seafood matrixes using a fast sample preparation method followed by analysis with GC/MS. The sample preparation method involves addition of ^{13}C-PAH surrogate mixture to homogenized samples and extraction by shaking with a water–ethyl acetate mixture. After phase separation induced by addition of anhydrous magnesium sulfate–sodium chloride (2 + 1, w/w) and centrifugation, an aliquot of the ethyl acetate layer is evaporated, reconstituted in hexane, and cleaned up using silica gel SPE. The analytes are eluted with hexane–dichloromethane (3 + 1, v/v), the clean extract is carefully evaporated, reconstituted in isooctane, and analyzed by GC/MS. To allow for the use of various GC/MS instruments, GC columns, silica SPE cartridges, and evaporation techniques and equipment, performance-based criteria were developed and implemented in the qualification phase of the collaborative study. These criteria helped laboratories optimize their GC/MS, SPE cleanup, and evaporation conditions; check and eliminate potential PAH contamination in their reagent blanks; and become familiar with the method procedure. Ten laboratories from five countries qualified and completed the collaborative study, which was conducted on three seafood matrixes (mussel, oyster, and shrimp) fortified with 19 selected PAH analytes at five different levels of benzo[*a*]pyrene (BaP) ranging from 2 to 50 µg/kg. Each matrix had a varying mixture of three different BaP levels. The other studied PAHs were at varying levels from 2 to 250 µg/kg to mimic typical PAH patterns. The fortified analytes in three matrixes were analyzed as blind duplicates at each level of BaP and corresponding other PAH levels. In addition, a blank with no added PAHs for each matrix was analyzed singly. Eight to 10 valid results were obtained for the majority of determinations. Mean recoveries of all tested analytes at the five different concentration levels were all in the range of 70–120%: 83.8–115% in shrimp, 77.3–107% in mussel, and 71.6–94.6% in oyster, except for a slightly lower mean recovery of 68.6% for benzo[*a*]anthracene fortified at 25 µg/kg in oyster (RSD_r: 5.84%, RSD_R: 21.1%) and lower mean recoveries for anthracene (Ant) and BaP in oyster at all three fortification levels (50.3–56.5% and 48.2–49.7%, respectively). The lower mean recoveries of Ant and BaP were linked to degradation of these analytes in oyster samples stored at –20°C, which also resulted in lower reproducibility (RSD_R values in the range of 44.5–64.7% for Ant and 40.6–43.5% for BaP). However, the repeatability was good (RSD_r of 8.78–9.96% for Ant and 6.43–11.9% for BaP), and the HorRat values were acceptable (1.56–1.94 for Ant and 1.10–1.45 for BaP). In all other cases, repeatability, reproducibility, and HorRat values were as follows: shrimp: RSD_r 1.40–26.9%, RSD_R 5.41–29.4%, HorRat: 0.22–1.34; mussel: RSD_r 2.52–17.1%, RSD_R 4.19–32.5%, HorRat: 0.17–1.13; and oyster: RSD_r 3.12–22.7%, RSD_R 8.41–31.8%, HorRat: 0.34–1.39. The results demonstrate that the method is fit-for-purpose to determine PAHs and their alkyl homologs in seafood samples. The method was approved by the Expert Review Panel on PAHs as the AOAC Official First Action Method 2014.08.

Received December 16, 2014.
The method was approved by the Expert Review Panel for Polycyclic Aromatic Hydrocarbons (PAHs) as First Action.
The Expert Review Panel for Polycyclic Aromatic Hydrocarbons (PAHs) invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
Corresponding author's e-mail: katerina.mastovska@covance.com
DOI: 10.5740/jaoacint.15-032

As a response to the 2010 oil spill in the Gulf of Mexico, AOAC INTERNATIONAL formed the Stakeholder Panel on Seafood Contaminants (SPSC) and later issued a call for methods for determination of polycyclic

Table 1. PAHs included in the collaborative study

Name	Abbreviation
1,7-Dimethylphenanthrene	1,7-DMP
1-Methylnaphthalene	1-MN
1-Methylphenanthrene	1-MP
2,6-Dimethylnaphthalene	2,6-DMN
3-Methylchrysene	3-MC
Anthracene	Ant
Benz[*a*]anthracene	BaA
Benzo[*a*] pyrene	BaP
Benzo[*b*]fluoranthene	BbF
Benzo[*g,h,i*]perylene	BghiP
Benzo[*k*]fluoranthene	BkF
Chrysene	Chr
Dibenz[*a,h*]anthracene	DBahA
Fluoranthene	Flt
Fluorene	Fln
Indeno[1,2,3-*cd*]pyrene	IcdP
Naphthalene	Naph
Phenanthrene	Phe
Pyrene	Pyr

aromatic hydrocarbons (PAHs) in seafood. The primary goal was to significantly reduce the time-to-signal (including sample preparation and extraction) in comparison with currently accepted analytical methods requiring 96–120 hours to complete. In addition, acceptable methods had to demonstrate an LOQ of 1 µg/kg for benzo[*a*]pyrene (BaP) in seafood. The SPSC PAH Working Group on Quantitative Methods evaluated about 30 methods that were submitted as a response to the call or found in the literature. The evaluation criteria included: fitness-for-purpose requirements (LOQ, speed, and scope), identification and quantification (compatibility with MS), quality of data to meet AOAC INTERNATIONAL single-laboratory validation requirements (e.g., accuracy, precision, and analysis of reference materials), and practical considerations, such as availability of equipment.

The Working Group selected a method developed for the determination of PAHs, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) in fish and seafood by Jana Hajslova's group at the Institute of Chemical Technology (ICT) in Prague, Czech Republic (1) within a European integrated project CONffIDENCE (Contaminants in Food and Feed: Inexpensive Detection for Control of Exposure; 2). This method was studied within the presented collaborative study, for which the analytes were narrowed down to include only PAHs and some of the relevant PAH alkyl homologs (*see* Table 1 for the list of 19 studied analytes and their abbreviations).

Table 2. PAH fortification levels (in µg/kg) in the shrimp, mussel, and oyster test samples

	Shrimp			Mussel			Oyster		
	Low	Mid	High	Low	Mid	High	Low	Mid	High
PAH	Level 1	Level 2	Level 4	Level 1	Level 3	Level 4	Level 2	Level 3	Level 5
1,7-DMP [a]	20	20	20	40	40	40	80	80	80
1-MN	20	75	200	20	100	200	75	100	250
1-MP	10	25	125	10	50	125	25	50	200
2,6-DMN	15	40	175	15	75	175	40	75	225
3-MC	10	30	145	10	90	145	30	90	225
Ant	5	10	40	5	15	40	10	15	60
BaA	5	15	60	5	25	60	15	25	100
BaP	2	5	25	2	10	25	5	10	50
BbF	5	10	75	5	30	75	10	30	100
BghiP	2	5	20	2	10	20	5	10	25
BkF	2	8	40	2	20	40	8	20	75
Chr	15	50	175	15	100	175	50	100	250
DBahA	2	5	15	2	10	15	5	10	20
Fln	5	15	50	5	25	50	15	25	75
Flt	10	25	100	10	50	100	25	50	150
IcdP	2	5	20	2	10	20	5	10	25
Naph	25	80	160	25	125	160	80	125	225
Phe	15	50	175	15	100	175	50	100	250
Pyr	15	40	125	15	75	125	40	75	200

[a] 1,7-DMP served as a homogenization check, which was added to the blank mussel and oyster matrix during the homogenization step (prior to fortification).

Collaborative Study

Purpose

The purpose of this study was to evaluate the method's intralaboratory and interlaboratory performance and submit the results to AOAC INTERNATIONAL for adoption as an Official Method for the determination of PAHs in seafood.

Study Design

This study evaluated the method performance for determination of 19 selected PAHs, including alkyl homologs relevant to an oil spill contamination (*see* Table 1), in three seafood matrixes: shrimp, oysters, and mussels, with five different levels of BaP ranging from 2 to 50 µg/kg. Each matrix had a varying mixture of three different BaP levels ("low," "mid," and "high"). The other studied PAHs were added at varying levels from 2 to 250 µg/kg to mimic typical PAH patterns (Table 2). The fortified analytes in the three matrixes were analyzed as blind duplicates at each level of BaP and corresponding other PAH levels. In addition, a blank with no added PAHs for each matrix was analyzed singly. The AOAC official method guidelines for collaborative study procedures (3) were followed for the preparation of the study and data analysis.

Test Sample Preparation

Blank mussel and oyster samples were homogenized with liquid nitrogen and tested in duplicate by an independent laboratory for potential contamination with the target PAHs. During homogenization, portions of the blank matrixes were spiked with 1,7-dimethylphenanthrene (1,7-DMP) at 40 and 80 µg/kg in the case of mussel and oyster, respectively. These were utilized as a homogenization check throughout the course of the study. The collaborators determined 1,7-DMP along with the other 18 analytes, which were spiked into 10 g sample portions placed in polypropylene centrifuge tubes by the study direction team. Five different spiking levels were made at varying PAH concentrations (Table 2), resulting in three different duplicate spiked samples/matrix in addition to a blank. Participants were supplied with the test samples ready for analysis labeled with unique identification numbers. All test samples were shipped frozen on dry ice with a material receipt document to be returned to the Study Directors. The test samples had to be stored in a freezer set to maintain at least –20 ± 10°C. Test samples were to be analyzed after completion of laboratory qualification and practice sample analysis.

Blank shrimp matrix (peeled, without head and tail, and uncooked) was homogenized without the use of liquid nitrogen using a blender. After testing for potential contamination with the target PAHs, 10 g blank sample portions were placed in polypropylene centrifuge tubes, which were sent to study participants together with spiking solutions labeled with unique identification numbers. Using instructions provided by the Study Directors, participants fortified the blank shrimp samples themselves on the day of the analysis.

Three different spiking levels were used at varying PAH concentrations (Table 2), resulting in three different duplicate spiked samples in addition to a blank (seven samples altogether). The blank shrimp samples were shipped frozen on dry ice with a material receipt document to be returned to the Study Directors. The test samples had to be stored in a freezer set to maintain at least –20 ± 10°C. The spiking solutions were to be stored in a refrigerator set to maintain 5 ± 3°C. (*Note*: This modification of the shrimp test sample preparation protocol (as compared to mussel and oyster) was made (after consultations with the SPSC PAH Working Group and the AOAC Methods Committee on PAHs) due to potential stability issues discovered during the practice sample analysis and follow-up experiments with fortified shrimp samples stored at different conditions. 3-Methylchrysene (3-MC) had to be replaced by 6-methylchrysene (6-MC) in the spiking and calibration solutions for shrimp samples due to the unavailability of a 3-MC reference standard at the time of preparation and shipment of the new set of shrimp samples to the study participants.)

Laboratory Qualification

During the laboratory qualification phase, the collaborators conducted the following seven steps. These steps were necessary because the Study Directors allowed the use of various GC/MS instruments, GC columns, silica SPE cartridges, and evaporation techniques and equipment. Therefore, performance-based criteria were developed to help laboratories optimize their GC/MS, SPE cleanup, and solvent evaporation conditions; check and eliminate potential PAH contamination in their reagent blanks; and become familiar with the method. Laboratory qualification and practice sample results had to be approved by the Study Directors before proceeding with the test sample analysis. Sixteen laboratories entered the qualification phase, but only 10 of them (listed in the *Acknowledgments* section) completed the qualification successfully and/or continued in the study.

(*1*) The first step was a GC separation test where participants analyzed a composite PAH solution by GC/MS/MS to obtain a baseline separation of BaP and benzo[*e*]pyrene (concentration ratio of 1:5); at least 50% valley separation of anthracene and phenanthrene (concentration ratio 1:2.5, evaluated for the anthracene peak); and at least 50% valley separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*] fluoranthene (concentration ratio of 1:1:1).

(*2*) The second step was a calibration range test where participants prepared calibration standards and obtained normalized calibration curves for the studied PAHs versus respective labeled internal standards (^{13}C-PAHs). Collaborators had to determine the linear range, test for carryover by injecting a solvent blank after the highest standard, and adjust injection conditions (such as injection volume, number of washes, syringe size, etc.) to achieve low detection limits, acceptable linearity for the tested concentration range, and minimum carryover. Coefficient of determination (r^2) values should be 0.990 or greater, and back-calculated concentrations of the calibration standards should not exceed ±20% of theoretical. For lower concentration levels, a limited calibration curve (without the higher-end concentration points) may be used for better accuracy. If a well characterized quadratic relationship occurs, then a best-fitted quadratic curve may be used for calibration. Otherwise, if the back-calculated concentrations exceed ±20% of theoretical, normalized signals of the nearest two calibration

standards that enclose the analyte signal in the sample could be used to interpolate the analyte concentration.

(*3*) The third step was a test of the solvent evaporation where participants determined absolute recoveries of both PAHs and ^{13}C-PAHs in two evaporation experiments (with three replicates each): (**a**) gentle evaporation of 5 mL of a PAH/^{13}C-PAH solution in ethyl acetate and reconstitution in isooctane and (**b**) gentle evaporation of 10 mL of a PAH/^{13}C-PAH solution in hexane–dichloromethane (3 + 1, v/v) and reconstitution in isooctane. The absolute recoveries of all analytes, including naphthalene, and ^{13}C-naphthalene had to be above 70%.

(*4*) The fourth step was the determination of the elution profiles of PAHs and fat on silica gel SPE columns chosen for the PAH analysis by the laboratory. The silica gel columns could be prepared in-house using the procedure described in the method or could be obtained commercially from different vendors. The elution volume of 10 mL hexane–dichloromethane (3 + 1, v/v) specified in the ICT method (1) was optimized for the analysis of PAHs, PCBs, and PBDEs using the in-house prepared silica gel minicolumns for which the silica gel deactivation (5% water added) and storage are controlled by the laboratory. For commercially available silica gel SPE cartridges, however, the deactivation and storage can vary, potentially resulting in different amounts of water in the silica thus potentially different retention characteristics. Therefore, it is important to test the elution profiles of PAHs and fat and determine the optimum volume of the elution solvent to ensure adequate analyte recoveries and fat cleanup. The PAH elution profile was determined by applying 1 mL of a PAH in hexane solution to the silica cartridge, collecting fractions of hexane–dichloromethane (3 + 1, v/v) eluting from the cartridge, exchanging the fractions to 0.5 mL isooctane, and analyzing them by GC/MS. The fat elution profile was checked gravimetrically by applying 1 mL of hexane containing 100 mg of fat (pure fish oil) onto the silica cartridge, collecting the optimum elution fraction determined for PAHs and three consecutive 1 mL fractions, and evaporating them to dryness.

(*5*) The fifth step was a reagent (procedure) blank test where participants determined concentrations of the target PAHs in three replicates of reagent (procedure) blank that was prepared the same way as the samples, except that 10 mL of water was used instead of the sample. The concentrations of all analytes in the reagent blanks had to be below the concentrations in the lowest calibration level standard. For naphthalene, levels below the second lowest calibration standard (equivalent to 5 ng/g of naphthalene in the sample) were still acceptable if the source of contamination could not be eliminated, such as by selection of a silica gel SPE column from a different vendor (or preparation of silica gel columns in-house), heating of glassware, addition of a hydrocarbon trap to the nitrogen lines used for solvent evaporation, etc.

(*6*) The sixth step was a low-level spike test where collaborators prepared and analyzed seven spiked samples using blank shrimp matrix and a mixed PAH spiking solution that were both supplied to them. The samples were spiked at PAH concentrations equivalent to the second lowest calibration level (1 µg/kg for BaP, which is a fitness-for-purpose LOQ requirement established for the study) to test instrument sensitivity and method precision. The shrimp matrix had to be stored in a freezer set to maintain at least –20 ± 10°C. The mixed PAH spiking solution was to be stored in a refrigerator set to maintain 5 ± 3°C.

(*7*) The seventh step was the analysis of practice samples. Three practice samples were supplied to the participants. Two of the three samples were shrimp blank matrix already spiked with two different mixed PAH solutions (BaP levels of 2–50 µg/kg, other PAHs at 2–250 µg/kg). The third sample was the National Institute of Standards and Technology Standard Reference Material 1974b, which is a mussel matrix with certified concentrations of incurred PAHs and other organic contaminants. All practice samples were shipped frozen on dry ice and had to be stored in a freezer set to maintain at least –20 ± 10°C.

Quality Assurance

The method uses a mixture of isotopically labeled ^{13}C-PAH surrogate standards that were added at 5 µg/kg to the samples prior to the extraction process. Quantification was based on calibration of analyte signals (peak areas or heights) divided by signals of respective ^{13}C-labeled internal standards plotted versus analyte concentrations. Eight concentration levels were used for the calibration, corresponding to 0.5, 1, 2, 5, 10, 20, 50, and 100 µg/kg for BaP and other lower level PAHs, and to 1.25, 2.5, 5, 12.5, 25, 50, 125, and 250 µg/kg for higher level PAHs, except for naphthalene that was present at levels corresponding to 2.5, 5, 10, 25, 50, 100, 250, and 500 µg/kg. Values of r^2 had to be 0.990 or greater, and back-calculated concentrations of the calibration standards should not exceed ±20% of theoretical. For lower concentration levels, a limited calibration curve (without the three higher-end concentration points) was used for better accuracy. In addition to reporting r^2 values, back-calculated calibration standard concentrations, and analyte concentrations, the collaborators were also required to report ion ratios as a means of verifying identification of the analyte peaks.

A solvent (isooctane) blank was injected before and after each calibration set. Reagent (procedural) blanks were analyzed with each set of samples. During homogenization, portions of the blank mussel and oyster matrixes were spiked with 1,7-DMP, which served as a homogenization check of the sample processing step.

Data Reporting

Participants supplied PAH and ^{13}C-PAH signals (peak areas or heights) in test samples, calibration standards, and blanks and other parameters as described above in Quality Assurance in Excel forms created by the Study Directors. They also had to provide details about their GC and MS instruments and method conditions, evaporation equipment and conditions, and silica gel SPE cartridge and optimum elution volume. Participants were asked to record all observations and any potential method deviations, investigate any potential unreasonable results (caused by, e.g., incorrect calculations and arithmetic errors, use of wrong units, transposition errors, incorrect standard preparation or contamination), and have all the results and

calculations reviewed by a peer, laboratory supervisor, or manager.

Data Analysis

The Study Directors reviewed and compiled all the data submitted by the participants. Statistical analysis was conducted using the AOAC spreadsheet for blind duplicates (4) to determine mean analyte concentrations, SD (S_r) and RSD (RSD_r) for repeatability (for blind duplicate data), SD (S_R) and RSD (RSD_R) for reproducibility, number of valid data points, HorRat value (RSD_R/predicted RSD_R), and percentage recovery for all data after removal of outliers (3). The following tests were used in the AOAC spreadsheet (4) to determine outliers: (*a*) the Cochran test for removal of laboratories showing significantly greater variability among replicate (within-laboratory) analyses than the other laboratories for a given material, and (*b*) the Grubbs' tests for removal of laboratories with extreme averages.

AOAC Official Method 2014.08
Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood
Gas Chromatography-Mass Spectrometry
First Action 2014

[Applicable for the determination of the following PAHs in mussel, oyster, and shrimp: 1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*k*]fluoranthene, chrysene, dibenz[*a,h*]anthracene, fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, naphthalene, phenanthrene, and pyrene. These were representative PAH analytes selected for the collaborative study. The method has been single-laboratory validated for 32 PAHs in fish and shrimp (1), and, therefore, is expected to be applicable to other GC-amenable PAHs and seafood matrices. The concentration ranges evaluated within the collaborative study are given in Table **2014.08A**.]

Caution: *See Appendix B: Laboratory Safety*. Use appropriate personal protective equipment such as laboratory coat, safety glasses or goggles, appropriate chemical-resistant gloves, and a fume hood. Dispose of solvents and solutions according to federal, state, and local regulations. Always handle open containers of solvents inside the fume hood, including the pouring, mixing, evaporating, and preparing standard solution. Keep containers covered or closed when not in use.

Hexane and isooctane.—Highly flammable, liquid irritants. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation.

Ethyl acetate.—Highly flammable, liquid irritants. Harmful if swallowed in quantity. Vapors may cause drowsiness.

Toluene.—Highly flammable, liquid irritant. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation. May cause drowsiness. Possible teratogen.

Dichloromethane.—Noncombustible, liquid irritant. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation. Asphyxiant. Causes central nervous system (CNS) depression. Possible carcinogen and mutagen.

PAHs.—Carcinogens, respiratory sensitizers, teratogens, reproductive hazard, mutagens. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation.

See Tables **2014.08B**–**D** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Homogenized seafood samples (10 g sample with a 5 µg/kg addition of ^{13}C-PAH surrogate mixture) are mixed with 5 mL water (or 10 mL water in the case of shrimp and other more viscous samples) and shaken vigorously by hand with 10 mL ethyl acetate in a 50 mL polypropylene centrifuge tube for 1 min. Subsequently, 4 g anhydrous magnesium sulfate and 2 g sodium chloride are added to the mixture to induce phase separation and force the analytes into the ethyl acetate layer. The tube is again shaken by hand for 1 min and then centrifuged for 10 min at >1500 rcf. A 5 mL aliquot of the ethyl acetate layer is evaporated, reconstituted in 1 mL hexane, and cleaned on an SPE column with 1 g silica gel and approximately 0.2 g anhydrous sodium sulfate on the top. The column is conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v)

Table 2014.08A. PAH and ^{13}C-PAH concentrations in the calibration standard solutions

	Concentration, µg/L				Equivalent concentration, µg/kg			
Calibration level	BaP and others[a]	Chr and others[b]	Naph[c]	^{13}C-PAHs	BaP and others	Chr and others	Naph	^{13}C-PAHs
1	5	12.5	25	50	0.5	1.25	2.5	5
2	10	25	50	50	1	2.5	5	5
3	20	50	100	50	2	5	10	5
4	50	125	250	50	5	12.5	25	5
5	100	250	500	50	10	25	50	5
6	200	500	1000	50	20	50	100	5
7	500	1250	2500	50	50	125	250	5
8	1000	2500	5000	50	100	250	500	5

[a] Analytes at 10 µg/mL in the mixed stock standard solution.

[b] Analytes at 25 µg/mL in the mixed stock standard solution.

[c] Analytes at 50 µg/mL in the mixed stock standard solution.

Table 2014.08B. Statistical results for the studied PAHs at three different concentration levels in shrimp after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	9	18	21.7	108.6	2.6	4.1	11.8	18.9	0.66
	9	18	22.7	113.7	1.8	4.2	8.0	18.7	0.66
	9	18	21.7	108.3	1.9	4.4	8.8	20.4	0.72
1-MN	9	18	23.1	115.4	6.2	6.8	26.9	29.4	1.04
	9	18	81.5	108.6	6.7	15.6	8.3	19.1	0.82
	9	18	203.2	101.6	17.8	55.4	8.8	27.3	1.34
1-MP	9	18	10.0	99.9	1.0	1.4	9.8	14.0	0.44
	9	18	25.0	99.9	1.9	3.7	7.6	14.9	0.53
	9	18	119.4	95.5	6.7	15.7	5.6	13.1	0.60
2,6-DMN	8	16	15.6	103.9	1.1	2.6	6.9	16.4	0.55
	8	16	37.8	94.6	4.8	7.4	12.6	19.5	0.74
	7	14	146.7	83.8	16.6	20.3	11.3	13.9	0.65
6-MC	9	18	11.1	110.5	0.6	1.5	5.8	13.1	0.42
	9	18	32.2	107.2	1.6	3.6	5.1	11.1	0.42
	9	18	145.2	100.1	8.7	13.7	6.0	9.4	0.44
Ant	9	18	4.9	98.5	0.3	0.5	6.7	10.3	0.29
	9	18	10.6	105.7	0.8	1.7	7.3	16.2	0.51
	9	18	38.9	97.4	2.4	4.6	6.2	11.7	0.45
BaA	9	18	4.8	95.9	0.3	0.5	7.0	9.7	0.27
	9	18	15.0	99.9	0.6	1.3	4.3	8.4	0.28
	9	18	56.6	94.4	2.5	5.2	4.5	9.2	0.37
BaP	9	18	1.9	96.2	0.1	0.2	6.5	12.1	0.29
	9	18	4.9	98.7	0.4	0.5	7.3	9.6	0.27
	8	16	23.1	92.3	1.1	1.6	4.6	7.0	0.25
BbF	9	18	4.8	96.7	0.3	0.5	6.6	10.1	0.28
	9	18	9.8	98.2	0.3	0.7	2.6	7.0	0.22
	9	18	71.6	95.5	3.9	6.3	5.5	8.8	0.37
BghiP	8	16	1.9	94.7	0.1	0.2	7.0	11.7	0.28
	8	16	4.9	98.5	0.2	0.5	4.3	9.9	0.28
	8	16	18.0	90.1	1.0	1.4	5.7	7.9	0.27
BkF	9	18	2.0	99.5	0.1	0.3	6.2	13.7	0.34
	9	18	8.1	101.7	0.4	0.7	4.9	8.7	0.26
	9	18	38.3	95.8	1.8	2.8	4.7	7.2	0.28
Chr	8	16	15.2	101.5	0.5	1.4	3.1	9.4	0.31
	9	18	50.7	101.4	2.2	4.1	4.4	8.1	0.32
	9	18	167.4	95.6	9.0	14.5	5.3	8.7	0.41
DBahA	9	18	1.9	95.9	0.2	0.3	10.9	13.5	0.33
	9	18	5.0	100.4	0.3	0.6	6.6	11.2	0.32
	9	18	13.8	91.8	0.9	1.2	6.4	8.4	0.28
Fln	8	16	5.2	103.0	0.1	0.5	1.4	10.0	0.28
	9	18	15.4	102.3	0.6	1.1	4.2	7.5	0.25
	9	18	47.3	94.7	2.1	4.1	4.5	8.7	0.34
Flt	9	18	9.7	97.2	0.6	1.0	6.0	10.4	0.32
	9	18	25.1	100.3	1.4	2.4	5.5	9.7	0.35
	9	18	93.9	93.9	4.9	8.7	5.2	9.3	0.41
IcdP	9	18	2.0	98.2	0.1	0.3	5.3	13.3	0.32
	9	18	5.1	102.2	0.5	0.6	9.1	11.0	0.31

Table 2014.08B. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	9	18	18.4	92.1	1.1	1.9	5.7	10.5	0.36
Naph	8	16	27.7	110.7	2.8	2.8	10.3	10.3	0.37
	9	18	84.1	105.1	5.6	8.8	6.7	10.5	0.45
	8	16	158.7	99.2	9.7	34.2	6.1	21.6	1.02
Phe	8	16	15.1	100.5	0.5	1.2	3.3	7.8	0.26
	9	18	49.7	99.4	1.5	3.0	3.1	6.0	0.24
	9	18	168.0	96.0	8.6	16.6	5.1	9.9	0.47
Pyr	9	18	14.8	98.5	0.9	1.3	6.1	8.8	0.29
	9	18	40.3	100.8	1.6	3.3	3.8	8.2	0.32
	8	16	118.7	95.0	2.9	6.4	2.5	5.4	0.25

and 4 mL hexane, followed by application of the 1 mL extract in hexane. The analytes are eluted with hexane–dichloromethane (3 + 1, v/v) using volume determined for the given silica gel SPE cartridges from the elution profiles of target analytes and fat, which are dependent on the silica deactivation. The clean extract is carefully evaporated, reconstituted in 0.5 mL isooctane, and analyzed by GC/MS. *See* Figure **2014.08A** for the method flow chart.

B. Apparatus

(**a**) *Homogenizer*.—WARING blender Model 38BL40 (Conair Corp., Stamford, CT) or equivalent.

(**b**) *Solvent evaporator*.—Any suitable solvent evaporator, such as a rotary vacuum evaporator, Kuderna-Danish evaporator, or a nitrogen blow-down system, may be used as long as it provides results meeting the laboratory qualification/method set-up requirements (absolute analyte recoveries >70% in both evaporation steps).

(**c**) *Centrifuge*.—Capable of centrifugation of 50 mL tubes at >1500 rcf for 10 min.

(**d**) *Furnace/oven*.—Capable of 600°C operation.

(**e**) *Balance(s)*.—Analytical, capable of accurately measuring weights from 1 mg to 10 g.

(**f**) *Gas chromatograph-mass spectrometer*.—Any GC/MS instrument [single quadrupole, triple quadrupole, time-of-flight (TOF), or ion trap] with electron ionization (EI) may be used as long as it provides results meeting the laboratory qualification requirements (to provide reliable results for the calibration range specified in Table **2014.08A**).

(**g**) *GC column*.—Capillary column BPX-50 (30 m, 0.25 mm id, 250 µm film thickness; Trajan Scientific, Austin, TX, USA) or equivalent (USP specification G3), such as Rxi-17Sil MS (Restek Corp., Bellefonte, PA, USA); DB-17MS, DB-17, or HP-50 (Agilent Technologies, Santa Clara, CA, USA); or any other column that enables adequate separation of PAHs as specified in the laboratory qualification requirements (*see* **G**).

C. Reagents and Materials

(**a**) *Hexane.*—>98.5%, mixture of isomers.

(**b**) *Isooctane.*—ACS or better grade.

(**c**) *Ethyl acetate.*—>99.5%, for GC residue analysis.

(**d**) *Dichloromethane.*—≥99.9%, for GC residue analysis.

(**e**) *Toluene.*—≥99.9%, for GC residue analysis.

(**f**) *Water.*—Purified, free of interfering compounds.

(**g**) *Anhydrous sodium sulfate (Na_2SO_4).*—≥99.0%, powder, heated at 600°C for 7 h and then stored in a desiccator before use (Na_2SO_4 prepared and stored as indicated can be used for 1 month from preparation).

(**h**) *Silica gel SPE column.*—Containing 1 g silica gel. Any commercially available silica gel SPE column can be used as long as it provides adequate fat cleanup and meets requirements for low background contamination specified in the laboratory qualification requirements: the concentrations of all analytes in the reagent blanks had to be below the concentrations in the lowest calibration level standard; for naphthalene, levels below the second lowest calibration standard (equivalent to 5 ng/g naphthalene in the sample) are still acceptable if the source of contamination could not be eliminated.

Silica gel SPE columns can be prepared in-house using the following procedure: Activate the silica gel by heating at 180°C for 5 h, and then deactivate it by adding 5% deionized water, shaking for 3 h. Store in a desiccator for 16 h before use (silica gel prepared and stored as indicated can be used for 14 days). Place a piece of deactivated glass wool in a Pasteur pipet (5 mL), add 1 g activated silica gel (Silica gel 60, 0.063–0.2 mm, 70–230 mesh or equivalent) and top it with approximately 0.2 g muffled anhydrous Na_2SO_4.

(**i**) *Anhydrous magnesium sulfate ($MgSO_4$).*—≥99.0%, powder, heated (muffled) at 600°C for 7 h, and then store in a desiccator before use ($MgSO_4$ prepared and stored as indicated can be used for 1 month from preparation). *Note*: A preweighed (commercially available) mixture of 2 g sodium chloride and 4 g anhydrous magnesium sulfate (muffled) in pouches or tubes can be used.

(**j**) *Sodium chloride (NaCl).*—≥99.0%.

(**k**) *Helium 5.0 or better, nitrogen 4.0 or better.*

(**l**) *Polypropylene centrifuge tubes.*—50 mL.

(**m**) *Glass Pasteur pipet.*—5 mL (for solvent transfers and/or in-house preparation of silica gel minicolumns).

(**n**) *Syringes/pipets.*—Capable of accurate measurement and transfer of appropriate volumes for standard solution preparation and sample fortification (50–1000 µL).

(**o**) *Volumetric flasks*.—5–100 mL.

(**p**) *Glassware for evaporation steps*.—Depending on the

Table 2014.08C. Statistical results for the studied PAHs at three different concentration levels in mussel after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	10	20	38.1	95.3	5.7	8.8	14.9	23.2	0.89
	9	18	39.4	98.6	4.6	8.3	11.7	21.1	0.81
	10	20	38.9	97.3	4.6	8.9	11.9	22.8	0.87
1-MN	10	20	19.3	96.5	3.1	4.5	16.2	23.2	0.80
	10	20	96.9	96.9	9.0	19.2	9.2	19.8	0.87
	8	16	208.7	104.4	26.6	26.6	12.7	12.7	0.63
1-MP	9	18	9.8	98.1	0.7	1.8	7.2	18.0	0.56
	9	18	45.8	91.6	5.3	9.6	11.6	21.0	0.82
	9	18	117.2	93.7	10.2	24.4	8.7	20.8	0.94
2,6-DMN	10	20	13.8	91.9	1.5	2.9	11.2	20.7	0.68
	8	16	65.4	87.2	3.1	13.6	4.8	20.7	0.86
	10	20	153.8	87.9	10.7	36.8	6.9	23.9	1.13
3-MC	10	20	9.9	98.6	0.7	1.7	7.6	16.7	0.52
	10	20	87.2	96.8	4.1	10.9	4.7	12.5	0.54
	10	20	138.0	95.2	3.8	16.7	2.8	12.1	0.56
Ant	10	20	3.9	77.9	0.3	1.3	6.7	32.5	0.88
	9	18	13.0	86.8	1.7	3.2	12.8	24.8	0.81
	9	18	31.5	78.8	1.4	7.9	4.6	25.0	0.93
BaA	10	20	4.3	85.1	0.3	0.7	6.9	15.9	0.44
	10	20	21.5	85.9	1.1	2.3	5.1	10.9	0.38
	8	16	52.6	87.7	1.3	2.2	2.5	4.2	0.17
BaP	9	18	1.6	79.2	0.1	0.3	5.6	20.5	0.49
	9	18	8.1	80.5	0.6	1.4	6.9	17.7	0.54
	9	18	19.3	77.3	0.6	2.6	3.0	13.4	0.46
BbF	10	20	4.7	94.4	0.3	0.5	7.2	10.6	0.30
	10	20	27.1	90.2	1.9	2.8	6.9	10.2	0.37
	10	20	69.1	92.1	2.1	5.8	3.0	8.4	0.35
BghiP	9	18	2.0	98.1	0.1	0.2	4.9	10.6	0.26
	10	20	9.3	92.7	0.4	1.1	4.4	12.1	0.37
	10	20	17.9	89.6	0.6	1.5	3.2	8.6	0.29
BkF	9	18	1.9	97.4	0.1	0.2	7.2	10.3	0.25
	10	20	18.5	92.7	1.1	2.4	6.2	12.8	0.44
	10	20	36.6	91.5	1.0	3.8	2.7	10.4	0.40
Chr	10	20	14.2	94.4	0.8	1.3	5.7	9.5	0.31
	10	20	91.6	91.6	4.1	8.6	4.5	9.4	0.41
	10	20	159.8	91.3	4.9	11.8	3.1	7.4	0.35
DBahA	10	20	1.9	93.1	0.2	0.2	9.1	11.2	0.27
	10	20	9.1	90.5	0.5	1.2	5.4	13.5	0.41
	10	20	13.6	90.8	0.5	1.2	4.0	8.5	0.28
Fln	10	20	5.4	107.7	0.2	0.6	3.5	10.6	0.30
	10	20	25.5	101.8	1.0	1.9	3.7	7.6	0.27
	9	18	48.1	96.2	1.7	2.4	3.5	4.9	0.20
Flt	9	18	10.2	102.4	1.1	1.4	10.7	13.2	0.41
	10	20	48.9	97.7	2.7	4.3	5.5	8.8	0.35
	9	18	93.3	93.3	4.9	6.6	5.3	7.1	0.31
IcdP	10	20	2.0	97.7	0.1	0.2	7.3	11.3	0.28
	10	20	9.4	93.7	0.5	1.1	5.6	11.9	0.37

Table 2014.08C. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	10	20	17.9	89.3	0.9	1.6	4.9	8.8	0.30
Naph	9	18	23.7	94.6	1.9	4.0	8.1	17.1	0.61
	10	20	105.9	84.7	10.1	26.7	9.5	25.2	1.12
	8	16	146.7	91.7	7.2	19.2	4.9	13.1	0.61
Phe	8	16	14.5	96.8	0.8	0.9	5.3	6.1	0.20
	8	16	93.1	93.1	3.9	8.5	4.1	9.2	0.40
	8	16	160.8	91.9	5.8	13.0	3.6	8.1	0.38
Pyr	10	20	14.2	94.6	0.7	1.3	5.0	9.3	0.31
	10	20	71.5	95.4	2.9	7.0	4.0	9.8	0.41
	10	20	116.5	93.2	4.5	9.6	3.8	8.3	0.37

evaporation technique (e.g., small round-bottom flasks, suitable tubes, or glassware for Kuderna-Danish evaporation). It is recommended to heat the glassware for at least 2 h at 250°C to remove potential contamination.

D. Reference Standards

(**a**) *PAH standards.*—High-purity reference standards of the PAH analytes (1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*k*]fluoranthene, chrysene, dibenz[*a,h*]anthracene, fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, naphthalene, phenanthrene, and pyrene).

(**b**) *U.S. Environmental Protection Agency (EPA) 16 PAH cocktail.*—(^{13}C, 99%), Product No. ES-4087 (5 µg/mL, 1.2 mL in nonane), Cambridge Isotope Labs (Tewksbury, MA, USA) or equivalent.

Containing: Acenaphthene ($^{13}C_6$, 99%), acenaphthylene ($^{13}C_6$, 99%), anthracene ($^{13}C_6$, 99%), benz[*a*]anthracene ($^{13}C_6$, 99%), benzo[*b*]fluoranthene ($^{13}C_6$, 99%), benzo[*k*]fluoranthene ($^{13}C_6$, 99%), benzo[*g,h,i*]perylene ($^{13}C_{12}$, 99%), benzo[*a*]pyrene ($^{13}C_4$, 99%), chrysene ($^{13}C_6$, 99%), dibenz[*a,h*]anthracene ($^{13}C_6$, 99%), fluoranthene ($^{13}C_6$, 99%), fluorene ($^{13}C_6$, 99%), indeno[1,2,3-*cd*]pyrene ($^{13}C_6$, 99%), naphthalene ($^{13}C_6$, 99%), phenanthrene ($^{13}C_6$, 99%), and pyrene ($^{13}C_6$, 99%).

E. Preparation of Standard Solutions

(**a**) *Individual stock solutions.*—Prepare individual PAH stock solutions at approximately 1000 or 2500 µg/mL in toluene.

(**b**) *Mixed stock standard solution.*—Use analyte individual stock solutions to obtain a mixed solution of each PAH at 10 µg/mL (for benzo[*a*]pyrene) and other low-level PAHs) or 25 µg/mL (for chrysene and other higher-level PAHs) or 50 µg/mL (for naphthalene) in isooctane. *See* Table **2014.08E** for analyte concentrations in the mixed stock standard solution.

(**c**) *Working PAH Solution A.*—Accurately transfer 0.5 mL of the mixed stock standard solution into a 5 mL volumetric flask and dilute to volume with isooctane.

(**d**) *Working PAH Solution B.*—Accurately transfer 0.5 mL of the Working PAH Solution A into a 5 mL volumetric flask and dilute to volume with isooctane.

(**e**) *Internal standard solution.*—Prepare 1 µg/mL solution of ^{13}C-PAHs in isooctane by 5-fold dilution of the 5 µg/mL EPA 16 ^{13}C-PAHs cocktail with isooctane.

(**f**) *Calibration standard solutions*—Prepare eight levels of calibration standard solutions (1 mL each) in 2 mL amber screw-cap vials. It is recommended to distribute small portions (enough for a single injection) of the calibration standard solutions into multiple crimp-top vials with 100 µL deactivated glass inserts. *See* Table **2014.08A** for analyte concentrations in the calibration standards and Table **2014.08F** for the dilution scheme.

(*1*) *For level 1 calibration standard*.–Accurately transfer 50 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 900 µL isooctane. Cap the vial and vortex mix briefly.

(*2*) *For level 2 calibration standard*.–Accurately transfer 100 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

(*3*) *For level 3 calibration standard*.–Accurately transfer 200 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 750 µL isooctane. Cap the vial and vortex mix briefly.

(*4*) *For level 4 calibration standard*.–Accurately transfer 500 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 450 µL isooctane. Cap the vial and vortex mix briefly.

(*5*) *For level 5 calibration standard*.–Accurately transfer 100 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

(*6*) *For level 6 calibration standard*.–Accurately transfer 200 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 750 µL isooctane. Cap the vial and vortex mix briefly.

(*7*) *For level 7 calibration standard*.–Accurately transfer 500 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 450 µL isooctane. Cap the vial and vortex mix briefly.

(*8*) *For level 8 calibration standard*.–Accurately transfer 100 µL of the mixed stock standard solution into the vial and

Table 2014.08D. Statistical results for the studied PAHs at three different concentration levels in oyster after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	8	16	72.3	90.4	4.1	11.7	5.7	16.2	0.68
	8	16	69.0	86.2	4.9	12.6	7.1	18.3	0.76
	8	16	65.6	82.0	5.5	13.2	8.4	20.1	0.83
1-MN	8	16	67.9	90.5	4.3	14.8	6.4	21.8	0.91
	9	18	90.8	90.8	20.6	28.9	22.7	31.9	1.39
	9	18	236.6	94.6	26.4	55.3	11.2	23.4	1.18
1-MP	7	14	20.2	80.8	1.8	4.8	8.8	23.9	0.83
	8	16	39.9	79.8	2.6	7.7	6.4	19.3	0.74
	8	16	154.7	77.3	16.9	34.7	10.9	22.4	1.06
2,6-DMN	7	14	30.5	76.2	3.0	5.3	9.8	17.5	0.65
	7	14	57.9	77.2	4.9	10.8	8.5	18.6	0.76
	7	14	161.3	71.7	12.7	20.9	7.9	13.0	0.62
3-MC	9	18	27.8	92.5	1.7	3.6	6.0	13.0	0.47
	9	18	79.8	88.6	5.1	10.3	6.4	13.0	0.55
	9	18	196.8	87.5	10.1	23.5	5.1	12.0	0.59
Ant	7	14	5.3	53.2	0.5	2.9	8.8	55.0	1.56
	7	14	7.5	50.3	0.8	4.9	10.0	64.7	1.94
	6	12	34.0	56.6	3.0	15.1	8.8	44.5	1.67
BaA	9	18	10.9	72.6	0.8	2.2	7.7	19.7	0.62
	9	18	17.2	68.6	1.0	3.6	5.8	21.1	0.71
	9	18	71.6	71.6	3.9	12.5	5.4	17.5	0.73
BaP	9	18	2.5	49.7	0.3	1.1	11.9	43.5	1.10
	9	18	4.8	48.2	0.4	2.0	9.0	42.2	1.18
	9	18	24.6	49.3	1.6	10.0	6.4	40.5	1.45
BbF	9	18	8.6	85.9	0.6	1.0	6.6	11.6	0.35
	9	18	24.6	81.8	1.7	2.7	7.1	11.2	0.40
	9	18	82.8	82.8	3.4	9.8	4.1	11.9	0.51
BghiP	9	18	4.1	82.4	0.2	0.5	5.9	12.3	0.34
	9	18	8.2	81.9	0.7	1.1	8.6	13.6	0.41
	9	18	19.6	78.4	1.0	2.3	4.9	11.7	0.41
BkF	9	18	6.9	85.9	0.5	1.1	7.7	16.3	0.48
	9	18	16.9	84.3	1.1	2.6	6.5	15.4	0.52
	9	18	62.9	83.8	3.8	8.2	6.1	13.1	0.54
Chr	9	18	43.0	85.9	2.8	4.3	6.5	9.9	0.39
	9	18	81.6	81.6	5.0	8.6	6.2	10.6	0.45
	9	18	204.1	81.6	8.7	19.0	4.3	9.3	0.46
DBahA	9	18	4.1	82.7	0.4	0.5	9.0	13.0	0.35
	8	16	8.2	82.2	0.6	1.1	7.5	13.4	0.41
	9	18	16.0	80.0	0.7	2.0	4.4	12.7	0.43
Fln	9	18	12.5	83.3	1.0	1.9	8.2	15.4	0.50
	9	18	20.3	81.2	1.4	3.0	6.8	14.6	0.51
	9	18	57.0	76.0	2.3	11.3	4.0	19.9	0.81
Flt	9	18	22.0	88.2	2.1	3.6	9.5	16.2	0.57
	9	18	42.0	83.9	5.1	7.5	12.2	17.9	0.69
	9	18	120.7	80.5	7.0	17.1	5.8	14.2	0.65
IcdP	9	18	4.3	86.8	0.4	0.6	9.6	13.1	0.36
	9	18	8.3	83.3	0.8	1.1	9.0	13.7	0.42

Table 2014.08D. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	9	18	20.0	80.1	1.0	2.2	4.8	10.7	0.37
Naph	9	18	71.0	88.7	5.3	9.4	7.5	13.2	0.55
	9	18	106.2	84.9	7.3	14.7	6.9	13.9	0.62
	8	16	193.9	86.2	6.0	29.8	3.1	15.4	0.75
Phe	9	18	41.6	83.2	3.0	5.5	7.2	13.2	0.51
	9	18	80.3	80.3	6.1	10.7	7.6	13.3	0.57
	8	16	203.9	81.6	9.5	22.5	4.7	11.0	0.54
Pyr	9	18	34.0	85.1	2.2	3.3	6.4	9.8	0.37
	8	16	63.2	84.3	2.2	5.3	3.5	8.4	0.35
	9	18	163.4	81.7	8.0	16.6	4.9	10.2	0.48

add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

F. Extraction and Cleanup Procedure

(*1*) Add 50 µL of the 1 µg/mL ^{13}C-PAHs solution to 10 ± 0.1 g of thoroughly homogenized seafood sample in a 50 mL polypropylene centrifuge tube.

(*2*) Vortex sample for 15 s and let equilibrate for 15 min.

(*3*) Add 5 mL (10 mL in the case of shrimp) of purified water and 10 mL ethyl acetate.

(*4*) Shake tube vigorously by hand for 1 min.

(*5*) Add 4 g of muffled anhydrous magnesium sulfate and 2 g sodium chloride, and seal the tube well (ensure that powder does not get into the screw threads or rim of the tube).

(*6*) Shake tube vigorously by hand for 1 min, ensuring that crystalline agglomerates are broken up sufficiently during shaking.

(*7*) Centrifuge tube at >1500 rcf for 10 min.

(*8*) Take a 5 mL aliquot of the upper ethyl acetate layer, add 50 µL isooctane as a keeper, and gently evaporate all ethyl acetate until only isooctane and co-extracted sample fat are left.

(*9*) Reconstitute in 1 mL hexane.

(*10*) Condition a silica SPE column (1 g silica gel with approximately 0.2 g of muffled anhydrous sodium sulfate on the top) with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane.

(*11*) Apply the extract in hexane onto the silica SPE cartridge.

(*12*) Elute with hexane–dichloromethane (3 + 1, v/v) using volume determined for the given silica gel SPE cartridges from the elution profiles of target analytes and fat, which are dependent on the silica deactivation, *see Note* (*4*) below. Collect the eluent.

(*13*) Add 0.5 mL isooctane (and 1-2 mL ethyl acetate) to the eluent as a keeper and gently evaporate down to 0.5 mL to remove hexane and dichloromethane from the final extract.

(*14*) Transfer the final extract into an autosampler vial for the GC/MS analysis.

Notes: (*1*) The fat capacity of the 1 g silica gel SPE column is approximately 0.1 g. If the 5 mL ethyl acetate extract aliquot contains more than 0.1 g fat, it is necessary to use a smaller aliquot volume to avoid sample breakthrough during the cleanup step.

(*2*) Ethyl acetate should not be present in the extract applied to the silica cartridge because it can affect the extract polarity, thus potentially retention of fat and analytes on the silica gel. The coextracted fat and 50 µL isooctane act as keepers during the first evaporation step (step *8*), thus the evaporation should be conducted gently until there is no significant change in the volume, i.e., until only the isooctane and coextracted fat are left in the evaporation tube or flask.

(*3*) Addition of 1-2 mL ethyl acetate to the eluent in step *13* is recommended for a better control of the evaporation process and higher absolute recoveries of volatile PAHs.

(*4*) The deactivation and storage of silica gel SPE cartridges can vary, potentially resulting in different amounts of water in the silica, thus its potentially different retention characteristics. Therefore, it is important to test the elution profiles of PAHs and fat and determine the optimum volume of the elution solvent to ensure adequate analyte recoveries and fat cleanup. The following procedure is recommended:

(**a**) Prepare a PAH solution in hexane by combining 50 µL of the Working PAH Solution A and 1 mL hexane in a vial. Mix well and apply onto a silica SPE column (1 g silica gel with approximately 0.2 g of muffled anhydrous sodium sulfate on the top), which was conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane.

(**b**) Elute with 10 mL hexane–dichloromethane (3 + 1, v/v),

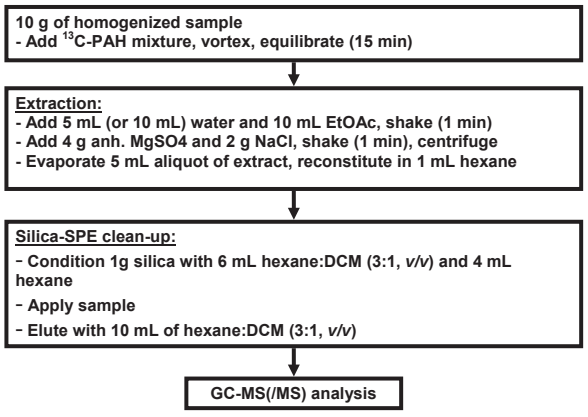


Figure 2014.08A. Flow chart of the method for determination of PAHs in seafood using GC/MS.

Table 2014.08E. Analyte concentrations in the mixed stock standard solution

Analyte	Concentration, µg/mL
Anthracene	10
Benz[*a*]anthracene	10
Benzo[*a*]pyrene	10
Benzo[*b*]fluoranthene	10
Benzo[*g,h,i*]perylene	10
Benzo[*k*]fluoranthene	10
Chrysene	25
Dibenz[*a,h*]anthracene	10
Fluoranthene	25
Fluorene	10
Indeno[1,2,3-*cd*]pyrene	10
Naphthalene	50
Phenanthrene	25
Pyrene	25
1-Methylnaphthalene	25
2,6-Dimethylnaphthalene	25
1-Methylphenanthrene	25
1,7-Dimethylphenanthrene	10
3-Methylchrysene	25

collecting 0.5 mL elution fractions in 20 evaporation tubes or flasks. Add 0.5 mL isooctane to each elution fraction and evaporate down to 0.5 mL using the optimized evaporation conditions. Analyze each fraction by GC/MS.

(**c**) Determine PAH elution profile by plotting analyte response (peak area or height) in a given fraction normalized to the sum of analyte responses in all tested fractions vs the elution volume. *See* Figure **2014.08B** for an example of a PAH elution profile. It is recommended to add an additional 0.5 mL on top of the determined elution fraction (corresponding to 100% recovery) as a safety margin ensuring good analyte recoveries in routine practice. This would result in the optimum elution volume of 7 mL for the silica cartridge tested in Figure **2014.08B**.

Table 2014.08F. Dilution scheme for preparation of the calibration standard solutions

Calibration level	Vol. of mixed stock standard solution, µL	Vol. of working PAH solution[a], µL	Vol. of working PAH solution B, µL	Vol. of ^{13}C-PAH 1 µg/mL solution, µL	Final vol.[a], µL
1	—	—	50	50	1000
2	—	—	100	50	1000
3	—	—	200	50	1000
4	—	—	500	50	1000
5	—	100	—	50	1000
6	—	200	—	50	1000
7	—	500	—	50	1000
8	100	—	—	50	1000

[a] Bring to volume using isooctane.

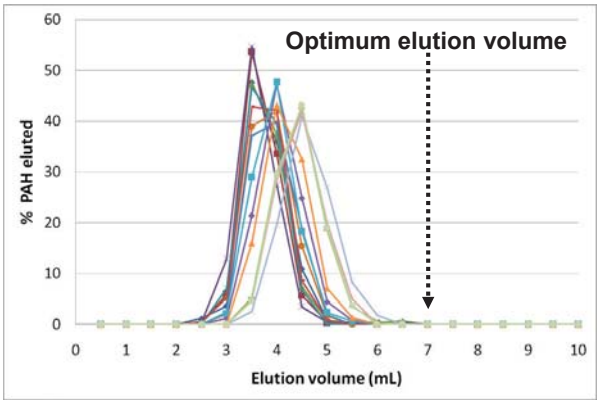


Figure 2014.08B. An example of elution profiles of PAHs on a silica gel SPE cartridge and determination of the optimum elution volume.

(**d**) To check the effectiveness of fat removal, dissolve 100 mg pure fish oil (or any suitable fat) in 1 mL hexane and apply it onto the silica gel cartridge, which was conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane. Elute with the optimum elution volume of hexane–dichloromethane (3 + 1, v/v), which was determined in the previous step (e.g., 7 mL for the example in Figure **2014.08B**). Collect this fraction in an evaporation tube or flask, which empty weight (after heating in an oven to remove moisture) was recorded to 4 decimal places using an analytical balance. Elute the cartridge with additional 3 × 1 mL hexane–dichloromethane (3 + 1, v/v) and collect in three evaporation tubes/flasks of known empty weight.

(**e**) Evaporate the four elution fractions to dryness and gravimetrically determine the amount of fat eluting in each fraction by subtracting the empty weights from newly recorded weights after solvent evaporation. There should be no fat eluting in the optimum elution fraction for PAHs (this can also be observed visually in the tubes).

(**f**) If there is fat coeluting with PAHs, then the PAH and fat elution profiles have to be reexamined to determine optimum elution volume for PAH and fat separation (potentially sacrificing up to 5% of late-eluting PAH amounts if necessary) or a different silica gel cartridge has to be used.

G. GC/MS Analysis

(**a**) *GC conditions.*—Table **2014.08G** provides GC conditions that were used by the collaborative study participants. Other conditions (e.g., column, temperature and flow program, and injection technique and volume) can be used as long as the laboratory qualification criteria for separation, sensitivity, and linearity are met. The injection temperature or program needs to be optimized to enable quantitative transfer of less volatile PAHs. If programmable temperature vaporizer (PTV) solvent vent mode is used, solvent venting parameters (temperature, time, flow, pressure) need to be carefully optimized to prevent losses of the volatile PAHs, especially naphthalene. The separation criteria (demonstrated in Figure **2014.08C**) include (*1*) a baseline separation of benzo[*a*]pyrene and benzo[*e*]pyrene (concentration ratio of 1:5), (*2*) at least 50% valley separation of anthracene and phenanthrene (concentration ratio 1:2.5; evaluated for the anthracene peak), and (*3*) at least 50% valley

Table 2014.08G. GC conditions used by collaborative study participants

Laboratory No.	1	2	3	4	5
GC Instrument	Thermo Trace GC Ultra	Agilent 6890N	Agilent 6890N	Agilent 6890	Agilent 7890A
Inlet type	PTV (Thermo 816, RP - 2004)	PTV (Gerstel CIS 4)	S/SL (Agilent)	PTV (Gerstel CIS 4)	Multi-mode (Agilent)
Column type	Thermo TR-50MS	Restek Rxi-17Sil MS	Agilent J&W DB-17MS	Restek Rxi-17Sil MS	Restek Rxi-17Sil MS
Column dimension	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm
Retention gap dimension	NA	NA	NA	2 m × 0.25 mm id, 0.25 µm	NA
Run time, min	40	49.97	47.17	46	39.33
Oven temperature program	60°C (1 min), 12°C/min to 210°C, 8°C/min to 340°C (10 min)	50°C (4.3 min), 30°C/min to 220°C, 2°C/min to 240°C, 5°C/min to 360°C (6 min)	80°C (1 min), 30°C/min to 220°C, 2°C/min to 270°C (1.5 min), 5°C/min to 320°C	65°C (4 min), 30°C/min to 220°C (0.5 min), 3°C/min to 320°C (5 min)	65°C (0.5 min), 15°C/min to 220°C, 4°C/min to 330°C (1 min)
He flow/pressure program	1.2 mL/min (constant flow)	1.3 mL/min (constant flow)	1.3 mL/min (constant flow)	1.3 mL/min (15 min), 50 mL/min^2 to 2 mL/min	2 mL/min (constant flow)
Injection mode	PTV solvent split	PTV solvent vent	Pulsed splitless	PTV solvent vent (stop flow)	PTV solvent vent
Injection volume, µL	8	8	1	8	5
Inlet temperature (program)	50°C (2.3 min), 6.7°C/s to 300°C (1 min), 10°C/s to 350°C (10 min)	50°C (2.3 min), 12°C/s to 340°C	320°C	15°C (0.5 min), 12°C/min to 275°C	70°C (0.15 min), 600°C/min to 325°C (5 min)
Liner	Thermo, LVI Silcosteel, 2 mm id	Gerstel, CIS4 deactivated baffled liner, 2 mm id	Restek, single-gooseneck splitless, 4 mm id	Gerstel, CIS4 deactivated baffled liner, 2 mm id	Agilent, dimpled deactivated, 2 mm id
PTV solvent vent time	0.1 min	138 s	NA	0.5 min	0.1 min
PTV solvent vent flow, mL/min	50	50	NA	100	50
PTV solvent vent pressure, psi	NA	50	NA	1.80	5
Pressure pulse and duration	NA	NA	50 psi for 0.2 min	NA	NA
Split vent purge flow and start	50 mL/min, 1 min	20 mL/min	50 mL/min, 1 min	50 mL/min, 2.5 min	60 mL/min, 2.6 min
Gas saver flow and start time	15 mL/min, 3 min	NA	15 mL/min, 6 min	30 mL/min, 3 min	NA
MS transfer line temperature, °C	250	280	280	280	300
	6	7	8	9	10
GC instrument	Agilent 7890	Agilent 7890	Agilent 6890N	Agilent 6890N	Agilent 6890N
Inlet type	S/SL (Agilent)	S/SL (Agilent)	S/SL (Agilent)	PTV (Apex, ProSep 800 Plus)	PTV (Gerstel CIS 4)
Column type	Restek Rxi-17Sil MS	Restek Rxi-17Sil MS	Agilent J&W DB-EUPAH	Phenomenex ZB-50	Agilent J&W DB-EUPAH
Column dimension	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	20 m × 0.18 mm id, 0.14 µm	30 m × 0.25 mm id, 0.25 µm	20 m × 0.18 mm id, 0.14 µm
Retention gap dimension	1 m × 0.25 mm	NA	NA	NA	NA
GC run time, min	46.33	41.63	31.83	48.27	36.17
Oven temperature program	65°C (0.5 min), 15°C/min to 220°C, 4°C/min to 330°C (8 min)	80°C (4.3 min), 30°C/min to 220°C, 2°C/min to 240°C, 5°C/min to 300°C (1 min), 30°C/min to 350°C (8 min)	80°C (1 min), 30°C/min to 130°C, 12°C/min to 240°C, 3.5°C/min to 310°C	80°C (4.6 min), 30°C/min to 130°C (5 min), 10°C/min to 260°C (15 min), 10°C/min to 320°C (3 min); Post run: 80°C (1 min)	55°C (3 min), 30°C/min to 220°C, 2°C/min to 240°C, 3°C/min to 285°C; 15°C/min to 310°C (1 min); Post run: 330°C (2 min)

Table 2014.08G. (*continued*)

Laboratory No.	6	7	8	9	10
He flow/pressure program	1.5 mL/min (13 min), 10 mL/min^2 to 2 mL/min	1.5 mL/min (constant flow)	1 mL/min (constant flow)	1.3 mL/min (19.5 min), 50 mL/min^2 to 2 mL/min	2 mL/min (constant flow)
Injection mode	Hot splitless	Hot splitless	Pulsed splitless	Splitless	PTV solvent vent
Injection volume, µL	1	1	1	10	5
Inlet temperature (program)	300°C	280°C	320°C	60°C (2.25 min), 300°C/min to 335°C (45 min)	50°C (0.5 min), 600°C/min 400°C (15 min), 30°C/min 70°C
Liner	SGE, Focus liner	Agilent, single taper, glass wool-packed, 4 mm id	Agilent, double taper, 4 mm id	Apex, ProSep taper liner, 2 mm id	Agilent, PTV multi-baffle, 2 mm id
PTV solvent vent time	NA	NA	NA	NA	0.5 min
PTV solvent vent flow	NA	NA	NA	NA	100 mL/min
PTV solvent vent pressure	NA	NA	NA	NA	50 kPa
Pressure pulse and duration	NA	NA	50 psi for 0.2 min	NA	NA
Split vent purge flow and start	50 mL/min, 1 min	50 mL/min, 1 min	50 mL/min, 1 min	50.0 mL/min, 2.30 min	100 mL/min, 3 min
Gas saver flow and start time	20 mL/min, 3 min	20 mL/min, 2 min	15 mL/min, 6 min	NA	15 mL/min, 6 min
MS transfer line temperature, °C	300	280	320	300	325

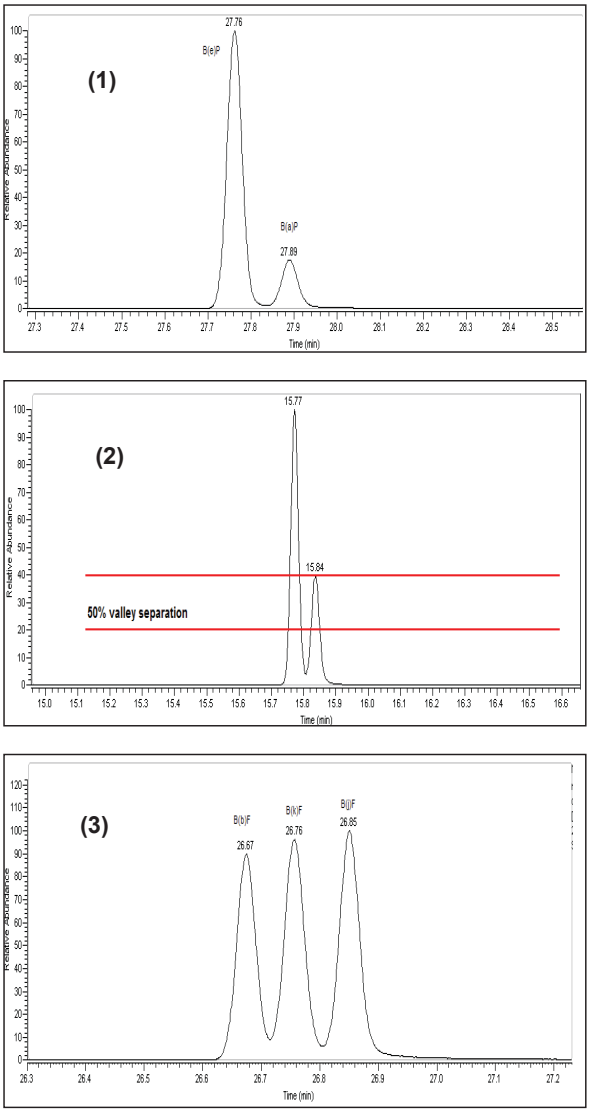


Figure 2014.08C. GC separation criteria: (1) A baseline separation of benzo[*a*]pyrene and benzo[*e*]pyrene (concentration ratio of 1:5), (2) at least 50% valley separation of phenanthrene and anthracene (concentration ratio 2.5:1; evaluated for the anthracene peak, which is the second peak in the figure), and (3) at least 50% valley separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*]fluoranthene (concentration ratio of 1:1:1).

separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*]fluoranthene (concentration ratio of 1:1:1). *Note*: Criteria for separation of chrysene and triphenylene (another PAH critical pair) were not set for the collaborative study. For accurate quantitation of chrysene, at least 50% valley separation is recommended, which can be achieved using selective stationary phases.

The maximum oven temperature program may not exceed the maximum temperature limit for a given column. Backbone-modified columns, such as Rxi-17Sil MS or DB-17MS, are recommended for their better temperature stability and also good selectivity for critical PAH pairs or groups, including the anthracene/phenanthrene pair or benzofluoranthenes. Conduct proper inlet and column

Table 2014.08H. MS ions (*m/z*) and MS/MS precursor to product ion transitions used by study participants for quantification (quant) and identification (qual) of target PAHs and ^{13}C-PAHs using single-stage MS (single quadrupole and TOF) and tandem MS/MS (triple quadrupole) instruments, respectively

	Single quad/TOF MS		Triple quad MS/MS	
Compound	Quant	Qual	Quant	Qual
1,7-DMP	206	191	206>190	206>205, 206>165
1-MN	142	115	142>115	142>141, 142>116
1-MP	192	189	192>191	192>165
2,6-DMN	156	141, 144	156>115	156>141
3-MC	242	241	242>239	242>226
Ant	178	177	178>176	178>177, 178>151
BaA	228	226	228>226	228>224, 228>202
BaP	252	253	252>250	250>248, 252>224
BbF	252	253	252>250	250>248, 252>224
BghiP	276	277	276>274	274>272, 276>275
BkF	252	253	252>250	250>248, 252>224
Chr	228	226	228>226	228>224, 228>202
DBahA	278	276	278>276	276>274, 278>274
Fln	166	165	166>165	166>164, 166>163
Flt	202	200	202>200	202>201
IcdP	276	277	276>274	274>272, 276>248
Naph	128	127	128>102	128>127
Phe	178	177	178>176	178>177, 178>151
Pyr	202	200	202>200	202>201
13C-Ant	184	183	184>183	184>182, 184>156
13C-BaA	234	232	234>232	234>206
13C-BaP	256	257	256>254	256>228
13C-BbF	258	259	258>256	258>255
13C-BghiP	288	289	288>286	288>287
13C-BkF	258	259	258>256	258>255
13C-Chr	234	232	234>232	234>206
13C-DBahA	284	282	284>282	284>280
13C-Fln	172	171	172>171	172>170
13C-Flt	208	205	208>206	208>207
13C-IcdP	282	283	282>280	282>281
13C-Naph	134	133	134>133	134>105
13C-Phe	184	183	184>183	184>156
13C-Pyr	205	203, 206, 208	205>203	205>204

maintenance to ensure adequate operation of the GC instrument. Perform system checks.

(**b**) *MS conditions.*—Any GC/MS instrument (single quadrupole, triple quadrupole, TOF, or ion trap) with EI may be used as long as it provides results meeting the laboratory qualification requirements. The 10 study participants used the following instruments: single quadrupole (Agilent 5973–Laboratory 4; Agilent 5975B XL Inert–Laboratories 3 and 8–10; Agilent 5975C–Laboratories 6 and 7), triple quadrupole (Agilent 7000B–Laboratory 5; Thermo TSQ–Laboratory 1), and time-of-flight (Leco Pegasus 4D–Laboratory 2). Pay special attention to the optimization of the MS transfer line and MS source temperature. Higher MS source temperatures are recommended (e.g., ≥280°C for Agilent sources) to provide optimum analysis (quantitative transfers, minimum peak tailing) for less volatile PAHs.

Table **2014.08H** provides MS ions (*m/z*) and MS/MS transitions used by the study participants for quantification and identification of target PAHs and ^{13}C-PAHs using single-stage MS (single quadrupole and TOF) and tandem MS/MS (triple quadrupole) instruments, respectively.

Use adequate data acquisition rate (dwell times in scanning instruments) and solvent delay time. Perform air/water checks and autotune to verify and obtain adequate operation

Table 2014.08I. PAH analytes and corresponding ^{13}C-PAHs used for PAH signal normalization

Analyte	^{13}C-PAH used for signal normalization
Anthracene	Anthracene ($^{13}C_6$)
Benz[*a*]anthracene	Benz[*a*]anthracene ($^{13}C_6$)
Benzo[*a*]pyrene	Benzo[*a*]pyrene ($^{13}C_4$)
Benzo[*b*]fluoranthene	Benzo[*b*]fluoranthene ($^{13}C_6$)
Benzo[*g,h,i*]perylene	Benzo[*g,h,i*]perylene ($^{13}C_{12}$)
Benzo[*k*]fluoranthene	Benzo[*k*]fluoranthene ($^{13}C_6$)
Chrysene	Chrysene ($^{13}C_6$)
Dibenz[*a,h*]anthracene	Dibenz[*a,h*]anthracene ($^{13}C_6$)
Fluoranthene	Fluoranthene ($^{13}C_6$)
Fluorene	Fluorene ($^{13}C_6$)
Indeno[1,2,3-*cd*]pyrene	Indeno[1,2,3-*cd*]pyrene ($^{13}C_6$)
Naphthalene	Naphthalene ($^{13}C_6$)
Phenanthrene	Phenanthrene ($^{13}C_6$)
Pyrene	Pyrene ($^{13}C_6$)
1-Methylnaphthalene	Naphthalene ($^{13}C_6$)
2,6-Dimethylnaphthalene	Phenanthrene ($^{13}C_6$)
1-Methylphenanthrene	Phenanthrene ($^{13}C_6$)
1,7-Dimethylphenanthrene	Phenanthrene ($^{13}C_6$)
3-Methylchrysene	Chrysene ($^{13}C_6$)

of the instrument. Verify identification of the analyte peaks by comparing the ion ratios of contemporaneously analyzed calibration standards, which have been analyzed under the same conditions.

(**c**) *Injection sequence.*—Bracket the seven test samples with two sets of calibration standards. Inject solvent blanks after the calibration level 8 (highest) standard and after the samples. In addition, analyze a reagent blank with each set of samples. Inject only once from each vial, thus preventing potential losses of volatile PAHs and/or contamination.

H. Calculations

Quantification is based on linear least-squares calibration of analyte signals (S_{PAH}) divided by signals ($S_{13C\text{-}PAH}$) of corresponding ^{13}C-labeled internal standards (*see* Table **2014.08I**) plotted versus analyte concentrations. Peak areas are generally preferred as signals used for the quantification, but peak heights should be used for peaks that are not well resolved, such as in the case of anthracene and phenanthrene. The analyte concentrations in the final extract (c_{PAH}, µg/L) are determined from the equation:

$$c_{PAH} = [(S_{PAH}/S_{13C\text{-}PAH}) - b]/a$$

where a is the slope of the calibration curve and b is the y-intercept.

The concentration of PAHs in the sample (C, µg/kg) is then calculated:

$$C = (c_{PAH}/c_{13C\text{-}PAH}) \times (X_{13C\text{-}PAH}/m)$$

where $c_{13C\text{-}PAH}$ is the concentration of the corresponding ^{13}C-PAH in the calibration standard solutions (in µg/L); $X_{13C\text{-}PAH}$ is the amount of the corresponding ^{13}C-PAH added to the sample (in ng); and m is the sample weight (in g). Based on the method procedure and preparation of the calibration standard solutions, $c_{13C\text{-}PAH}$ is 50 µg/L, $X_{13C\text{-}PAH}$ is 50 ng, and m for the test samples is 10 g.

In the collaborative study, eight concentration levels were used for the calibration, corresponding to 5, 10, 20, 50, 100, 200, 500, and 1000 µg/L for benzo[*a*]pyrene and other lower-level PAHs, to 12.5, 25, 50, 125, 250, 500, 1250, and 2500 µg/L for higher-level PAHs, except for naphthalene that was present at 25, 50, 100, 250, 500, 1000, 2500, and 5000 µg/L. Coefficients of determination (r^2) should be 0.990 or greater and back-calculated concentrations of the calibration standards should not exceed ±20% of theoretical. For lower concentration levels, a limited calibration curve (without the higher-end concentration points) may be used for better accuracy. If a well-characterized quadratic relationship occurs, then a best-fitted quadratic curve may be used for calibration. Otherwise, if the back-calculated concentrations exceed ±20% of theoretical, normalized signals of the nearest two calibration standards that enclose the analyte signal in the sample can be used to interpolate the analyte concentration in the final extract.

Results and Discussion

Laboratory Qualification Phase

The analysis of PAHs poses several difficulties due to their physicochemical properties and occurrence in the environment and various materials that can lead to contamination issues. PAH properties, such as their volatility, polarity, and structure, affect their GC separation, MS determination/identification, and recoveries during solvent evaporation and silica SPE steps. To allow for flexibility and the use of various instruments, equipment, and columns, the Study Directors did not want to prescribe the use of a specific GC/MS instrument, GC column and separation conditions, silica SPE cartridge, and evaporation technique, equipment, or conditions. For this reason, they developed performance-based criteria for the GC/MS analysis (including separation of critical PAH pairs/groups, calibration range, or carryover), optimum elution volume in the SPE step (based on the elution profiles of PAHs and fat dependent on the silica deactivation), and evaporation conditions (to avoid significant loses of volatile PAHs, mainly naphthalene). These criteria were part of the laboratory qualification phase to help laboratories optimize conditions independent of their instrument/equipment choice or availability. This was also a very important consideration for the future implementation of the method in other laboratories.

Another essential step in the laboratory qualification phase involved check of reagent blanks for potential PAH contamination. The concentrations of all analytes in the reagent blanks had to be below the concentrations in the lowest calibration level standard. For naphthalene, levels below the second lowest calibration standard (equivalent to 5 ng/g of naphthalene in the sample) were still acceptable if the source of contamination could not be eliminated, such as by selection of a silica gel SPE column from a different vendor (or preparation of silica gel columns in-house), heating of glassware, addition of a hydrocarbon trap to the nitrogen lines used for solvent evaporation, etc. Some laboratories found that their reagent

Table 3. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the low PAH level in shrimp

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC S10	17.4	17.9	24.9	14.1	21.4	21.2	18.5	23.4	26.4
	SFC S11	23.2	25.6	27.0	14.2	21.4	20.8	23.1	23.1	27.4
1-MN	SFC S10	44.8	33.3	23.5	17.9	23.6	22.1	17.8	18.6	20.5
	SFC S11	24.6	18.0	25.4	19.6	26.4	20.2	22.3	19.3	17.5
1-MP	SFC S10	9.1	8.4	10.4	7.5	9.9	10.7	8.8	9.8	11.5
	SFC S11	10.7	10.5	12.3	7.5	9.8	10.4	11.2	9.3	11.9
2,6-DMN	SFC S10	32.0[a]	16.8	17.4	21.4	14.8	13.7	13.1	13.1	14.8
	SFC S11	35.3[a]	15.4	16.7	20.1	14.2	14.0	16.7	13.0	13.9
6-MC	SFC S10	7.8	11.6	12.5	10.9	10.6	10.8	9.7	11.9	11.8
	SFC S11	8.4	10.8	13.6	11.0	10.6	10.7	11.9	12.4	11.9
Ant	SFC S10	3.8	5.2	5.6	4.7	4.7	5.1	4.0	5.0	5.2
	SFC S11	4.6	5.2	5.9	4.7	4.7	5.0	5.1	5.1	5.0
BaA	SFC S10	3.9	5.5	4.6	4.7	4.6	4.7	4.2	5.1	4.9
	SFC S11	4.1	5.2	5.5	4.7	4.6	4.6	5.2	5.2	4.9
BaP	SFC S10	1.4	2.2	2.0	1.9	1.8	1.9	1.7	2.2	1.9
	SFC S11	1.6	2.0	2.2	1.9	1.8	1.9	2.1	2.2	1.9
BbF	SFC S10	3.7	5.2	5.1	4.8	4.6	4.7	4.3	5.1	4.7
	SFC S11	4.3	5.4	5.8	4.8	4.5	4.7	5.2	5.1	5.0
BghiP	SFC S10	1.4	2.2	1.9	1.9	1.8	1.9	1.7	2.0	2.7[a]
	SFC S11	1.6	2.1	2.2	1.9	1.8	1.9	2.0	2.0	3.5[a]
BkF	SFC S10	1.4	2.3	2.2	2.0	1.9	1.7	1.8	2.2	2.0
	SFC S11	1.6	2.2	2.3	2.0	1.9	1.6	2.3	2.2	2.0
Chr	SFC S10	12.3	17.6	15.5	14.9	14.2	14.7	13.2[a]	15.7	15.9
	SFC S11	13.1	16.8	16.9	15.0	14.3	14.5	16.2[a]	16.3	16.0
DBahA	SFC S10	1.3	2.1	2.2	2.0	1.8	2.0	1.7	2.1	2.3
	SFC S11	1.6	2.0	1.9	1.9	1.7	1.9	2.2	2.2	1.6
Fln	SFC S10	4.3	5.3	5.5	4.9	4.8	5.0	4.2[a]	5.6	6.0
	SFC S11	4.3	5.4	5.6	4.9	4.8	5.0	5.2[a]	5.4	5.9
Flt	SFC S10	7.2	10.6	9.8	9.6	9.2	9.5	8.5	10.6	10.8
	SFC S11	8.2	10.7	10.7	9.7	9.2	9.5	10.4	10.6	10.1
IcdP	SFC S10	1.4	2.2	1.9	2.0	1.7	2.0	1.7	2.3	2.1
	SFC S11	1.6	2.1	2.0	2.0	1.7	2.0	2.1	2.3	2.1
Naph	SFC S10	35.8	29.6	28.9	26.0	27.3	26.1	23.3	28.1	33.9[a]
	SFC S11	27.1	26.2	27.7	26.3	27.1	24.7	29.5	29.0	42.5[a]
Phe	SFC S10	12.2	16.1	14.4	14.7	15.0	14.9	12.7[b]	15.6	16.2
	SFC S11	13.2	16.3	16.1	14.6	14.9	15.2	15.9[b]	15.5	16.4
Pyr	SFC S10	12.0	16.0	14.4	14.8	14.2	14.6	12.6	15.6	16.0
	SFC S11	12.8	15.8	16.6	14.9	14.2	14.4	15.7	15.7	15.7

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 4. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the mid PAH level in shrimp

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC S9	28.6	17.4	26.2	14.9	21.6	21.0	20.3	23.9	25.1
	SFC S14	27.9	23.8	28.4	14.9	23.3	21.1	22.4	21.8	26.6
1-MN	SFC S9	84.6	111.5	89.1	59.8	83.7	70.1	72.8	75.4	56.9
	SFC S14	99.7	101.4	101.7	66.1	85.9	74.9	81.9	83.3	67.6
1-MP	SFC S9	26.8	17.8	29.6	18.6	24.5	24.7	23.7	26.1	27.1
	SFC S14	26.6	23.9	30.8	18.5	25.6	25.0	26.5	29.2	24.2
2,6-DMN	SFC S9	80.0[a]	46.5	40.8	40.3	38.5	33.4	38.1	31.3	23.4
	SFC S14	102.1[a]	43.2	42.9	51.7	37.8	31.7	42.3	27.0	36.8
6-MC	SFC S9	27.0	33.9	37.2	30.0	30.9	30.5	30.7	37.5	33.6
	SFC S14	24.5	33.0	38.7	30.2	31.3	30.8	33.6	32.0	33.2
Ant	SFC S9	9.2	10.8	13.4	9.4	9.8	9.8	9.3	11.1	11.8
	SFC S14	8.3	10.4	15.2	9.5	9.8	9.7	10.8	12.2	10.0
BaA	SFC S9	13.3	17.0	16.2	14.3	14.4	14.0	14.1	17.0	15.4
	SFC S14	12.4	16.2	15.5	14.3	14.6	14.2	15.6	15.2	15.9
BaP	SFC S9	4.5	5.7	5.1	4.9	4.7	4.7	4.7	4.5	5.5
	SFC S14	3.9	5.2	5.9	4.9	4.7	4.7	5.1	5.3	5.0
BbF	SFC S9	8.8	10.8	10.6	9.8	9.3	9.2	9.4	10.2	10.1
	SFC S14	8.8	10.5	11.1	9.7	9.4	9.4	10.1	10.0	9.5
BghiP	SFC S9	4.6	5.6	5.8	4.9	4.6	4.7	4.6	4.5	11.5[b]
	SFC S14	4.3	5.3	5.9	4.8	4.6	4.8	4.9	5.1	2.5[b]
BkF	SFC S9	7.9	8.6	9.5	7.7	7.7	8.0	7.6	7.6	8.5
	SFC S14	6.5	8.6	9.6	8.0	7.7	8.1	8.3	8.2	8.3
Chr	SFC S9	46.8	52.4	55.8	48.4	48.2	47.7	47.8	57.6	54.7
	SFC S14	42.8	50.2	57.8	48.5	49.1	48.1	52.3	51.4	53.1
DBahA	SFC S9	4.2	6.1	5.1	5.0	4.8	4.9	4.7	6.0	4.3
	SFC S14	4.0	5.3	5.7	5.0	4.8	5.0	5.2	5.4	4.9
Fln	SFC S9	14.8	15.8	16.6	14.4	14.6	14.8	14.0	16.9	17.0
	SFC S14	13.3	15.7	17.2	14.6	14.7	14.4	15.5	15.4	16.4
Flt	SFC S9	21.7	26.6	27.5	24.6	23.8	23.5	23.4	26.7	29.1
	SFC S14	19.9	26.7	28.9	24.6	24.0	23.2	26.7	25.6	25.0
IcdP	SFC S9	4.4	5.7	4.5	5.0	4.8	5.3	4.8	5.1	6.7
	SFC S14	4.1	5.4	5.5	5.0	4.8	5.1	5.2	5.4	5.1
Naph	SFC S9	86.2	88.1	86.8	75.9	80.2	71.9	79.4	75.3	90.1
	SFC S14	104.3	86.4	90.6	73.6	80.6	76.2	90.2	80.2	97.8
Phe	SFC S9	46.8	50.9	54.2	47.6	48.9	47.9	46.5	48.7	51.3
	SFC S14	44.7	50.3	56.2	47.8	50.0	47.4	51.3	50.7	53.5
Pyr	SFC S9	36.5	41.8	45.3	39.3	39.2	38.6	38.2	38.8	43.6
	SFC S14	32.6	42.0	46.6	39.4	39.7	38.5	42.3	41.5	42.1

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 5. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the high PAH level in shrimp

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC S8	26.5	19.3	21.1	16.2	20.0	19.9	16.1	23.0	28.2
	SFC S13	32.4	17.9	22.2	16.9	21.2	20.7	20.4	21.7	26.2
1-MN	SFC S8	318.7	195.9	243.8	145.0	202.9	197.9	146.0	236.8	105.8
	SFC S13	307.7	180.7	241.9	179.9	213.2	208.7	177.5	205.6	149.7
1-MP	SFC S8	122.8	103.8	122.0	102.3	120.8	115.2	92.3	125.8	150.3
	SFC S13	123.3	104.4	124.5	101.4	121.5	118.7	114.6	141.7	143.1
2,6-DMN	SFC S8	311.3[a]	148.1	167.6	128.2[a]	148.2	154.2	128.7	134.4	153.8
	SFC S13	706.5[a]	101.8	183.3	275.1[a]	166.9	144.4	155.3	130.4	136.0
6-MC	SFC S8	127.0	162.0	154.6	140.1	141.3	138.3	115.6	145.4	164.3
	SFC S13	126.7	149.8	152.3	151.5	142.6	143.9	137.8	166.5	153.4
Ant	SFC S8	35.8	42.4	47.1	37.6	37.7	36.7	28.6	40.6	44.6
	SFC S13	35.0	39.4	40.6	36.1	37.8	37.6	34.9	44.0	44.4
BaA	SFC S8	54.8	65.7	57.9	55.0	55.4	53.1	44.3	58.6	64.7
	SFC S13	52.5	63.3	55.5	54.0	55.0	55.1	53.1	60.2	61.0
BaP	SFC S8	22.9	25.1	25.0	22.9	22.8	22.1	18.8	23.9	26.0[a]
	SFC S13	21.9	24.3	23.9	22.0	22.7	23.2	22.3	25.3	37.8[a]
BbF	SFC S8	67.6	83.4	74.2	70.0	69.6	68.8	57.0	72.1	82.9
	SFC S13	70.4	73.4	69.6	67.6	69.0	70.5	67.4	76.5	79.1
BghiP	SFC S8	17.2	20.5	19.7	17.8	17.5	17.5	14.4	18.6	52.0[a]
	SFC S13	18.0	18.3	18.8	17.4	17.4	18.3	17.2	19.9	15.8[a]
BkF	SFC S8	37.4	41.9	41.4	35.9	38.2	37.3	30.7	38.5	41.4
	SFC S13	36.3	39.4	39.6	37.3	37.2	39.1	36.3	41.6	40.1
Chr	SFC S8	160.8	186.8	174.4	164.4	164.0	159.7	131.8	167.6	194.0
	SFC S13	155.1	175.0	165.3	161.1	163.6	164.4	157.3	186.2	181.5
DBahA	SFC S8	12.8	16.2	14.5	14.3	13.5	13.0	11.3	14.3	13.6
	SFC S13	12.4	14.4	13.3	13.4	13.4	13.4	13.6	15.5	15.1
Fln	SFC S8	48.5	53.2	48.6	46.2	46.5	46.2	35.6	47.8	52.7
	SFC S13	45.7	50.9	45.8	46.5	46.3	46.8	42.9	49.9	52.0
Flt	SFC S8	86.3	103.6	96.7	92.8	92.1	87.9	72.1	96.0	111.4
	SFC S13	89.6	96.8	96.0	92.2	92.6	90.8	87.4	103.2	102.9
IcdP	SFC S8	17.3	20.9	18.6	18.2	17.5	17.6	14.4	19.1	22.9
	SFC S13	17.0	19.7	17.3	17.7	18.0	17.9	16.9	20.5	20.1
Naph	SFC S8	244.7	137.6	174.1	143.1	147.9	147.2	112.0	166.8	260.0[a]
	SFC S13	218.7	129.4	166.1	141.6	149.4	148.9	137.6	173.7	184.9[a]
Phe	SFC S8	155.7	186.0	166.5	165.2	165.1	162.3	126.0	171.2	201.3
	SFC S13	153.8	176.9	173.8	164.2	169.0	165.8	153.7	183.0	185.2
Pyr	SFC S8	109.3	125.9	122.7	117.6	115.6	113.6	91.3[a]	119.9	123.2
	SFC S13	106.4	123.6	118.3	116.3	115.3	116.4	110.4[a]	128.6	127.4

[a] Outliers removed by Cochran test.

Table 6. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the low PAH level in mussel

		Laboratory No.									
PAH	Sample ID	1	2	3	4	5	6	7	8	9	10
1,7-DMP	SFC M1	40.8	41.5	44.4	23.9	45.4	39.2	40.9	44.5	30.9	37.9
	SFC M6	18.2	47.1	51.3	25.9	44.4	38.9	43.2	43.8	29.0	31.6
1-MN	SFC M1	17.0	21.2	23.2	10.4	19.1	17.5	22.1	24.6	16.2	16.0
	SFC M6	27.9	16.5	27.6	12.6	18.9	18.8	21.2	20.2	16.8	18.6
1-MP	SFC M1	33.7[a]	8.3	9.9	5.7	10.4	9.6	10.4	10.8	9.5	13.1
	SFC M6	18.0[a]	8.0	10.7	6.6	10.1	10.1	10.9	11.0	10.6	10.8
2,6-DMN	SFC M1	21.8	9.5	13.8	12.3	14.8	11.7	13.4	14.5	11.8	12.0
	SFC M6	17.8	10.8	14.6	16.5	14.8	12.4	11.9	15.7	11.0	14.6
3-MC	SFC M1	6.4	10.4	10.5	8.0	10.2	10.6	11.0	10.9	8.9	11.4
	SFC M6	6.1	8.1	11.3	10.2	10.0	10.8	11.1	10.8	8.8	11.8
Ant	SFC M1	4.9	4.4	3.1	1.2	2.3	5.0	4.7	4.9	3.8	4.9
	SFC M6	4.6	3.6	3.6	1.5	2.3	5.0	4.8	5.2	3.6	4.5
BaA	SFC M1	3.9	4.7	4.0	2.5	4.1	4.6	4.6	4.7	3.9	5.2
	SFC M6	4.2	4.0	4.9	3.1	4.1	4.5	4.7	4.5	3.8	5.1
BaP	SFC M1	1.3	2.1	1.8	1.0	1.3	0.1[b]	1.8	1.9	1.5	1.6
	SFC M6	1.4	2.0	1.9	1.2	1.2	0.1[b]	1.8	1.8	1.3	1.6
BbF	SFC M1	4.4	5.3	4.8	3.9	4.8	4.9	4.8	4.8	4.0	5.6
	SFC M6	4.7	4.3	5.1	4.7	4.7	4.3	4.8	4.7	4.1	5.7
BghiP	SFC M1	1.8	2.3[a]	2.0	1.7	2.0	2.0	2.0	2.1	1.7	2.3
	SFC M6	1.7	1.7[a]	2.2	2.0	1.9	2.0	2.2	1.9	1.7	2.3
BkF	SFC M1	1.8	1.8	2.2	1.6	2.0	1.8	1.9	2.1	47.0[a]	2.5
	SFC M6	1.8	1.9	2.1	2.0	1.9	2.0	1.8	2.0	1.6[a]	2.1
Chr	SFC M1	12.7	14.5	14.3	11.7	14.6	14.4	14.3	14.9	12.9	16.9
	SFC M6	13.2	12.3	15.3	14.2	14.3	14.4	14.4	14.0	12.9	17.0
DBahA	SFC M1	1.7	2.2	2.0	1.5	1.8	1.9	1.9	1.9	2.1	2.2
	SFC M6	1.7	1.7	1.9	1.7	1.8	1.8	1.9	1.8	1.6	2.2
Fln	SFC M1	5.3	6.2	5.6	5.5	5.1	5.1	5.0	5.1	4.5	6.2
	SFC M6	5.2	6.4	5.7	6.1	5.0	5.3	4.8	5.2	4.6	5.9
Flt	SFC M1	8.9	10.1	9.9	19.5[b]	10.0	12.4	9.6	10.2	10.8	12.0
	SFC M6	11.0	8.1	10.9	20.7[b]	9.7	12.5	9.8	9.8	7.5	11.2
IcdP	SFC M1	1.8	2.4	1.9	1.6	2.0	1.9	1.9	2.0	1.9	2.3
	SFC M6	1.6	1.9	2.1	1.9	2.0	2.0	2.0	1.9	1.7	2.4
Naph	SFC M1	20.3[a]	27.6	26.7	20.2	23.1	20.2	26.2	26.4	23.8	14.8
	SFC M6	36.0[a]	28.9	30.6	18.7	23.3	20.9	26.5	23.2	23.9	20.8
Phe	SFC M1	15.8	27.5[b]	14.2	12.7	15.2	14.2	14.5	15.3	14.1	21.2[b]
	SFC M6	13.9	26.7[b]	15.6	14.3	14.9	14.5	14.8	15.4	12.9	17.5[b]
Pyr	SFC M1	14.9	14.4	14.7	11.8	14.5	13.7	14.1	14.7	13.0	16.8
	SFC M6	14.2	11.9	15.4	13.2	14.2	14.0	14.4	14.0	12.9	16.8

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 7. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the mid PAH level in mussel

		Laboratory No.									
PAH	Sample ID	1	2	3	4	5	6	7	8	9	10
1,7-DMP	SFC M3	5.7[a]	48.0	45.9	27.4	46.1	40.5	42.9	43.8	28.2	36.6
	SFC M5	39.0[a]	38.5	41.7	31.0	44.1	38.9	42.1	57.2	28.8	28.1
1-MN	SFC M3	79.1	92.7	136.2	97.2	95.5	90.3	105.4	110.0	103.5	72.1
	SFC M5	78.2	105.0	117.3	87.2	97.2	103.0	109.8	119.5	87.4	50.7
1-MP	SFC M3	60.2[a]	55.2	54.3	32.2	52.2	47.1	50.6	50.5	31.5	50.9
	SFC M5	870.8[a]	41.7	48.0	30.1	49.1	44.9	51.0	63.2	31.7	40.3
2,6-DMN	SFC M3	21.5[a]	64.6	69.5	92.3	79.9	63.1	57.6	62.3	43.0	71.7[a]
	SFC M5	59.2[a]	63.6	62.1	85.7	74.9	59.4	61.7	64.0	43.3	92.5[a]
3-MC	SFC M3	70.3	81.5	101.6	80.7	93.8	93.4	93.3	91.4	78.6	98.9
	SFC M5	69.8	86.6	95.8	70.4	87.4	90.3	95.4	97.3	69.5	96.9
Ant	SFC M3	22.5	17.5	16.5	7.1	8.2	16.5	14.5	13.8	12.3	13.5
	SFC M5	14.9	13.6	14.4	11.0	6.6	13.7	14.8	15.2	10.8	14.7
BaA	SFC M3	22.3	21.0	24.8	18.4	21.5	23.2	22.7	22.3	19.6	23.9
	SFC M5	22.2	21.3	23.1	16.3	18.8	21.7	23.3	22.3	17.1	23.5
BaP	SFC M3	7.4	9.1	10.0	6.6	6.8	1.1[b]	9.2	9.8	7.7	8.4
	SFC M5	7.6	8.5	8.8	5.6	5.9	1.1[b]	9.4	9.9	6.4	7.8
BbF	SFC M3	28.2	27.3	29.2	24.0	28.9	31.6	27.9	27.6	24.3	29.9
	SFC M5	28.3	27.3	27.1	21.0	26.9	25.0	28.4	26.9	21.3	29.8
BghiP	SFC M3	8.8	9.1	10.8	8.1	9.5	9.8	10.0	9.7	7.5	11.0
	SFC M5	9.5	9.0	9.8	7.1	8.9	9.3	10.1	9.3	7.3	10.8
BkF	SFC M3	18.7	18.1	22.0	15.2	19.5	21.0	19.3	19.2	16.1	21.9
	SFC M5	18.8	18.3	20.0	14.0	18.2	17.2	19.0	18.9	13.9	21.4
Chr	SFC M3	92.7	86.1	100.8	79.7	98.0	95.2	93.2	92.0	86.0	105.2
	SFC M5	94.5	91.3	94.0	70.8	91.4	91.7	94.9	95.5	76.0	102.0
DBahA	SFC M3	8.8	9.4	9.7	8.0	9.4	9.9	9.6	9.5	6.3	11.0
	SFC M5	8.5	9.2	8.9	7.1	8.8	8.9	10.0	9.5	7.5	11.2
Fln	SFC M3	24.8	25.0	26.8	29.2	25.2	24.9	24.5	24.5	23.9	28.5
	SFC M5	24.3	25.6	25.1	29.6	23.6	24.1	25.1	26.0	21.9	26.5
Flt	SFC M3	47.8	45.5	49.7	46.6	50.8	55.3	48.1	48.2	39.2	55.6
	SFC M5	53.7	47.6	46.8	42.1	47.2	54.5	49.0	49.5	46.6	53.3
IcdP	SFC M3	9.0	9.6	10.1	8.1	9.7	10.1	9.5	9.7	8.6	11.3
	SFC M5	9.5	9.3	9.8	7.2	9.1	9.2	9.9	9.5	6.9	11.4
Naph	SFC M3	91.8	125.4	134.8	84.0	104.1	97.1	124.2	129.6	123.9	71.7
	SFC M5	84.6	122.2	127.8	65.1	107.7	114.8	124.8	135.3	109.7	40.0
Phe	SFC M3	92.1	206.3[b]	102.0	81.9	102.1	93.7	95.1	94.3[a]	84.3	98.8
	SFC M5	97.1	191.3[b]	93.9	76.3	96.2	90.5	96.6	119.2[a]	82.5	107.0
Pyr	SFC M3	79.2	71.9	80.3	62.0	75.6	71.2	72.4	72.2	64.2	80.2
	SFC M5	75.4	74.6	75.8	55.5	70.4	69.3	73.3	72.0	58.2	76.7

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 8. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the high PAH level in mussel

		Laboratory No.									
PAH	Sample ID	1	2	3	4	5	6	7	8	9	10
1,7-DMP	SFC M2	34.5	39.8	41.4	26.1	44.7	40.4	46.8	43.6	30.2	37.6
	SFC M7	22.4	34.0	42.2	26.5	47.0	39.3	48.5	56.7	30.7	45.8
1-MN	SFC M2	183.7	222.9[a]	211.8	199.1	193.9	195.7	196.9	190.0	206.8	387.8[b]
	SFC M7	274.7	459.3[a]	218.8	241.5	198.7	208.8	199.6	220.5	199.4	62.0[b]
1-MP	SFC M2	431.7[b]	113.4	122.8	81.6	128.5	115.8	135.8	123.1	79.6	125.9
	SFC M7	1752.2[b]	102.7	129.2	81.9	133.2	112.6	137.7	146.5	79.7	159.3
2,6-DMN	SFC M2	139.5	128.0	138.5	214.2	177.8	143.4	142.1	161.2	98.7	184.6
	SFC M7	121.6	135.2	158.1	244.0	187.9	141.7	149.4	144.7	94.2	171.3
3-MC	SFC M2	100.6	133.6	147.7	127.2	146.5	146.7	150.4	138.0	127.4	149.4
	SFC M7	103.5	130.3	156.7	128.4	151.9	144.9	151.9	145.6	121.6	157.7
Ant	SFC M2	35.3	44.7[b]	39.9	19.9	20.9	37.7	37.1	33.2	31.2	29.0
	SFC M7	31.7	33.9[b]	42.8	16.3	20.3	38.9	37.5	33.7	31.7	29.9
BaA	SFC M2	51.1	49.3	52.2	43.4[b]	49.9	53.3	54.9	50.0	47.7[a]	54.1
	SFC M7	52.0	50.7	55.0	41.7[b]	51.2	52.9	55.8	53.3	476.3[b]	56.1
BaP	SFC M2	19.1	19.8	22.3	15.6	16.6	3.0[a]	22.4	22.4	19.8	17.6
	SFC M7	18.3	19.4	22.9	15.1	17.0	3.0[a]	22.6	20.8	18.5	17.5
BbF	SFC M2	74.8	68.2	65.5	59.0	71.1	74.8	70.4	66.6	62.7	71.9
	SFC M7	71.8	67.2	68.5	58.8	74.1	77.2	71.2	69.5	60.3	78.1
BghiP	SFC M2	18.2	18.0	18.2	15.1	17.9	18.3	18.9	17.4	16.3	19.5
	SFC M7	18.8	17.6	19.7	14.9	18.7	18.0	19.3	18.4	15.2	20.3
BkF	SFC M2	38.8	33.8	38.8	30.8	37.3	36.3	38.3	36.9	30.5	41.7
	SFC M7	37.9	35.0	37.8	30.6	37.9	39.5	38.2	37.7	30.4	43.8
Chr	SFC M2	159.8	158.5	153.2	137.7	166.6	163.9	164.1	152.1	150.9	174.8
	SFC M7	166.8	152.4	165.9	139.2	172.3	161.0	165.6	161.0	145.3	184.1
DBahA	SFC M2	13.7	13.7	13.6	12.1	13.7	13.3	14.8	13.2	13.2	15.0
	SFC M7	13.9	13.0	14.5	10.6	14.3	13.2	14.8	14.0	12.3	15.4
Fln	SFC M2	50.2	49.0	45.8	59.2[a]	47.5	47.0	47.3	46.0	49.4	50.7
	SFC M7	49.8	49.1	49.8	59.4[a]	48.0	46.6	48.4	43.0	45.1	53.0
Flt	SFC M2	92.6	88.7	86.7	66.5[a]	96.2	96.5	93.4	89.7	93.1	100.2
	SFC M7	104.7	88.4	93.6	63.4[a]	98.1	93.7	94.7	84.1	79.6	104.3
IcdP	SFC M2	17.8	18.3	17.2	15.5	18.7	18.6	18.4	17.3	16.7	19.0
	SFC M7	17.0	16.6	18.7	15.3	19.7	19.3	18.5	18.3	15.2	21.3
Naph	SFC M2	125.2[b]	149.8	143.7	108.7	134.0	138.5	159.5	165.5	164.9	246.4[b]
	SFC M7	197.9[b]	160.7	160.0	104.6	141.0	144.4	159.0	165.6	146.5	34.3[b]
Phe	SFC M2	159.7	452.2[b]	159.4	142.5	173.2	161.0	166.2	162.4	143.6	164.0[b]
	SFC M7	178.0	366.3[b]	170.3	142.9	177.6	159.0	169.8	167.6	139.0	200.9[b]
Pyr	SFC M2	133.5	119.2	114.3	102.2	119.8	114.8	118.6	108.3	104.4	124.3
	SFC M7	123.0	117.5	122.6	102.9	125.1	113.3	120.1	119.2	97.4	128.6

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 9. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the low PAH level in oyster

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC O2	30.7[a]	85.4	74.9	52.1	69.6	52.7	79.5	76.4	73.9
	SFC O4	74.6[a]	85.7	80.1	58.6	82.0	56.1	76.2	81.5	72.1
1-MN	SFC O2	68.1	61.7[a]	81.6	51.7	59.4	71.7	82.0	76.2	38.0
	SFC O4	64.2	299.8[a]	90.5	56.7	69.1	75.2	81.5	73.7	46.5
1-MP	SFC O2	45.1[a]	36.7[a]	21.8	12.1	19.9	18.0	25.0	24.6	12.9
	SFC O4	103.1[a]	22.5[a]	24.4	14.3	23.2	19.2	24.9	25.0	17.5
2,6-DMN	SFC O2	88.8[a]	29.9	30.5	61.9[a]	30.3	23.8	36.0	30.7	24.7
	SFC O4	231.7[a]	28.6	35.8	30.2[a]	36.9	26.1	35.6	36.4	21.3
3-MC	SFC O2	24.6	29.7	34.2	21.1	25.0	27.5	30.4	28.1	23.0
	SFC O4	27.4	27.0	34.6	24.6	29.2	28.0	30.6	29.4	25.2
Ant	SFC O2	4.0[a]	6.2	7.5	2.3	1.4	5.4	9.1	4.7	—[b]
	SFC O4	9.8[a]	6.6	8.8	1.3	1.5	5.5	8.9	5.1	10.4[b]
BaA	SFC O2	9.8	12.7	12.6	5.6	8.6	11.1	13.5	11.3	9.5
	SFC O4	10.9	11.7	13.4	8.1	9.5	11.6	13.4	12.0	10.8
BaP	SFC O2	1.5	4.3	2.7	1.4	1.5	1.2	4.3	2.6	2.3
	SFC O4	1.8	3.3	3.0	1.8	1.7	1.4	4.3	2.8	2.8
BbF	SFC O2	7.3	10.0	8.8	6.6	7.7	8.9	9.3	8.8	7.4
	SFC O4	8.0	9.3	9.5	7.6	9.2	9.8	9.3	9.2	7.9
BghiP	SFC O2	3.3	4.7	4.4	3.2	3.6	4.0	4.7	4.2	4.0
	SFC O4	3.8	4.5	4.7	3.6	4.2	4.2	4.7	4.3	3.9
BkF	SFC O2	5.4	8.0	8.2	5.1	6.1	7.2	7.4	6.8	5.3
	SFC O4	6.4	7.4	9.1	5.9	7.3	7.8	7.3	7.4	5.8
Chr	SFC O2	39.8	46.7	45.9	32.7	39.2	43.7	46.2	42.4	37.0
	SFC O4	44.9	44.9	48.5	38.2	46.6	44.8	46.0	45.5	40.3
DBahA	SFC O2	3.4	5.2	4.1	3.1	3.7	4.0	4.8	4.2	4.7
	SFC O4	3.8	4.3	4.3	3.6	4.4	4.1	4.7	4.4	3.8
Fln	SFC O2	11.4	13.1	14.6	8.1	10.6	10.3	14.1	12.8	12.2
	SFC O4	12.4	14.6	14.6	10.4	12.4	10.9	13.9	14.0	14.5
Flt	SFC O2	22.2	23.2	23.2	19.5	19.4	26.5	22.9	21.0	16.2
	SFC O4	24.5	22.3	25.1	13.5	23.0	28.2	23.5	22.6	19.8
IcdP	SFC O2	3.4	5.0	4.5	3.3	3.7	4.3	4.7	4.3	4.8
	SFC O4	4.0	4.5	5.0	3.7	4.4	5.4	4.6	4.4	4.2
Naph	SFC O2	64.1	67.4	79.8	61.6	59.3	71.1	79.2	74.2	74.4
	SFC O4	49.1	81.0	79.7	63.7	62.8	72.8	76.5	80.8	79.6
Phe	SFC O2	35.0	47.6	44.2	40.7	39.6	33.7	46.0	43.5	38.8
	SFC O4	37.9	47.7	49.1	36.8	47.2	36.7	45.4	46.4	32.6
Pyr	SFC O2	33.3	34.6	36.6	26.0	30.3	32.8	37.6	33.8	30.0
	SFC O4	34.8	36.6	39.0	30.1	36.5	34.3	37.1	35.8	33.3

[a] Outliers removed by the Cochran test.

[b] Removed due to the reported integration issues.

Table 10. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the mid PAH level in oyster

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC O1	96.8[a]	81.8	69.8	40.2	63.5	56.5	75.6	74.5	78.2
	SFC O7	31.3[a]	80.0	82.6	47.5	71.7	61.6	76.9	73.7	69.8
1-MN	SFC O1	84.8	86.4	109.9	35.5	75.9	96.1	107.4	102.2	45.4
	SFC O7	79.3	116.3	152.8	104.4	77.8	100.9	101.2	111.3	46.0
1-MP	SFC O1	500.3[a]	40.9	45.4	24.2	37.8	37.2	47.0	48.5	30.5
	SFC O7	91.8[a]	44.7	48.7	31.1	42.3	37.3	44.2	46.9	31.3
2,6-DMN	SFC O1	573.4[a]	71.6	54.5	14.6[a]	53.0	46.2	64.0	63.9	50.9
	SFC O7	179.1[a]	84.1	56.2	93.4[a]	58.4	49.1	58.3	54.9	45.2
3-MC	SFC O1	73.9	89.6	94.0	57.5	72.3	83.8	88.7	83.2	67.5
	SFC O7	75.8	79.1	98.6	74.5	74.8	82.3	83.9	86.6	69.8
Ant	SFC O1	21.9[a]	11.0	11.9	1.7	1.5	8.5	13.7	6.2	11.0 [b]
	SFC O7	6.4[a]	9.4	13.0	1.8	0.5	7.2	12.6	6.6	24.8 [b]
BaA	SFC O1	15.6	20.6	19.6	10.3	12.9	19.3	21.9	18.5	15.4
	SFC O7	15.3	19.6	21.1	12.8	11.4	18.4	20.3	19.0	16.9
BaP	SFC O1	3.4	7.0	5.7	2.7	2.8	3.1	9.0	4.7	5.6
	SFC O7	2.9	5.7	6.3	3.2	3.0	2.7	8.2	5.0	5.8
BbF	SFC O1	21.3	28.7	26.1	18.5	22.8	28.3	27.0	24.7	21.4
	SFC O7	23.2	26.1	27.4	23.9	23.6	25.9	25.0	25.9	22.1
BghiP	SFC O1	6.5	9.8	8.6	5.9	7.3	8.8	9.4	8.7	8.8
	SFC O7	6.7	8.2	9.4	7.7	7.5	8.5	9.0	9.0	7.5
BkF	SFC O1	13.9	19.8	19.6	12.2	15.1	19.0	18.1	17.2	14.0
	SFC O7	14.2	18.4	19.9	15.9	16.0	21.0	17.1	17.7	14.3
Chr	SFC O1	75.1	93.0	87.7	61.2	74.7	87.9	89.8	85.8	71.7
	SFC O7	75.4	84.6	93.1	78.3	79.2	86.4	83.8	87.1	73.5
DBahA	SFC O1	6.9	10.4	8.6	6.2	7.3	8.5	9.5	8.7	11.3[a]
	SFC O7	7.0	8.5	9.2	7.4	7.5	8.3	8.9	8.6	7.3[a]
Fln	SFC O1	18.1	25.1	22.5	14.6	16.8	18.0	23.2	21.5	19.7
	SFC O7	17.8	23.1	24.2	18.2	18.7	18.0	21.7	21.4	22.4
Flt	SFC O1	42.0	46.5	44.4	23.0	37.4	58.4	47.5	42.4	33.0
	SFC O7	40.6	43.1	47.2	40.0	40.5	47.2	42.8	45.4	34.0
IcdP	SFC O1	6.9	9.9	9.0	6.1	6.9	9.1	9.4	8.8	9.7
	SFC O7	6.9	8.8	9.5	7.8	7.7	8.2	8.7	8.9	7.8
Naph	SFC O1	89.8	118.9	110.0	77.4	87.6	112.1	117.3	115.8	113.6
	SFC O7	82.6	112.8	125.0	101.6	88.4	113.9	110.2	117.7	116.4
Phe	SFC O1	65.0	96.6	87.5	58.3	76.0	71.8	89.9	89.1	75.3
	SFC O7	68.1	87.2	93.6	77.9	84.0	72.0	83.8	88.3	81.7
Pyr	SFC O1	59.2	71.1	68.8	45.2[a]	55.2	64.9	69.3	64.6	56.2
	SFC O7	57.8	65.9	70.5	59.1[a]	59.4	62.8	64.6	63.6	57.9

[a] Outliers removed by the Cochran test.

[b] Removed due to the reported integration issues.

Table 11. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the high PAH level in oyster

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC O5	136.0[a]	56.6	66.6	38.2	65.5	54.5	78.4	72.0	75.8
	SFC O6	16.4[a]	53.3	66.8	53.7	68.3	56.5	84.9	85.3	73.2
1-MN	SFC O5	296.9	289.7	289.0	214.7	182.5	222.5	275.3	309.5	125.4
	SFC O6	212.6	280.2	285.0	231.3	194.8	218.6	253.2	243.9	134.3
1-MP	SFC O5	4082.3[a]	121.1	173.3	108.1	148.6	138.2	184.2	185.9	103.7
	SFC O6	219.8[a]	130.0	184.0	150.7	160.5	130.8	203.2	217.5	135.1
2,6-DMN	SFC O5	2790.3[a]	190.4	154.6	339.2[a]	164.7	134.1	179.9	145.3	130.8
	SFC O6	331.7[a]	187.9	167.7	541.4[a]	168.9	127.6	173.9	159.6	173.0
3-MC	SFC O5	191.0	180.2	218.1	146.1	172.2	195.0	215.8	215.8	184.4
	SFC O6	214.7	199.9	233.3	165.6	185.1	194.0	226.0	217.2	187.6
Ant	SFC O5	59.3[a]	35.3	39.8	14.9[b]	9.1	26.8	52.1	30.0	12.0[b]
	SFC O6	136.2[a]	41.1	47.3	41.4[b]	10.3	29.0	54.7	32.1	44.1[b]
BaA	SFC O5	66.3	67.8	80.4	47.1	55.0	71.6	87.3	78.5	71.7
	SFC O6	74.8	79.2	82.3	50.0	60.7	71.9	91.1	80.8	72.4
BaP	SFC O5	16.9	25.5	32.0	14.7	14.8	12.7	42.4	25.8	28.4
	SFC O6	20.2	28.4	33.9	16.8	17.4	11.7	43.8	27.0	31.0
BbF	SFC O5	75.1	76.8	85.2	62.9	74.8	98.2	87.4	87.0	79.6
	SFC O6	78.2	86.3	87.9	70.4	78.8	101.7	91.6	88.7	79.5
BghiP	SFC O5	17.4	18.5	21.7	14.9	17.2	19.7	22.4	20.6	19.0
	SFC O6	19.0	21.2	22.2	16.5	18.6	19.8	23.5	20.9	19.8
BkF	SFC O5	55.5	59.2	65.7	46.9	54.2	71.3	67.3	66.5	60.5
	SFC O6	59.7	69.6	69.9	52.5	58.3	78.7	70.4	66.7	58.4
Chr	SFC O5	197.8	191.9	214.9	157.9	183.2	207.8	220.3	218.3	199.0
	SFC O6	214.2	210.7	226.5	176.6	194.5	207.2	231.4	219.4	201.5
DBahA	SFC O5	15.0	15.8	19.2	12.0	13.8	14.8	18.3	16.5	15.4
	SFC O6	16.2	17.4	18.4	13.4	15.0	15.5	19.2	16.8	15.3
Fln	SFC O5	51.0	63.5	61.1	36.9	48.3	48.8	66.4	62.0	70.8
	SFC O6	55.5	68.0	65.2	36.2	49.7	46.3	69.0	57.4	70.5
Flt	SFC O5	132.5	121.3	124.8	76.4	106.9	136.5	131.5	129.9	102.6
	SFC O6	127.3	135.3	130.6	92.8	113.9	126.6	137.8	131.5	115.1
IcdP	SFC O5	18.1	19.1	22.4	15.4	18.1	20.1	21.8	21.6	18.8
	SFC O6	19.5	21.4	22.3	17.1	19.2	22.0	23.1	21.6	19.2
Naph	SFC O5	214.6[a]	201.4	205.5	146.5	157.7	180.5	209.7	213.3	238.0
	SFC O6	171.1[a]	215.4	210.1	146.2	158.2	175.8	215.3	207.8	221.1
Phe	SFC O5	137.6[a]	208.6	203.1	192.7	183.8	168.7	222.5	224.5	196.8
	SFC O6	214.4[a]	227.0	217.7	189.6	194.1	158.2	232.7	222.1	220.4
Pyr	SFC O5	160.0	158.4	169.5	125.3	142.3	164.7	179.8	175.1	161.1
	SFC O6	173.2	179.1	179.6	140.1	151.2	154.6	187.4	176.0	163.4

[a] Outliers removed by the Cochran test.

[b] Removed due to the reported integration issues.

Table 12. Recoveries (%) of the studied PAHs in oyster obtained by a collaborator storing the test samples SFC O1–O7 at –20°C

PAH	SFC O1	SFC O2	SFC O3	SFC O4	SFC O5	SFC O6	SFC O7	Mean	RSD, %
1,7-DMP	93	95	93	102	90	107	92	96	6.3
1-MN	102	102		98	124	98	111	106	9.5
1-MP	97	99		100	93	109	94	99	5.8
2,6-DMN	85	77		91	65	71	73	77	13
3-MC	92	94		98	96	97	96	95	2.1
Ant	**41**	**47**		**51**	**50**	**53**	**44**	**48**	**9.6**
BaA	74	75		80	78	81	76	77	3.5
BaP	**47**	**51**		**56**	**52**	**54**	**50**	**52**	**6.3**
BbF	82	88		92	87	89	86	87	3.7
BghiP	87	83		87	82	84	90	86	3.4
BkF	86	85		92	89	89	88	88	2.9
Chr	86	85		91	87	88	87	87	2.5
DBahA	87	84		89	82	84	86	85	2.7
Fln	86	86		93	83	77	86	85	6.3
Flt	85	84		90	87	88	91	87	3.2
IcdP	88	85		88	86	86	89	87	1.6
Naph	93	93		101	95	92	94	95	3.4
Phe	89	87		93	90	89	88	89	2.2
Pyr	86	85		89	88	88	85	87	2.2

Table 13. Recoveries (%) of the studied PAHs in oyster obtained bya collaborator storing the test samples SFC O1–O7 at –70°C

PAH	SFC O1	SFC O2	SFC O3	SFC O4	SFC O5	SFC O6	SFC O7	Mean	RSD, %
1,7-DMP	94	99	108	95	98	106	96	100	5.4
1-MN	107	109		109	110	101	101	106	3.8
1-MP	94	100		100	92	102	88	96	5.5
2,6-DMN	85	90		89	80	77	78	83	6.8
3-MC	99	101		102	96	100	93	99	3.5
Ant	**92**	**91**		**89**	**87**	91	84	89	3.3
BaA	88	90		90	87	91	81	88	4.0
BaP	**90**	**87**		**86**	**85**	88	82	86	3.0
BbF	90	93		93	87	92	83	90	4.2
BghiP	94	95		94	89	94	90	93	2.7
BkF	91	92		91	90	94	85	90	3.1
Chr	90	92		92	88	93	84	90	3.8
DBahA	95	96		95	92	96	89	94	3.1
Fln	93	94		93	88	92	87	91	3.2
Flt	95	92		94	88	92	86	91	4.1
IcdP	94	93		93	87	92	87	91	3.5
Naph	94	99		96	93	96	88	94	3.8
Phe	90	92		91	89	93	84	90	3.6
Pyr	92	94		93	90	94	86	91	3.3

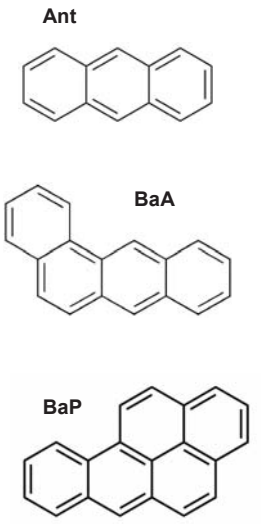


Figure 1. Structures of anthracene (Ant), benzo[*a*]anthracene (BaA), and benzo[*a*]pyrene (BaP).

blank PAH levels were too high to participate in the collaborative study and to conduct low-level PAH analysis in general. This was typically due to their location (high environmental contamination) and/or their laboratory contamination. Some participants were able to reduce the reagent (procedure) blank contamination by moving the sample preparation (extraction, cleanup, and evaporation) away from oil pumps, such as MS rough pumps. The method requires heating of the used salts and recommends heating of glassware. Solvents, plastic material, and equipment may also be sources of PAHs, including polypropylene centrifugation/extraction tubes. As one collaborator discovered, simple testing of polypropylene tubes using ethyl acetate wash/extraction may not reveal PAH contamination. However, the extraction dynamic during the actual procedure can release potentially present PAHs into the extract when the tubes get heated due to the exothermic reaction caused by addition of $MgSO_4$ to the water-containing extraction mixture. For this and other potential contamination reasons, it is highly important to analyze a reagent blank with every sample batch.

In addition to the contamination issues, another problem faced by laboratories less experienced in PAH analysis was optimization of the evaporation conditions to prevent losses of volatile analytes, especially naphthalene. Isooctane is used as a keeper in both evaporation steps, but it did not prevent significant losses of volatile PAHs in the second evaporation step in certain laboratories. For this reason, the study direction team recommended addition of 1–2 mL of ethyl acetate to the SPE eluent for a better control of the final evaporation process, which helped in most cases and was added as a recommendation to the method procedure.

Sixteen laboratories entered the qualification phase, but only 10 of them (listed in the *Acknowledgments* section) completed the qualification successfully and/or continued in the study. In many cases, the reason why a participant did not complete the qualification phase was the availability of resources and not the ability to qualify for the study.

Collaborators' Comments

Most of the study participants commented very positively on the speed and ease of use of the method, especially laboratories currently analyzing PAHs with much more labor-intensive and time-consuming methods.

The most frequently reported sources of PAH contamination were salts (which have to be muffled and stored appropriately). Some participants had problems with PAH sources in their laboratories caused by the use of oil pumps in the vicinity of the space used for the sample preparation. As noted above, one collaborator discovered PAHs in polypropylene centrifuge tubes used for practice sample analysis (*Note*: All tubes and containers used for the test sample storage and preparation were pretested by the study direction team).

Several collaborators initially had problems with optimization of the evaporation steps to prevent losses of volatile PAHs. As noted above, the addition of ethyl acetate in the second evaporation step resolved this issue in most cases. The majority of study participants used evaporation with a gentle stream of nitrogen at room temperature or a maximum of 40°C.

Collaborators noticed differences in the color of extracts of oyster blank versus fortified samples stored for several months in a freezer at –20°C. The blank sample produced a dark green extract, whereas the same blank sample fortified with PAHs gave a yellow-brown extract. This was not observed for oyster samples stored for a shorter period of time and/or stored at –70°C. As discussed below, this observation could be linked to degradation issues in oysters. Also, the participants noted that oyster extracts were generally dirtier than shrimp and mussel extracts, which affected chromatography in some cases.

One collaborator reported the use of a mechanical shaker instead of hand-shaking. Mechanical shaking is generally preferred by routine testing laboratories, and this method modification is acceptable as long as a vigorous and effective shaking (up and down in the tube) is ensured.

Collaborative Study Results

Tables 3–11 provide the collaborative study results obtained by the participating laboratories in three blind duplicates (low, mid, and high fortification level) in shrimp, mussel, and oyster. Results from Laboratory No. 10 are presented only for mussel because the Study Directors excluded their oyster and shrimp data sets due to calibration (standard preparation) issues.

1,7-DMP was used as a homogenization check and was added to blank mussel and oyster samples at 40 and 80 µg/kg, respectively, during the homogenization step. The mean concentration value obtained for 1,7-DMP by all participants (except for sample SFC M4 lost by Laboratory No. 6 and the result for sample SFC M3 from Laboratory No. 1, which was removed as an apparent outlier) in all seven test mussel samples (three blind duplicates and one blank) was 38.8 µg/kg (RSD = 21.5%, n = 68), which corresponds to mean recovery of 97.0%. In the case of oysters, the mean concentration value obtained for 1,7-DMP by all participants (except for Laboratories 1 and 10, for which all 1,7-DMP results were eliminated as outliers in the Grubbs' tests applied

to the blind duplicates) in all seven test samples was 70.5 µg/kg (RSD = 16.3%, n = 56), which corresponds to mean recovery of 88.1%. These results indicate that the mussel and oyster samples sent to the study participants had good homogeneity. 1,7-DMP was also added to all shrimp test samples at 20 µg/kg during the fortification step conducted by the study participants. The mean concentration value was 22.1 µg/kg (RSD = 19.0%, n = 63), corresponding to mean recovery of 111%. Statistical results for 1,7-DMP obtained in blind duplicate samples are summarized together with the other analytes in Tables **2014.08B–D**.

Tables **2014.08B–D** provide statistical results obtained for the studied analytes at three different concentration levels in shrimp, mussel, and oyster after elimination of statistical outliers (highlighted in Tables 3–11).

Eight to 10 valid results were obtained for the majority of determinations. Mean recoveries of all tested analytes at the total of five different concentration levels were all in the range of 70–120%: 83.8–115% in shrimp, 77.3–107% in mussel, and 71.6–94.6% in oyster, except for a slightly lower mean recovery of 68.6% for BaA fortified at 25 µg/kg in oyster (RSD_r: 5.84%, RSD_R: 21.1%) and lower mean recoveries for Ant and BaP in oyster at all three fortification levels (50.3–56.5% and 48.2–49.7%, respectively).

The lower mean recoveries of Ant and BaP were linked to degradation of these analytes in oyster samples stored at –20°C (*see* the discussion about degradation issues below), which also resulted in lower reproducibility (RSD_R values in the range of 44.5–64.7% for Ant and 40.6–43.5% for BaP). However, the repeatability was good (RSD_r of 8.78–9.96% for Ant and 6.43–11.9% for BaP), and the HorRat values were acceptable (1.56–1.94 for Ant and 1.10–1.45 for BaP).

In all other cases, repeatability, reproducibility and HorRat values were as follows:

(*1*) Shrimp: RSD_r 1.40–26.9%, RSD_R 5.41–29.4%, HorRat 0.22–1.34;

(*2*) Mussel: RSD_r 2.52–17.1%, RSD_R 4.19–32.5%, HorRat 0.17–1.13; and

(*3*) Oyster: RSD_r 3.12–22.7%, RSD_R 8.41–31.8%, HorRat 0.34–1.39.

Overall, the results of the collaborative study demonstrate that the method is fit-for-purpose to determine PAHs and their alkyl homologs in seafood samples.

Degradation Issues

The Study Directors reported problems with lower recoveries for Ant and BaP in oyster samples stored at –20°C to the SPSC PAH Working Group and the AOAC Methods Committee on PAHs when they discovered a significant difference between results for these two analytes obtained in two different participating laboratories storing the samples at two different freezer temperatures of –20 and –70°C (*see* recovery results in Tables 12 and 13, respectively). Due to the limited availability and cost of oyster samples, it was decided to continue with the study and not proceed with preparation of new study test samples that would be fortified by collaborators on the day of the analysis as was done for shrimp (*Note*: in the case of shrimp, overall lower recoveries were obtained for all studied PAHs depending on the shrimp sample storage conditions).

The laboratory storing samples at –20°C analyzed another set of oyster samples about 1.5 months after the first set and confirmed the lower recoveries for Ant and BaP. This collaborator also made another interesting observation related to the color difference between extracts obtained in the first set (all dark green) and the second set (the dark green extract was produced only for the blank sample), whereas all extracts of fortified samples were yellow-brown. This observation, which was later confirmed by additional study participants, indicates matrix changes caused by the presence of PAHs and accompanied by selective losses of Ant and BaP. In addition to these two analytes, BaA also showed lower recoveries (around 70%) in oysters when compared to the rest of the studied PAHs.

Furthermore, a closer examination of the results obtained for Ant, BaP, and BaA in mussel also show somewhat lower mean recoveries of these three analytes (around 80%) when compared to the other analytes. These mean results do not include data obtained for BaP in mussel test samples by Laboratory No. 6, which analyzed the test samples about a year later than most other participants. All Laboratory No. 6 BaP results in mussel test samples showed very good repeatability within the duplicates but were eliminated as Grubbs' test outliers because they were significantly lower than the results obtained by other laboratories. No other data obtained by Laboratory No. 6 were identified as outliers using the Cochran or Grubbs' tests.

Figure 1 provides structures of Ant, BaA, and BaP showing that these PAHs contain the same moiety in terms of the linear 3-ring (anthracene) structure. This structural commonality and similar degradation behavior indicate that they could be substrates for the same enzyme(s). The significant differences in recoveries obtained for samples stored at –20°C versus –70°C (Tables 12 and 13, respectively) represent more supporting evidence for an enzymatic degradation being the most probable cause for the lower recoveries observed for these analytes in oyster (and mussel) test samples. To prevent degradation of these analytes in seafood matrixes during long-term storage, the Study Directors recommend storing homogenized samples at –70°C or lower. Unfortunately, this was not a feasible requirement for this collaborative study because the majority of the collaborating laboratories did not have this storage capability.

Acknowledgments

The Study Directors wish to thank the SPSC PAH Working Group (chaired by Gina Ylitalo from National Oceanic and Atmospheric Administration), the AOAC Methods Committee on PAHs (chaired by Tom Phillips, Maryland Department of Agriculture, Annapolis, MD), and AOAC INTERNATIONAL staff for the discussions about the study design, analyte selection, and stability issues as well as for the overall support provided to the collaborative study. Special acknowledgment goes to Jack Cochran (Restek Corp., Bellefonte, PA) for useful discussions about the PAH GC/MS analysis.

John Schmitz and Jack Jabusch (Covance Laboratories, Madison, WI) and Lucie Drabova and Jana Pulkrabova (ICT, Prague, Czech Republic) are acknowledged for technical advice and support during the preparation and the entire course of the study.

The Study Directors thank the Nutritional Chemistry and Food Safety business unit at Covance Laboratories for supplying and preparing PAH standards and spiking solutions; obtaining

blank mussel samples; homogenizing, weighing, and fortifying all samples; and shipping samples to the study participants.

Cambridge Isotope Laboratories (Tewksbury, MA) is acknowledged for providing discounted ^{13}C-PAH standard mixtures, Shrimp Alliance for supplying blank shrimp samples, and Jo Marie Cook (Florida Dept. of Agriculture and Consumer Services, Tallahassee, FL) for securing blank oyster samples for the study.

The Study Directors are very grateful to all laboratories that participated in the study and especially to the following collaborators, listed alphabetically based on their institution name:

Rolando Perez, Jason Betz, and Steven Perez, Adpen Laboratories, Inc., Jacksonville, FL

Ken Keide, Mark Misunis, and Jian Wang, Canadian Food Safety Inspection Agency, Calgary Laboratory, AB, Canada

Jack Jabusch and John Schmitz, Covance Laboratories Inc., Madison, WI

Jason Stepp, Walter Hammack, and Jo Marie Cook, Florida Dept. of Agriculture and Consumer Services, Tallahassee, FL

Patricia Lopez-Sanchez, Radoslaw Lizak, and Thomas Wenzl, Institute for Reference Materials and Measurements, EU PAH Reference Laboratory, Geel, Belgium

Lucie Drabova and Jana Pulkrabova, Institute of Chemical Technology, Prague, Czech Republic

Joe Binkley, Doug Staples, and Scott Pugh, Leco Corporation, St. Joseph, MI

Bonita Taffe, Michigan Department of Community Health Bureau of Labs, Lansing, MI

Jan Rosmus, State Veterinary Institute, National PAH Reference Laboratory, Prague, Czech Republic

Katerina Bousova and Klaus Mittendorf, Thermo Fisher Scientific Food Safety Response Center, Dreieich, Germany

References

(1) Kalachova, K., Pulkrabova, J., Drabova, L., Cajka, T., Kocourek, V., & Hajslova, J. (2011) *Anal. Chim. Acta* **707**, 84–91. http://dx.doi.org/10.1016/j.aca.2011.09.016

(2) CONffIDENCE (2010) *Contaminants in Food and Feed: Inexpensive Detection for Control of Exposure*. http://www.conffidence.eu

(3) *Official Methods of Analysis* (2002) *Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis*, Appendix D. http://www.aoac.org/Official_Methods/Collaborative_Study_Validation_Guidelines.pdf

(4) AOAC Spreadsheet for Blind Duplicates 2.0. http://www.aoac.org/stats/

Expert Review Panel on Polycyclic Aromatic Hydrocarbons (PAHs)

OFFICIAL CHAIR'S EXPERT REVIEW PANEL REPORT

ACKNOWLEDGMENT

The undersigned chair hereby confirms that the following document has been reviewed and constitutes the final version of the Official Chair's Report for the Expert Review Panel on Polycyclic Aromatic Hydrocarbons (PAHs) held on Monday, September 8, 2014 during the AOAC Annual Meeting and Exposition at the Boca Raton Resort and Club, 501 East Camino Reel, Boca Raton, Florida 33432.

TOM PHILLIPS, MD DEPARTMENT OF AGRICULTURE

Expert Review Panel Chair

30 October 2014

Date

AOAC RESEARCH INSTITUTE
2275 Research Blvd, Suite 300
Rockville, Maryland 20850
UNITED STATES

Contact:
La'Kia Phillips, Conformity Assessment Coordinator at lphillips@aoac.org
Deborah McKenzie, Sr. Director, DMcKenzie@aoac.org

EXPERT REVIEW PANEL MEETING ATTENDEES

Expert Review Panel Chair(s)
Tom Phillips, MD Department of Agriculture

Expert Review Panel Members
Mark Crosswhite, Florida Department of Agriculture
Julie Kowalski, RESTEK
Cheryl Lassitter, DOC, NOAA, NMFS, NSIL
Kai Liu, Eurofins
Jian Wang, Canadian Food Inspection Agency (CFIA)
Xiaoyan Wang, United Chemical Technologies, Inc. (UCT)
Stephen A. Wise, National Institute of Standards and Technology (NIST)

Not Present: Tracy Collier - (Alternate), NOAA (retired)
Not Present: Lowri de Jager, U.S. Food and Drug Administration

Method Authors
Kate Mastovska, Covance Laboratories
Jana Hajslova, Institute of Chemical Technology

AOAC Staff
Scott Coates
Deborah McKenzie
Tien Milor
La'Kia Phillips

Observers
Jana Huckrabolt, Institute of Chemical Technology
Nick Kosa, Bioo Scientific
Laszlo Torma,
Suramya Waidyanatha, NPT/NIEHS

EXPERT REVIEW PANEL, METHOD BACKGROUND, AND CONCLUSIONS

Criteria for Vetting Methods to be considered:
AOAC convened the *Official Methods of Analysis*^{SM} (OMA) Expert Review Panel for Polycyclic Aromatic Hydrocarbons (PAHs) on Monday, September 8, 2014 from 1:30pm to 4:00pm during the AOAC Annual Meeting and Exposition in Boca Raton, Florida. The purpose of the meeting was to 1) Review the Collaborative Study Manuscript/ OMAMAN-15: Determination Of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry (Study Director: Katerina Mastovska, Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA) and to 2) discuss First to Final Action requirements and feedback mechanisms. The candidate method was reviewed against the approved collaborative study protocol. Supplemental information was also provided to the reviewers which included the collaborative study manuscript, response to AOAC statistical review, summary shrimp corrected, DBAHA shrimp mid s9&s14 corrected, ICDP oyster mid o1&o7 corrected, and additional experiments with shrimp matrix.

Criteria for Vetting Experts and Selection Process:
The following nine (9) candidates and one (1) alternate were submitted for consideration by the Official Methods Board to evaluate candidate methods for Polycyclic Aromatic Hydrocarbons (PAHs) methods as per the Expert Review Panel (ERP) Policies and Procedures. The candidates were highly recommended by the Chemical Contaminants and Residues in Foods Community, have participated in various AOAC activities, including but limited to, Method Centric Committees that were formed under the legacy OMA pathway, and were vetted by the Official Methods Board. The experts are Tom Phillips, Cheryl Lassitter, Tracy Collier (Alternate Member), Kai Liu, Mark Crosswhite, Jian Wang, Lowri de Jager, Xiaoyan Wang, Julie Kowalski, and Stephen Wise. Tom Philips was vetted as the Expert Review Panel Chair.

ERP Orientation:
The ERP members have completed the mandatory AOAC Expert Review Panel Orientation Webinar on Wednesday, July 16, 2014.

Expert Review Panel Meeting Quorum
The meeting of the Expert Review Panel was held in person. A quorum is the presence of seven (7) members or 2/3 of the total vetted ERP, whichever is greater. Eight (8) out of the nine (9) voting members were present and therefore met a quorum to conduct the meeting.

Standard Method Performance Requirements (SMPRs): N/A

Conclusion:
The Expert Review Panel reviewed OMAMAN-15: Determination of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry and adopted this method for First Action Official Method status by a unanimous decision.

Subsequent ERP Activities:
ERP members will continue to evaluate the method for 2 years.

MEETING MINUTES

I. Welcome and Introductions

The Expert Review Panel Chair, Tom Phillips welcomed Expert Review Panel members and initiated introductions. The Chair discussed with the panel the goal of the meeting.

II. Review of AOAC Volunteer Policies

Deborah McKenzie presented an overview of AOAC Volunteer Policies, Volunteer Acceptance Agreement and and Expert Review Panel Policies and Procedures which included Volunteer Conflicts of Interest, Policy on the Use of the Association, Name, Initials, Identifying Insignia, Letterhead, and Business Cards, Antitrust Policy Statement and Guidelines, and the Volunteer Acceptance Form (VAF). All members of the ERP were required to submit and sign the Volunteer Acceptance Form.

III. Expert Review Panel Process Overview and Guidelines

Deborah McKenzie presented an overview of the Expert Review panel process. The presentation included information regarding method submission, recruitment of ERP members, composition and vetting expertise, method assignments, meeting logistics, consensus, First Action to Final Action requirements, method modifications, publications, and documentation.

IV. Review of Methods

All members of the ERP presented a review and discussed the proposed collaborative study manuscript for the Determination of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry. The method authors are Katerina Mastovska of Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA. A summary of comments was provided to the ERP members.[1]

MOTION:
Motion by Kowalski; Second by Crosswhite to adopt this method as a First Action Official Method.
Consensus demonstrated by: 7 in favor, 1 opposed, and 0 abstentions.
Motion failed.[2]

Negative Vote Discussion: One member of the expert review panel voted against the motion. Due to the reviewer's comments, he inquired about the method not using a certified reference material for PAHs in seafood. Standard Reference Material (SRM) 1974b Mussel Tissue is mentioned as part of the qualification of the labs as a practice sample, but no data was reported using SRM 1974b for validation of the proposed method. The availability of SRM 1974c (which has replaced SRM 1974b) provided an excellent opportunity to use a CRM to validate an AOAC method. The discussion of the Expert Review Panel concluded that the use of a certified reference material was not required and did not delineate scientific reasoning to not move the method forward. This method was created in an effort to address an emergency response to the gulf oil spill. The information provided in reference to the selection of the 19 target PAH compounds and the matrices selected were noted in the Fitness for Purpose statement established by the Stakeholder Panel on Petroleum Contaminants in Seafood in 2010. The ERP captured a revote.

[1] Attachment 1: Summary of Expert Reviewer Comments for OMAMAN-15

[2] *Method must be adopted by unanimous decision of ERP on first ballot, if not unanimous, negative votes must delineate scientific reasons. Negative voter(s) can be overridden by 2/3 of voting ERP members after due consideration.*

MOTION:
Motion by Kowalski; Second by Crosswhite to adopt this method as a First Action Official Method.
Consensus demonstrated by: 8 in favor, 0 opposed, and 0 abstentions (Unanimous).
Motion Passed.

V. Discuss Final Action Requirements for First Action Official Methods *(if applicable)*
No further action was discussed at this time.

VI. Adjournment

AOAC RESEARCH INSTITUTE

AOAC *OFFICIAL METHODS OF ANALYSIS* (OMA)

OMAMAN-15: Determination Of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry: A Collaborative Study*

Study Director: Katerina Mastovska, Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA

	Summary of Method
ER 1	PAHs in homogenized seafood's are extracted with EtOAc:water. Extracts are cleaned with SPE technique before GC-MS analysis.
ER 2	This method utilizes solvent extraction of a homogenized sample followed by a silica-SPE procedure. The eluant is introduced into a GC-MS or GC-MS/MS in either SIM or MRM modes. Issues encountered by participating laboratories are typical of PAH analyses including loss of more volatile PAHs during evaporation and background PAH contamination. This method achieved good sensitivity, accuracy and precision and has Limits of detection/quantification that are lower than the levels of concern in seafood samples set by regulatory agencies.
ER 3	The method describes analysis of 19 PAHs and alkylated PAHs via GC-MS. Sample preparation involves solvent extraction followed by salting out partitioning with silica SPE cleanup. Method performance criteria are set instead of prescribing specific products/instruments needed to successfully complete analysis. Criteria address analytes recovery, matrix cleanup, calibration quality, chromatographic separation and detector sensitivity.
ER 4	This method presented a procedure to determine 19 selected polycyclic aromatic hydrocarbons (PAHs) and their relevant alkyl homologous in seafood using fast sample preparation followed by GC-MS analysis. The sample preparation included two steps: (1) extraction using water-ethyl acetate and salt-out by anhydrous magnesium sulfate and sodium chloride; (2) clean-up by silica gel SPE. GC-MS was very common and practical instrumentation for PAHs analysis. The method is simple, fast, accurate, robust and is easy to follow.
ER 5	Collaborative study conducted to determine selected PAHs and relevant alkyl homologues in seafood matrices using a fast sample preparation method followed by analysis with gas chromatography-mass spectrometry (GC-MS).
ER 6	This is a GC-MS method with C13 labeled internal standards; sample preparation is a QuEChERS approach followed by SPE cleanup. The method was designed around performance-based criteria regarding the GC separation, SPE, and evaporation steps.
ER 7	PAHs in seafood samples (10 g, hydrated with 5 or 10 mL water) are extracted into 10 mL ethyl acetate with the aid of partitioning salts (4 g magnesium sulfate and 2 g sodium chloride). 5 mL of the ethyl acetate extract is concentrated down and cleaned with 1 g silica gel SPE cartridge, the eluate is concentrated and solvent exchanged to 0.5 mL isooctane, and analyzed by GC/MS.
ER 8	good

	Method Scope/Applicability
ER 1	Applicable to seafood's such as mussel, oyster, and shrimp.
ER 2	This method provides determination of PAH and PAH analogues in shrimp, mussels and finfish which are representative of this class of compounds. The method achieves limits of detection well below the regulatory levels of concern.
ER 3	Scope of the method includes 19 specific PAHs in seafood. The matrices tested, shrimp, oyster and mussel, are typically 5% lipid content and below (USDA Nutrient database). Lipid content of commodities amendable for this method is an important consideration and should be addressed in the text of the method. Higher fat samples are addressed briefly in the method, indicating that a reduction of volume of extract should be applied to the silica SPE cartridge. This is a reasonable modification to the method but has implications for overall detectability, especially for BaP. It is possible that to meet fat removal criteria, modifications for calibration curves and/or sample preparation will need to be made. Modifications may be significant and therefore some comment on an upper limit of the lipid content applicable for the method as written would be useful.
ER 4	The method covers 19 selected PAHs in shrimp, mussel, and oyster with analytical ranges of fit for purpose. The scope should be easily expanded to include more analytes and applied to a verity of seafood or processed ones.
ER 5	All Federal, State and Commercial laboratories analyzing PAHs in seafood.
ER 6	The methods is intended for seafood that would accumulate PAHs and has been tested using mussel, oyster, and shrimp matrices.
ER 7	The method is capable to detect 19 selected PAHs quantitatively in shrimp, oyster and mussel samples.
ER 8	good

OMAMAN-15: Determination Of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry: A Collaborative Study*

Study Director: Katerina Mastovska, Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA

	General Comments
ER 1	This study successfully addressed the urgent need for a reliable approach to analyzing various PAHs in potentially contaminated seafood's. Method procedures are easy to follow and not time-consuming.
ER 2	The validation procedure outlined in this document was comprehensive and the results demonstrated that the method was accurate and rugged. This method is a significant improvement over current regulatory methods. It is significantly faster, requires less organic solvent and produces less waste, less labor intensive and is easier to perform that the current method, while fulfilling required method performance benchmarks.
ER 3	none
ER 4	The collaborative study report was a very clear presentation. The experiment or study was thorough and well designed. Performance-based criteria led to a robust method for analysis of PAHs in seafood. The method was practical and fit for purpose. Results from collaborative study supported the method performance and demonstrated that the method was fit-for-purpose to determine PAHs and their alkyl homologues in seafood.
ER 5	Method is well-written but needs more specificity in select sections. For example, on page 6, under (5), it is stated all analytes of reagent blanks must be below the concentrations in the lowest calibration standard. Needs more clarification....how far below? Also, since stability of some PAHs was questionable, a Stability Study needs to be carried out with PAH standards stored at varying temperatures and times. The Safety Section must be in the front of the method since safety is more important than any other part of the protocol.
ER 6	None
ER 7	The method is quick, easy to use, and allows flexibility in method development. It demonstrated good GC separations of isomer pairs, excellent recoveries and reproducibility were achieved except for 2 compounds in oyster which might degrade at -20 C, no degradation was observed when oyster was stored at -70 C, however it's not very practical for many labs to maintain such low storing temperature.
ER 8	Very good method

	Method Clarity
ER 1	Good clarity throughout the manuscript.
ER 2	The method is well written and easily understood. The instructions are clear and I found no ambiguities.
ER 3	The method as written is clean with only a few instances for improvement. Performance criteria and how to evaluate the criteria are nicely described.
ER 4	The method procedure is well described and steps are easy to follow.
ER 5	Method is generally clear but needs more specificity in select areas.
ER 6	The method is clear and all the necessary information provided to reproduce and use the method. The authors should use correct nomenclature for the PAHs, i.e., Benzo[ghi]perylene not benzo[g,h,i]perylene
ER 7	The method is clearly described in the manuscript.
ER 8	good

OMAMAN-15: Determination Of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry: A Collaborative Study*

Study Director: Katerina Mastovska, Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA

	Pros/Strengths
ER 1	The organizer took great effort setting up the procedures to allow for individual lab's choice of various instruments, columns, SPE vendors, and evaporation techniques, as long as the lab passed the performance requirements. The procedures are straight forward and not hard to follow.
ER 2	Fairly easy method that requires significantly less sample preparation compared to other methods. Utilizes equipment and instrumentation that is widely available. Uses less flammable and toxic solvents than other methods. Sample handling is minimized which decreases the probability of environmental contamination.
ER 3	Strengths include: 1. easy to follow criteria for sample preparation evaluation 2. easy to follow instructions for solution and calibration solution preparation 3. some allowance for environmental background of the naphthalene 4. importance of monitoring blank is clearly stated
ER 4	The method is simple, fast, accurate, robust and is easy to follow.
ER 5	Well written, encompasses analyses of PAH compounds deleterious to humans at low levels, the calculations outlines on pages 14-15 are well written.
ER 6	A major strength is that the method is an isotope dilution (ID) GC-MS methods using C13 labeled internal standards for 13 of the 19 target PAHs. The sample preparation appears to be simplified compared to normal solvent extraction methods (Soxhlet, ACE, MAE). Another strength is the performance criteria required for the choice of GC column and the requirements to separate critical PAH isomers such as the benzofluoranthenes.
ER 7	The method is simple, fast, and easy to use. High sample throughput with little lab ware needed. Applicable to a variety of seafood matrices. Overall method performance are acceptable.
ER 8	no comment

	Cons/Weaknesses
ER 1	Isotope-Labeled mixed standards may be expensive or could be unavailable occasionally. Precision of results (all three levels) may have some room for improvement.
ER 2	Still requires some sample clean up, including a dry down step which if performed incorrectly could cause artificially low calculated concentrations for low molecular weight PAHs. Does not incorporate many alkyl homolog PAH compounds. These are often present at higher concentration in oil contamination and have similar toxicity to the PAHs. Addition of these compounds to the GC-MS method would increase the applicability and impact of the method. This could perhaps be done in the future
ER 3	Weaknesses include: 1. Method scope of 1 ug/kg LOQ of BaP was not tested as a fortification level. As I read the method, the lowest fortification level for BaP was 2 ug/kg. 2. Polypropylene tubes used for extraction will likely cause users of the method issues with PAH contamination. Discussion of alternatives would be helpful. 3. PAH GC-MS analysis has significant differences than typical analysis of most other types of compounds. Guidance for GC-MS parameters would likely be helpful for users of the method. These include parameters like inlet temperature, transfer line temperature, ion source temperature, column loadability and efficient flow conditions. 4. There is no recommendation on how to report data on chrysene and triphenylene if the recommended, but not required, 50% valley separation is not met. Can chrysene and triphenylene be reported together? 5. Ion ratios are mentioned as a requirement for identification but there is no indication as to the RSD value that is acceptable or some other qualification. 6. When a linear calibration curve is not possible, allowance for a "well-characterized" quadratic formula is made but with no discussion of what "well-characterized" means. Some guidance would be useful because some user will not be accustomed using quadratic calibration curves.
ER 4	A commercially available mix of standards suitable for the method is beneficial.
ER 5	The Safety Section must be in the front of the method since safety is more important than any other part of the protocol. Method must be more specific. Under Degradation Issues on page 18, the discussion emphasizes the need for a Stability Study. 18.2 megaohm water should be used for any GC/MS method (page 9, Section C). Need a statement that documented calibrations/reference checks were performed on all analytical equipment and instrumentation used in the collaborative study.
ER 6	A major weakness is that there is no validation using a certified reference materials for PAHs in seafood. Standard Reference Material (SRM) 1974b Mussel Tissue is mentioned as part of the qualification of the labs (p. 6) as a practice sample, but no data are reported using SRM 1974b for validation of the proposed method. The availability of SRM 1974c (which has replaced SRM 1974b) provided an excellent opportunity to use a CRM to validate an AOAC method. The use of only fortified/spiked samples for the method validation is a weakness. Spiked samples are sometimes the only option but in this

OMAMAN-15: Determination Of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry: A Collaborative Study*

Study Director: Katerina Mastovska, Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA

	case with SRM 1974 available, it could have been handled differently. The selection of the 19 target PAHs to determine could be questioned. The authors comment on criteria for separation of chrysene and triphenylene and also BaP and BeP, which is good, but perhaps triphenylene and BeP should have been included. There were no performance criteria for the separation of the alkyl-PAHs from potential isomers, e.g., with 3-MeChr targeted, how do you know if you are separating it from other methylchrysene or methyl-BaA isomers? The inclusion of several alkyl-PAHs is good, but will this really provide a method to look at the alkyl-PAHs, which in petroleum contaminated samples may be more abundant than the parent PAHs. Should there be a provision for looking at the alkyl-PAHs as a group by MS? Perhaps this is beyond the intended scope of this method.
ER 7	Fish, one of the most common seafood, was not covered in this method. Oyster needs to be stored at -70 C, which is not very easy for many labs to maintain.
ER 8	no comment

	Supporting Data Comments
ER 1	Great summary of results and provided complete statistical information. Would like to see more on linearity results.
ER 2	The supporting data indicates that the method is rugged and accurate.
ER 3	Nicely organized.
ER 4	na
ER 5	More specificity needed in quantitative parameters.
ER 6	In general, this is a good method, but needs further validation.
ER 7	Tested real samples. Additional experiments with shrimp matrix performed.
ER 8	good

	Method Optimization
ER 1	N/A
ER 2	Was well described and performed appropriately.
ER 3	Good.
ER 4	na
ER 5	The Safety Section must be in the front of the method since safety is more important than any other part of the protocol. Method must be more specific when outlining quantitative parameters. Under Degradation Issues on page 18, the discussion emphasizes the need for a Stability Study. 18.2 megaohm water should be used for any GC/MS method (page 9, Section C). Need a statement that documented calibrations/reference checks were performed on all analytical equipment and instrumentation used in the collaborative study.
ER 6	The method optimization is well done in particular the SPE cleanup. Could the use of an aminopropyl SPE be less sensitive than silica regarding deactivation by moisture content?
ER 7	Elution solvent volume for silica gel SPE cleanup is optimized. Increased water amount (10 mL) is used in shrimp samples to help shake and extract PAHs from more viscous shrimp samples.
ER 8	good

OMAMAN-15: Determination Of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry: A Collaborative Study*

Study Director: Katerina Mastovska, Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA

	Analytical Range
ER 1	Varies depending on the analyte. Generally, low is from 2 ppb to 25 ppb. High is 20 ppb to 250 ppb.
ER 2	Range varies and is dependent on the specific analyte. This is appropriate as lower molecular weight PAHs are generally found in higher concentrations than higher molecular weight PAHs. It is acceptable for this application as levels of concern for low molecular weight PAHs are significantly higher.
ER 3	Sufficient.
ER 4	BaP: 0.5 - 100 µg/kg (0.5, 1, 2, 5, 10, 20, 50, 100) Other PAHs: 1.25 - 250 µg/kg (1.25, 2.5, 5, 12.5, 25, 50, 125, 250) naphthalene: 2.5 - 500 µg/kg (2.5, 5, 10, 25, 50, 100, 250, 500)
ER 5	Good
ER 6	Suitable
ER 7	0.5 to 100 ug/kg for BaP and other lower-level PAHs; 1.25 to 250 ug/kg for higher-level PAHs; and 2.5 to 500 ug/g for naphthalene.
ER 8	good

	LOQ
ER 1	Not discussed in the method. Likely in the low ppbs.
ER 2	LOQ varies and is dependent on the specific analyte. This is appropriate as lower molecular weight PAHs are generally found in higher concentrations than higher molecular weight PAHs. It is acceptable for this application as levels of concern for low molecular weight PAHs are significantly higher.
ER 3	Please see comment above about spike levels and LOQ of BaP.
ER 4	Bap: 0.5 µg/kg Other PAHs: 1.25 µg/kg naphthalene: 2.5 µg/kg
ER 5	Good
ER 6	Suitable
ER 7	1 ug/kg for BaP and other lower-level PAHs; 2.5 ug/g for higher-level PAHs; and 5 ug/g for naphtalene.
ER 8	good

	Accuracy/Recovery
ER 1	Varies depending on the analyte. 70-120% mostly.
ER 2	Accuracy/Recovery is high and meets validation criteria.
ER 3	Good
ER 4	In shrimp: 83.8-115% In mussel: 77.3-107% In oyster: 71.6-94.6%, except for a lower mean recovery of 68.6% for benzo[α]anthracene (BaA) in oyster, and 50.3-56.5% and 48.2-49.7% for anthracene and beno[α]pyrene, respectively.
ER 5	Good
ER 6	As noted by the authors, the recoveries for the oyster tissue are low and probably inadequate.
ER 7	After excluding the outliers, the mean recoveries (8-10 labs) are in the range of 70-120% with a few exceptions which may due to the compound degradation in oyster samples stored at -20 C.
ER 8	good

OMAMAN-15: Determination Of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry: A Collaborative Study*

Study Director: Katerina Mastovska, Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA

	Precision
ER 1	Varies. Mostly around or below 10%, with one exception of 27% for low level 1-MN.
ER 2	Precision and reproducibility varies and is dependent on the specific analyte. The reported values meet the validation criteria
ER 3	Good
ER 4	In Shrimp: 1.40-26.9% In mussel: 2.52-17.1% In oyster: 3.12-22.7%
ER 5	Good
ER 6	I am not familiar with the expectations for precision for an AOAC method; however, the precision here appears to be adequate.
ER 7	Precision was excellent.
ER 8	good

	Reproducibility
ER 1	Varies. Mostly between 10%-20%.
ER 2	Precision and reproducibility varies and is dependent on the specific analyte. The reported values meet the validation criteria
ER 3	Good
ER 4	In Shrimp: 5.41-29.4% In mussel: 4.19-32.5% In oyster: 8.41-31.8%
ER 5	Good
ER 6	I am not familiar with the expectations for reproducibility for AOAC method; however, the reproducibility for this study appears to be inadequate for many of the more volativle PAHs (e.g., naphthalene) and particularly in the oyster tissue.
ER 7	Reproducibility was good except for a few compounds in oyster stored at -20 C.
ER 8	good

	System Suitability
ER 1	Not discussed.
ER 2	System is suitable
ER 3	Good
ER 4	na
ER 5	Were IDLs, MDLs and PQLs carried out on all instrumentation used in the Collaborative Study? If not, this should be performed and documented in the Method. Are there records of Intra-day, Inter-day variability? Are there records of Analyst variability? If so, the Method should state.
ER 6	No comments
ER 7	System check samples were analyzed.
ER 8	very good

OMAMAN-15: Determination Of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry: A Collaborative Study*

Study Director: Katerina Mastovska, Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA

	First Action Recommendation
ER 1	Yes.
ER 2	Yes
ER 3	Yes
ER 4	I recommend that the method, which has been gone through AOAC collaborative study successfully, for determination of PAHs in seafood using GC-MS be adopted Official Fist Action
ER 5	No
ER 6	Not yet....I think it needs validation with a natural matrix CRM such as SRM 1974c.
ER 7	Yes, with minor modifications (please see After First Action Recommendation)
ER 8	yes

	After First Action Recommendation
ER 1	Explore for ways to improve inter-lab precision RSD(R)%
ER 2	NO
ER 3	See comments above.
ER 4	na
ER 5	It may be helpful to refer to the FDA's LIB # 4475 to get a better feel for how the Method should be formatted and important quantitative data to include. The only exception here is the Safety Section is not in the front of this FDA LIB.
ER 6	As mentioned above, information on the method performance using SRM 1974c
ER 7	Fish samples should be analyzed in the future to see if this method is applicable to fish as well, especially those with high fat content. Was matrix effect significant? or the internal standards (13C PAHs) added to samples before extraction corrected the matrix effect of their corresponding PAHs? How about the alkyl PAHs that did not include their isotope labeled standards in this study? Should matrix matched calibration be more appropriate? I would suggest the study group to compare the PAH recoveries using this method and one of the other currently accepted methods to test an oyster reference material stored at - 20 C to show if the degradation of Ant and BaP is method dependent.
ER 8	no

AOAC Official Method 2014.09
Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea
GC/MS, GC/MS/MS, and LC/MS/MS
First Action 2014

Forty representative pesticides were selected based on guidance from the AOAC Method-Centric Committee on Pesticide Residues to conduct a multilaboratory validation study of the single-laboratory validated (SLV) method using two representative brands of tea. The representative pesticides selected for the multilaboratory validation study of the SLV method are as follows: 2,4′-DDE, 4,4′-DDE, benalaxyl, bifenthrin, bromophos-ethyl, bromopropylate, chlorfenapyr, diflufenican, dimethenamid, fenchlorphos, picoxystrobin, pirimicarb, pirimiphos-methyl, procymidone, propyzamide, pyrimethanil, quinoxyfen, tefluthrin, tolclofos-methyl, and trifluralin by GC/MS and GC/MS/MS; and acetochlor, benalaxyl, bensulide, butralin, chlorpyrifos, clomazone, diazinon, ethoprophos, flutolanil, imidacloprid, indoxacarb, kresoxim-methyl, monolinuron, picoxystrobin, pirimiphos-methyl, propoxur, quinoxyfen, tebufenpyrad, triadimefon, and trifloxystrobin by LC/MS/MS.

LOQs for the 653 pesticides included in the SLV ranged from 0.03 to 1210 µg/kg. LODs of the GC/MS method ranged from 1.0 to 500 µg/kg, and the corresponding LOQs ranged from 2.0 to 1000 µg/kg. LODs of the GC/MS/MS method ranged from 1.0 to 900 µg/kg, and the corresponding LOQs ranged from 2.0 to 1800 µg/kg. LODs of the LC/MS/MS method ranged from 0.03 to 4820 µg/kg, and the corresponding LOQs ranged from 0.06 to 9640 µg/kg.

A total of 482 of the 653 pesticides can be analyzed by GC/MS and GC/MS/MS, while 417 of the 653 pesticides can be analyzed by LC/MS/MS with LODs ≤100 µg/kg. There are 264 out of the 653 pesticides that can be analyzed by GC/MS, and 325 out of 653 by LC/MS/MS with LODs ≤10 µg/kg. There are 270 pesticides that can be analyzed by both GC/MS and LC/MS/MS. Of these, there are 264 pesticides that can be analyzed by GC/MS and 247 by LC/MS/MS, with LODs ≤100 µg/kg for the GC/MS method. There are, however, 133 pesticides that can be analyzed by GC/MS and 200 by LC/MS/MS, with LODs ≤10 µg/kg.

A. Principle

Test samples are extracted with acetonitrile using a homogenizer, and the extracts are cleaned up with Cleanert TPT (Agela Technologies, Tianjin, China), EnviCarb/PSA or ECPSACB506 (UCT, Bristol, PA, USA), BE Carbon 500 g/PSA (Agilent Technologies, Santa Clara, CA, USA), or InerSep GC/PSA (GL Sciences, Tokyo, Japan), or equivalent cartridges. The pesticides are eluted with acetonitrile–toluene (3 + 1, v/v), concentrated, dried, dissolved in the recommended solution, and analyzed by GC/MS, GC/MS/MS, or LC/MS/MS. Quantification is with a matrix-matched standard calibration curve.

B. Reagents

(a) *Solvents*.—Acetonitrile, toluene, and *n*-hexane (HPLC grade).

(b) *Acetonitrile–toluene*.—3 + 1 (v/v).

(c) *Ultrapure water*.—Obtained from a Milli-RO plus system together with a Milli-Q system (EMD Millipore Corp., Billerica, MA, USA).

(d) *Anhydrous sodium sulfate*.—Analytically pure. Baked at 650°C for 4 h and stored in a desiccator.

C. Materials

(a) *SPE cartridges*.—Cleanert TPT (2000 mg, 12 mL; Agela Technologies), or EnviCarb/PSA (500 mg/500 mg, 6 mL; Supelco, Bellefonte, PA, USA), or equivalent.

(b) *SPE tube adapter*.—For 12 mL SPE tubes (57267), for 6 mL SPE tubes (57020-U; Sigma-Aldrich Shanghai Trading Co., Ltd, Shanghai, China), or equivalent.

(c) *Disposable flow control valve liners*.—For Visiprep TM-DL (57059; Sigma-Aldrich Shanghai Trading Co., Ltd).

(d) *Pear-shaped flask*.—80 mL (Z680346-1EA; Sigma-Aldrich Shanghai Trading Co., Ltd), or equivalent. *See* Figure **2014.09**.

(e) *Reservoir*.—30 mL (A82030; Agela), or equivalent. *See* Figure **2014.09**.

(f) *Centrifuge tube*.—80 mL.

(g) *Millipore filter membrane (nylon)*.—13 mm × 0.2 µm.

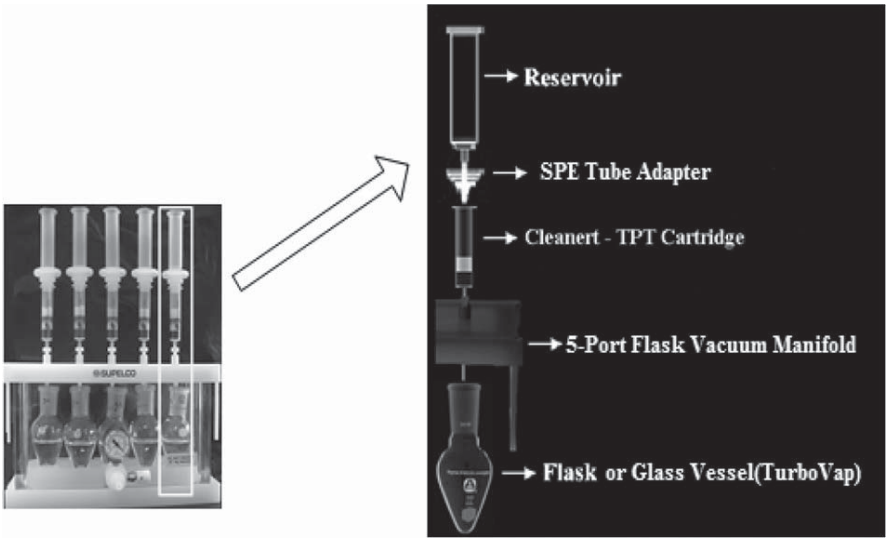


Figure 2014.09. SPE equipment.

Table 2014.09A. GC/MS retention times, quantifying ions, qualifying ions, ion abundances, LODs, and LOQs for the 20 pesticides

No.	Pesticide	Retention time, min	Quantifying ion, *m/z*	Qualifying ion 1, *m/z*	Qualifying ion 2, *m/z*	LOQ, µg/kg	LOD, µg/kg
ISTD[a]	Heptachlor-epoxide	22.15	353(100)[b]	355(79)	351(52)		
1	Trifluralin	15.43	306(100)	264(72)	335(7)	20.0	10.0
2	Tefluthrin	17.35	177(100)	197(26)	161(5)	10.0	5.0
3	Pyrimethanil	17.43	198(100)	199(45)	200(5)	10.0	5.0
4	Propyzamide	18.94	173(100)	255(23)	240(9)	10.0	5.0
5	Pirimicarb	19.00	166(100)	238(23)	138(8)	20.0	10.0
6	Dimethenamid	19.77	154(100)	230(43)	203(21)	10.0	5.0
7	Tolclofos-methyl	19.83	265(100)	267(36)	250(10)	10.0	5.0
8	Fenchlorphos	19.90	285(100)	287(69)	270(6)	40.0	20.0
9	Pirimiphos-methyl	20.37	290(100)	276(86)	305(74)	20.0	10.0
10	2,4'-DDE	22.75	246(100)	318(34)	176(26)	25.0	12.5
11	Bromophos-ethyl	23.12	359(100)	303(77)	357(74)	10.0	5.0
12	4,4'-DDE	23.95	318(100)	316(80)	246(139)	10.0	5.0
13	Procymidone	24.57	283(100)	285(70)	255(15)	10.0	5.0
14	Picoxystrobin	24.79	335(100)	303(43)	367(9)	20.0	10.0
15	Chlorfenapyr	27.40	247(100)	328(54)	408(51)	200.0	100.0
16	Quinoxyfen	27.15	237(100)	272(37)	307(29)	10.0	5.0
17	Benalaxyl	27.68	148(100)	206(32)	325(8)	10.0	5.0
18	Bifenthrin	28.62	181(100)	166(32)	165(35)	10.0	5.0
19	Diflufenican	28.73	266(100)	394(25)	267(14)	10.0	5.0
20	Bromopropylate	29.46	341(100)	183(54)	339(51)	20.0	10.0

[a] ISTD = Internal standard.

[b] Numbers in parentheses are ion abundances.

D. Preparation of Standard Solutions

(a) *Prep*aration *of stock solutions*.—Accurately weigh 5–10 mg individual pesticide and chemical pollutant standards (accurate to 0.1 mg) into a 10 mL volumetric flask. Dissolve and dilute to volume with methanol, toluene, acetone, acetonitrile, isooctane, etc., depending on each individual compound's solubility. All standard stock solutions are stored in the dark at 0–4°C and can be used for 1 year.

(b) *Preparation of mixed standard solution.*—Depending on properties and retention times of compounds, all compounds are divided into a series of groups. The concentration of each compound is determined by its sensitivity on the instrument for analysis. Mixed standard solutions are stored in the dark below 4°C.

(c) *Working standard mixed solution in matrix.*—Prepare working standard mixture solution in matrix of pesticide and chemical pollutants by diluting an appropriate amount of mixed standard solution with blank extract, which has been taken through the method with the rest of the samples. Mix thoroughly. Used for plotting the standard curve. Working standard mixture solution in matrix must be freshly prepared.

E. Apparatus and Conditions

(a) *GC/MS analysis.*—(*1*) *System*.—Model 7890A gas chromatograph connected to a Model 5975C mass selective detector with electron ionization (EI) source and equipped with a Model 7683 autosampler and Chemstation data processing software system (Agilent Technologies), or equivalent.

(*2*) *Column*.—DB-1701 capillary column (30 m × 0.25 mm × 0.25 µm; J&W Scientific, Folsom, CA, USA), or equivalent.

(*3*) *Column temperature*.—40°C hold 1 min, 30°C/min to 130°C, 5°C/min to 250°C, 10°C/min to 300°C, hold 5 min.

(*4*) *Carrier gas*.—Helium, purity ≥99.999%, flow rate 1.2 mL/min.

(*5*) *Injection port temperature*.—290°C.

(*6*) *Injection volume*.—1 µL.

(*7*) *Injection mode*.—Splitless, purge on after 1.5 min.

(*8*) *Ionization mode*.—EI.

(*9*) *Ion source polarity*.—Positive ion.

(*10*) *Ionization voltage*.—70 eV.

(*11*) *Ion source temperature*.—230°C.

(*12*) *GC/MS interface temperature*.—280°C.

(*13*) *Solvent delay*.—14 min.

(*14*) *Ion monitoring mode*.—Selected ion monitoring (SIM); one quantifying ion and two qualifying ions are selected for each compound. The retention times, quantifying ions, qualifying ions, and the expected ion abundances for each of the 20 pesticides included in the study and heptachlor epoxide are listed in Table **2014.09A**. SIM acquisition parameters for ions monitored by GC/MS are shown in Table **2014.09B**.

(b) *GC/MS/MS analysis.*—(*1*) *GC/MS/MS system*.—Model 7890A gas chromatograph connected to a Model 7000B triple quadrupole mass spectrometer with EI source and equipped with a Model 7693 autosampler and Mass Hunter data processing software system (Agilent Technologies), or equivalent.

Table 2014.09B. SIM acquisition parameters by GC/MS for the 20 pesticides of interest in this study

Group	Start time, min	Monitored ions, *m/z*	Dwell time, ms
1	14.85	306, 264, 335	80
2	16.85	177, 197, 161, 198, 199, 200	80
3	17.97	173, 255, 240, 166, 238, 138	80
4	19.43	154, 230, 203, 285, 287, 270, 265, 267, 250	40
5	20.00	290, 276, 305	80
6	21.77	246, 318, 176, 353, 355, 351	80
7	22.93	359, 303, 357, 318, 316, 246	80
8	24.20	335, 303, 367, 283, 285, 255	80
9	25.87	237, 272, 307, 247, 328, 408, 148, 206, 325	40
10	28.49	181, 166, 165, 266, 394, 267, 341, 183, 339	40

(*2*) Operating conditions are the same as for GC/MS with the exception that the ion monitoring mode is by selected reaction monitoring (SRM) and monitoring one precursor ion and two product ion transitions.

(*3*) The monitored ion transitions and the collision energies for the 20 pesticides of interest in the study and heptachlor epoxide are shown in Table **2014.09C**. The SRM acquisition parameters for the precursor and product ion transitions monitored by GC/MS/MS are shown in Table **2014.09D**.

(c) *LC/MS/MS analysis.*—(*1*) *LC/MS/MS system*.—An Agilent Series 1200 HPLC system directly coupled to a 6430 triple quadrupole mass spectrometer equipped with an electrospray ionization source and Model G1367D autosampler with a Mass Hunter data processing software system (Agilent Technologies).

(*2*) *Column*.—Zorbax SB-C18, 2.1 × 100 mm × 3.5 µm, or equivalent.

(*3*) *Mobile phase gradient elution program and flow rate*.—*See* Table **2014.09E**.

(*4*) *Column temperature*.—40°C.

(*5*) *Injection volume*.—10 µL.

(*6*) *Ionization mode*.—ESI.

(*7*) *Ion source polarity*.—Positive ion.

(*8*) *Nebulizer gas*.—Nitrogen gas.

(*9*) *Nebulizer gas pressure*.—0.28 Mpa.

Table 2014.09C. GC/MS/MS retention times, monitored ion transitions, collision energies, LODs, and LOQs for the 20 pesticides

No.	Pesticide	Retention time, min	Quantifying precursor/production transition, *m/z*	Qualifying precursor/production transition, *m/z*	Collision energy, V	LOQ, µg/kg	LOD, µg/kg
ISTD	Heptachlor-epoxide	22.15	353/263	353/282	17;17		
1	Trifluralin	15.41	306/264	306/206	12;15	4.8	2.4
2	Tefluthrin	17.40	177/127	177/101	13;25	0.8	0.4
3	Pyrimethanil	17.42	200/199	183/102	10;30	6.0	3.0
4	Propyzamide	18.91	173/145	173/109	15;25	1.0	0.5
5	Pirimicarb	19.02	238/166	238/96	15;25	4.0	2.0
6	Dimethenamid	19.73	230/154	230/111	8;25	2.0	1.0
7	Fenchlorphos	19.83	287/272	287/242	15;25	16.0	8.0
8	Tolclofos-methyl	19.87	267/252	267/93	15;25	10.0	5.0
9	Pirimiphos-methyl	20.36	290/233	290/125	5;15	10.0	5.0
10	2,4'-DDE	22.79	318/248	318/246	15;15	6.0	3.0
11	Bromophos-ethyl	23.16	359/303	359/331	10;10	10.0	5.0
12	4,4'-DDE	23.90	318/248	318/246	25;25	4.0	2.0
13	Procymidone	24.70	283/96	283/255	10;10	2.0	1.0
14	Picoxystrobin	24.75	335/173	335/303	10;10	10.0	5.0
15	Quinoxyfen	27.18	237/208	237/182	25;25	80.0	40.0
16	Chlorfenapyr	27.37	408/59	408/363	15;5	140.0	70.0
17	Benalaxyl	27.66	148/105	148/79	15;25	2.0	1.0
18	Bifenthrin	28.63	181/166	181/165	10;5	10.0	5.0
19	Diflufenican	28.73	266/218	266/246	25;10	20.0	10.0
20	Bromopropylate	29.46	341/185	341/183	15;15	8.0	4.0

Table 2014.09D. SRM acquisition parameters by GC/MS/MS analysis for the 20 pesticides of interest

Group	Start time, min	Monitored ion transitions, *m/z*	Dwell time, ms
1	14.76	306/264, 306/206	50
2	15.87	177/127, 177/101, 200/199, 183/102	50
3	18.06	173/145, 173/109, 238/166, 238/96	50
4	19.26	230/154, 230/111, 287/272, 287/242, 267/252, 267/93	25
5	20.07	290/233, 290/125	50
6	21.87	353/282, 353/263	50
7	22.60	359/331, 359/303, 318/248, 318/246	50
8	23.59	335/303, 335/173, 318/248, 318/246, 283/96, 283/255	50
9	26.71	148/105, 148/79, 408/363, 408/59, 237/208, 237/182	25
10	27.88	266/246, 266/218, 181/166, 181/165	50
11	28.96	341/185, 341/183	50

(*10*) *Ion spray voltage*.—4000 V.

(*11*) *Dry gas temperature*.—350°C.

(*12*) *Dry gas flow rate*.—10 L/min.

(*13*) Monitored ion transitions, collision energies, and fragmentation energies for the 20 pesticides and chlorpyrifos methyl are shown in Table **2014.09F**, and SRM acquisition parameters by LC/MS/MS for the precursor and product ion transitions monitored are shown in Table **2014.09G**.

(d) *Homogenizer*.—Rotational speed higher than 13 500 r/min (report also in *g*-force units; T-25B, Janke & Kunkel, Staufen, Germany), or equivalent.

(e) *Rotary evaporator*.—Buchi EL131 (Flawil, Switzerland) or equivalent.

(f) *Centrifuge*.—Centrifugal force higher than 2879 × *g* (Z320; B. HermLe AG, Gosheim, Germany), or equivalent.

(g) *Nitrogen evaporator*.—EVAP 112 (Organomation Associates, Inc., New Berlin, MA, USA), or equivalent.

(h) *TurboVap*.—LV Evaporation System (Caliper Life Sciences, Hopkinton, MA, USA), or equivalent.

(i) *Visiprep 5-port flask vacuum manifold*.—RS-SUPELCO 57101-U (Sigma-Aldrich Shanghai Trading Co., Ltd). *See* Figure **2014.09**.

(j) *Variable volume pipets*.—10 µL, 200 µL, and 1 mL.

(k) *Balance*.—Capable of accurately measuring weights from 0.05 to 100 g within ±0.01 g.

F. Extraction and Cleanup Procedure

(a) *Sample extraction.*—(*1*) Weigh 5 g dry tea powder (accurate to 0.01 g) into an 80 mL centrifuge tube.

(*2*) Add 15 mL acetonitrile.

(*3*) Homogenize at 13 500 rpm for 1 min.

(*4*) Centrifuge at 2879 × *g* for 5 min at room temperature.

(*5*) Transfer the supernatant into a pear-shaped flask.

(*6*) Re-extract the sample with 15 mL acetonitrile, homogenize, centrifuge, and combine the supernatants from the two extractions.

(*7*) Concentrate the extract to approximately 1 mL in a rotary evaporator (or TurboVap) in a 40°C water bath.

(*8*) Place a pear-shaped flask in the vacuum manifold.

(*9*) Mount a Cleanert TPT cartridge onto the manifold.

(*10*) Add anhydrous sodium sulfate (approximately 2 cm) onto the Cleanert TPT packing material.

(*11*) Add 10 mL acetonitrile–toluene (3 + 1, v/v) to activate the cartridge.

(*12*) Stop the flow through the cartridge when the liquid level in the cartridge barrel has just reached the top of the sodium sulfate packing.

(*13*) Discard the waste solution collected in the pear-shaped flask and replace with a clean pear-shaped flask.

(b) *SPE cleanup*.—(*1*) Load the concentrated extract from **F**(**a**)(*7*) into the conditioned Cleanert TPT cartridge collecting the eluate into the clean pear-shaped flask.

(*2*) Rinse the pear-shaped flask that contained the concentrated extract with 3 × 2 mL acetonitrile–toluene (3 + 1, v/v).

Table 2014.09E. Gradient elution conditions for LC/MS/MS analysis

Step	Time, min	Flow rate, µL/min	Mobile phase A (0.1% formic acid in water, %)	Mobile phase B (acetonitrile, %)
0	0.00	400	99.0	1.0
1	3.00	400	70.0	30.0
2	6.00	400	60.0	40.0
3	9.00	400	60.0	40.0
4	15.00	400	40.0	60.0
5	19.00	400	1.0	99.0
6	23.00	400	1.0	99.0
7	23.01	400	99.0	1.0

Table 2014.09F. LC/MS/MS retention times, ion transitions, collision energies, LODs, and LOQs for the 20 pesticides of interest in this study

No.	Pesticide	Retention time, min	Quantifying precursor/ production transition, *m/z*	Qualifying precursor/ product ion transition, *m/z*	Collision energy, V	Fragmentation, V	LOQ, µg/kg	LOD, µg/kg
ISTD	Chlorpyrifosmethyl	16.01	322.0/125.0	322.0/290.0	15; 15	80		
1	Imidacloprid	3.81	256.1/ 209.1	256.1/175.1	10; 10	80	22.0	11.0
2	Propoxur	5.89	210.1/111.0	210.1/168.1	10; 5	80	24.4	12.2
3	Monolinuron	6.83	215.1/126.0	215.1/148.1	15; 10	100	3.6	1.8
4	Clomazone	8.3	240.1/125.0	240.1/89.1	20; 50	100	0.4	0.2
5	Ethoprophos	11.37	243.1/173.0	243.1/215.0	10; 10	120	2.8	1.4
6	Triadimefon	11.64	294.2/69.0	294.2/197.1	20; 15	100	7.9	3.9
7	Acetochlor	12.94	270.2/224.0	270.2/148.2	5; 20	80	47.4	23.7
8	Flutolanil	13.25	324.2/262.1	324.2/282.1	20; 10	120	1.1	0.6
9	Benalaxyl	14.40	326.2/148.1	326.2/294.0	15; 5	120	1.2	0.6
10	Kresoxim-methyl	14.58	314.1/267	314.1/206.0	5; 5	80	100.6	50.3
11	Picoxystrobin	14.99	368.1/145.0	368.1/205.0	20; 5	80	8.4	4.2
12	Pirimiphos-methyl	15.05	306.2/164.0	306.2/108.1	20; 30	120	0.2	0.1
13	Diazinon	15.20	305.0/169.1	305.0/153.2	20; 20	160	0.7	0.4
14	Bensulide	15, 45	398.0/158.1	398.0/314.0	20; 5	80	34.2	17.1
15	Quinoxyfen	16.60	308.0/197.0	308.0/272.0	35; 35	180	153.4	76.7
16	Tebufenpyrad	16.82	334.3/147.0	334.3/117.1	25; 40	160	0.3	0.1
17	Indoxacarb	16.76	528.0/150.0	528.0/218.0	20; 20	120	7.5	3.8
18	Trifloxystrobin	16.82	409.3/186.1	409.3/206.2	15; 10	120	2.0	1.0
19	Chlorpyrifos	17.65	350.0/198.0	350.0/97.0	20; 35	100	53.8	26.9
20	Butralin	17.98	296.1/240.1	296.1/222.1	10; 20	100	1.9	1.0

(*3*) Load the rinse into the cartridge when the level of the loading solution in the cartridge reaches the top of the anhydrous sodium sulfate packing.

(*4*) Connect a 30 mL reservoir onto the upper part of the cartridge using an adapter (*see* Figure **2014.09**).

(*5*) Elute the cartridge with 25 mL acetonitrile–toluene (3 + 1, v/v).

(*6*) Evaporate the eluate to approximately 0.5 mL using a rotary evaporator (or TurboVap) in a 40°C water bath.

For GC/MS and/or GC/MS/MS analysis only:

(*7*) Add 40 µL heptachlor epoxide (internal standard; ISTD) working standard solution to the sample in **F**(**b**)(*6*).

(*8*) Evaporate to dryness under a stream of nitrogen in a 35°C water bath (or Turbo Vap).

(*9*) Dissolve the dried residue in 1.5 mL hexane, ultrasonicate the samples to mix, and filter through a 0.2 µm membrane filter. The sample is ready for GC/MS or GC/MS/MS analysis.

For LC/MS/MS analysis only:

(*10*) Add 40 µL chlorpyrifos methyl (ISTD) working standard solution to the sample prepared in **F**(**b**)(*6*).

(*11*) Evaporate to dryness under a stream of nitrogen in a 35°C water bath (or Turbo Vap).

(*12*) Dissolve the dried residue in 1.5 mL acetonitrile–water (3 + 2, v/v), ultrasonicate the samples to mix, and filter through a 0.2 µm membrane filter. The sample is ready for LC/MS/MS analysis.

G. Qualitative and Quantitative Analysis

(a) *Criteria for qualitative identification and confirmation*.—(*1*) Measure the retention time of the monitored peaks and match them with the same peaks in the pesticide standard chromatograms.

Table 2014.09G. SRM acquisition parameters by LC/MS/MS analysis for the 20 pesticides

Group	Start time, min	Monitored ion transitions, *m/z*	Dwell time, ms
1	0	256.1/209.1, 256.1/175.1, 210.1/111.0, 210.1/168.1, 240.1/125.0, 240.1/89.1, 243.1/173.0, 243.1/215.0, 294.2/69.0, 294.2/197.1, 215.1/126.0, 215.1/148.1	30
2	12	270.2/224.0, 270.2/148.2, 306.2/164.0, 306.2/108.1, 324.2/262.1, 324.2/282.1, 326.2/148.1, 326.2/294.0, 305.0/169.1, 305.0/153.2, 314.1/267.0, 314.1/206.0, 322.0/125.0, 322.0/290.0, 368.1/145.0, 368.1/205.0, 398.0/158.1, 398.0/314.0	20
3	16.4	334.3/147.0, 334.3/117.1,528.0/150.0,528.0/218.0, 409.3/186.1, 409.3/206.2,296.1/240.1, 296.1/222.1, 350.0/198, 350.0/97.0, 308.0/197.0, 308.0/272.0	25

Table 2014.09H. Recommended maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques

Relative intensity (% of base peak), %	GC/MS (relative), %	GC/MS/MS, LC/MS/MS (relative), %
>50	±10	±20
>20–50	±15	±25
>10–20	±20	±30
≤10	±50	±50

(*2*) Measure the ion abundances for the qualifier ions for the detected pesticides and verify that they are within the expected limits. If the peaks match, the presence of the pesticide is confirmed. *See* Table **2014.09H**.

(b) *Quantitative calculations.*—(*1*) Use instrument data processing software for GC/MS (SIM), GCMS/MS, and/or LC/MS/MS to calculate a response ratio (measured abundance of pesticide/measured abundance of heptachlor epoxide for GC and chlorpyrifos methyl for LC) and construct a five-point matrix-matched calibration curve of response ratio versus concentration of pesticide in standard solution.

(*2*) Using the regression data from the appropriate matrix-matched calibration curve, calculate the concentration of each pesticide found in the samples.

(*3*) If a validated computer system is not being used for calculations, follow the steps below:

(*a*) Measure the peak area of each respective standard level for each pesticide and the peak area of corresponding ISTD.

(*b*) Calculate the ratio of the analyte response to that of the ISTD.

(*c*) Run a linear regression analysis using the ratio of each pesticide at five different levels with no weighting or 1/x weighting, where x = concentration.

(*d*) Measure the peak area of each pesticide found in the sample and the peak area of corresponding ISTD.

(*e*) Calculate the amount of each pesticide in the solution injected from the standard curve.

(*f*) Calculate the amount of each pesticide present in the sample.

Test results should be reported to two decimal places or four significant digits.

Reference: *J. AOAC Int*. **98**, 1428(2015)
DOI: 10.5740/jaoacint.15021

Posted: October 13, 2015

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-07-08 08:20:27
Please indicate your perspective	Expert Review Panel Member
AOAC Official Methods Number (XXXX.XX)	2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	Yes
Please specify the information to be provided to support the reproducibility of this method as written.	https://sampleprep.unitedchem.com/media/at_assets/tech_doc_info/Pesticides_in_tea_AOAC

6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No
Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Xiaoyan Wang
Organization	UCT
E-mail Address	xwang@unitedchem.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-07-29 16:30:33
Please indicate your perspective	Expert Review Panel Member
AOAC Official Methods Number (XXXX.XX)	AOAC 2014.09
Method Name or Manuscript Title	High Throughput Analytical Techniques for the Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea by GC/MS, GC/MS/MS, and LC/MS/MS: Collaborative Study, First Action 2014.09
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Pang, Guo-Fang; Fan, Chun-Lin; Cao, Yan-Zhong; Yan, Fang; Li, Yan; Kang, Jian; Chen, Hui; Chang, Qiao-Ying Journal of AOAC International, Volume 98, Number 5, September-October 2015, pp. 1428-1454(27) I, nor my Agency Laboratories have not had the opportunity to use the described method, so I don't really have any experimental feedback to provide. I will wait until I hear feedback from other actual users to make any comments I may have.
Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Joe Boison
Organization	Canadian Food Inspection Agency (CFIA)
E-mail Address	joe.boison@inspection.gc.ca

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-30 09:56:36
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea GC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	Yes
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	Yes

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Chen Wang
Organization	Tea research Institute, Chinese Academy of Agricultural Sciences
E-mail Address	wangchen@tricaas.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-30 09:54:23
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	Yes
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	Yes
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	Yes

If Yes, please note any recommended changes to the AOAC First Action Method as written. Please note that this information can be uploaded as a file attachment.	hoping to use QuEChERs method
Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Jia Ren
Organization	DENO TESTING Service (Shanghai)
E-mail Address	renjia@shdenuo.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-30 09:51:35
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea GC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	Yes
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
Upload documentation to support the Single Laboratory Validation Information	TPT.pdf
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	Yes

6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No
Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	JianjunWang
Organization	Shanghai Bino Testing Service Co., Ltd
E-mail Address	bino_wjj@126.com

Recoveries for 41 pesticides in green tea and black tea determined by GC-MS: 70%-94%

No.	Pesticides	Recovery
1.	Phorate	84%
2.	Quintozene	81%
3.	Diazinon	85%
4.	Fonofos	80%
5.	Etrimfos	79%
6.	Dimethoate	70%
7.	Aldrin	81%
8.	Vinclozolin	79%
9.	Buprofezin	82%
10.	Metalaxyl	81%
11.	Methyl-parathion	85%
12.	Chlorpyrifos	90%
13.	Chlorpyrifosmethyl	93%
14.	Triazophos	84%
15.	Fenthion	82%
16.	Malathion	82%
17.	Fenitrothion	84%
18.	Triadimefon	78%
19.	Pendimethalin	81%
20.	Quinalphos	88%
21.	Phenthoate	81%

No.	Pesticides	Recovery
22.	Procymidone	92%
23.	Dieldrin	80%
24.	Methidathion	80%
25.	Fenamiphos	80%
26.	Terbufos	81%
27.	Bifenthrin	94%
28.	Fenpropathrin	89%
29.	Cypermethrin	88%
30.	Fenvalerate	90%
31.	Deltamethrin	87%
32.	Trifluralin	84%
33.	Sulfotep	81%
34.	A-HCH	84%
35.	Δ-HCH	88%
36.	B-HCH	83%
37.	Dicofol	85%
38.	p,p'-DDD	79%
39.	o,p'-DDD	80%
40.	o,p'-DDT	80%
41.	p,p'-DDE	80%

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-30 09:37:06
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	Yes
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	No
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Hong Chang
Organization	BENGBU Center for Disease Control and prevention
E-mail Address	494283390@qq.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:59:59
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Daliang Huang
Organization	Dalian entry-exit inspection and quarantine bureau
E-mail Address	ciqzhhdl@163.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:57:41
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	No
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Amadeo R. Fernández-Alba
Organization	EURL-FV, University of Almería
E-mail Address	amadeo@ual.es

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:54:35
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	Yes
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	Yes
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Li Zhou
Organization	Tea research institute Chinese Academy of agricultural sciences
E-mail Address	lizhou@tricaas.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:49:03
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	No
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	No
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	YU-Ju Huang
Organization	Tunding substation, tea research and extension station, executive yuan, ROC(Taiwan)
E-mail Address	Tds511@ttes.gov.tw

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:47:01
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	No
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Naoki Kanamaru
Organization	Japan grain inspection association central research laboratory
E-mail Address	n-kanamaru@kokken.or.jp

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:45:42
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Yanzhong Cao
Organization	Qinhuangdao Entry-exit Inspection and Quarantine Bureau
E-mail Address	qhdciq@aliyun.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:44:07
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Liqi Xie
Organization	Food inspection central of CIQ ShenZhen
E-mail Address	Ms64@163.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-07-18 03:30:47
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea GC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
Upload documentation to support the Single Laboratory Validation Information	Appendix-Fujian.docx
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No

6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No
Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Fang Yang
Organization	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau
E-mail Address	yffjciq@gmail.com

Appendix

Determination results of multi-residues in tea by GC-MS

No.	Pesticides	Retention time/min	Green tea										
			Calibration curve			Spike levels (μg/kg)	Recovery/%						RSD/%
			R^2	Slope	Intercept		No.1	No.2	No.3	No.4	No.5	AVE	
IS	heptachlor	18.787											
1	trifluralin	9.940	0.9997	1.0858	-0.0155	200	82.2	85.5	83.7	84.7	94.2	86.1	5.47
2	tefluthrin	12.609	0.9982	1.8509	0.3906	100	88.1	89.7	87.6	87.3	97.3	90.0	4.66
3	pyrimethanil	14.156	0.9977	2.6666	0.5547	100	81.3	83.3	80.9	80.8	94.5	84.2	6.96
4	propyzamide	13.246	0.9984	0.9642	0.2457	100	83.8	85.5	80.3	81.5	87.2	83.7	3.37
5	pirimicarb	15.174	0.9981	1.5951	0.4131	100	91.6	94.2	83.1	91.0	103.7	92.7	7.99
6	dimethenamid	15.305	0.9963	0.4662	0.1679	40	86.7	95.2	90.8	89.8	102.3	93.0	6.52
7	tolclofos-methyl	16.264	0.9971	1.3229	0.3183	100	88.3	90.9	88.5	87.9	97.4	90.6	4.40
8	fenchlorphos	16.017	0.9965	1.2458	0.3545	200	95.1	99.2	95.4	96.7	103.6	98.0	3.62
9	pirimiphos-methyl	16.337	0.9977	0.5001	0.1095	100	90.1	92.0	89.7	89.1	98.1	91.8	4.01
10	2,4-DDE	20.000	0.9977	4.0213	0.7936	400	113.4	101.0	86.4	84.7	94.6	96.0	12.18
11	bromophos-ethyl	19.611	0.9973	0.3133	0.0691	100	90.5	92.3	89.6	89.4	99.1	92.2	4.36
12	4,4-DDE	21.820	0.9978	3.1875	0.6239	400	83.9	86.1	83.9	83.8	92.9	86.1	4.56
13	procymidone	19.907	0.9975	0.3892	0.0890	100	87.7	92.6	90.8	87.6	98.0	91.3	4.68
14	picoxystrobin	21.198	0.9979	0.7657	0.1335	200	88.1	91.4	87.6	87.7	95.2	90.0	3.68
15	chlorfenapyr	23.250	0.9976	0.4209	0.0998	800	97.2	99.1	95.9	95.4	102.6	98.0	3.01
16	quinoxyfen	27.183	0.9980	1.1099	0.2239	100	81.3	84.3	82.1	81.7	91.9	84.2	5.27
17	benalaxyl	26.357	0.9976	1.5987	0.3213	100	85.9	90.7	87.0	86.2	93.9	88.7	3.91
18	bifenthrin	28.293	0.9978	3.0963	0.8757	100	85.0	88.4	85.7	84.2	92.7	87.2	3.97
19	diflufenican	27.751	0.9982	2.0066	0.3759	100	86.3	89.8	86.5	85.2	94.3	88.4	4.20
20	bromopropylate	29.781	0.9978	1.8023	0.3328	200	87.0	91.5	88.5	87.3	95.5	90.0	3.99

Determination results of multi-residues in tea by LC-MS/MS

No.	Pesticides	Retention time/min	Green tea										
			Calibration curve			Spike levels (μg/kg)	Recovery/%						RSD/%
			R2	Slope	Intercept		No.1	No.2	No.3	No.4	No.5	AVE	
IS	chlorpyrifos-methyl	17.618											
1	imidacloprid	5.663	0.9987	0.0850	0.0856	45	115.4	103.8	114.8	111.4	105.3	110.1	4.87
2	propoxur	8.264	0.9989	1.8593	31.8969	50	108.6	93.3	114.0	108.4	108.1	106.5	7.30
3	monolinuron	9.409	0.9980	0.0116	-0.0314	20	87.3	91.6	93.1	86.3	91.1	89.9	3.28
4	clomazone	11.107	0.9991	0.7681	0.9358	20	93.8	94.0	100.2	96.6	105.5	98.0	5.00
5	ethoprophos	13.899	0.9991	0.3678	1.0466	20	101.1	92.7	103.0	97.1	104.0	99.6	4.65
6	triadimefon	14.096	0.9985	0.2186	-1.0063	20	103.6	94.2	102.9	98.2	104.2	100.6	4.28
7	acetochlor	15.138	0.9990	0.2468	-0.3841	40	99.4	94.0	102.2	99.4	105.2	100.0	4.14
8	flutolanil	15.671	0.9969	0.4262	-0.8631	20	105.4	96.1	105.9	101.6	105.0	102.8	4.01
9	benalaxyl	16.24	0.9990	2.5914	18.3183	20	99.9	92.5	102.5	98.9	104.0	99.6	4.44
10	kresoxim-methyl	16.571	0.9946	0.1833	5.8772	200	107.1	96.0	92.0	103.6	103.7	100.5	6.21
11	picoxystrobin	17.119	0.9965	1.4234	1.7725	20	133.5	97.5	97.4	102.5	110.3	108.2	13.93
12	pirimiphos-methyl	16.453	0.9999	1.2458	5.7368	20	93.1	90.0	94.3	91.7	98.5	93.5	3.45
13	diazinon	17.09	0.9997	2.9582	-2.6557	20	105.9	95.5	97.4	97.1	108.8	101.0	5.93
14	bensulide	17.405	0.9952	0.2903	0.2172	60	116.7	93.3	99.7	103.2	106.0	103.8	8.30
15	quinoxyfen	17.491	0.9961	0.6027	4.4482	100	81.6	95.6	89.3	93.8	99.6	92.0	7.50
16	tebufenpyrad	18.016	0.9941	0.0441	-0.3803	20	114.5	99.8	97.9	105.3	106.4	104.8	6.22
17	indoxacarb	18.262	0.9992	0.0263	-0.0882	20	100.6	94.3	110.6	104.2	104.9	102.9	5.84
18	trifloxystrobin	18.311	0.9978	0.4936	-1.0594	20	101.7	98.6	101.7	100.2	108.9	102.2	3.85
19	chlorpyrifos	18.884	0.9928	0.0072	-0.0321	200	111.4	126.6	92.8	87.2	87.1	101.0	17.26
20	butralin	19.292	0.9956	0.0022	-0.0035	20	72.1	84.2	82.1	99.2	128.0	93.1	23.37

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-07-18 03:40:57
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea GC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Yanzhong Cao
Organization	Qinhuangdao Entry-exit Inspection and Quarantine Bureau
E-mail Address	qhdciq@aliyun.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-07-18 03:59:25
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea GC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Liqi Xie
Organization	Food Inspection Center of Shenzhen Entry-exit Inspection and Quarantine Bureau
E-mail Address	ms64@163.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-07-18 04:02:15
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea GC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method''s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	No
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Naoki Kanamaru
Organization	JAPAN GRAIN INSPECTION ASSOCIATION CENTRAL RESEARCH LABORATORY
E-mail Address	n-kanamaru@kokken.or.jp

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:34:17
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	No
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	Yes

If Yes, please note any recommended changes to the AOAC First Action Method as written. Please note that this information can be uploaded as a file attachment.	sample preparation is time consuming, i.e., rotatory evaporation.
Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Ying Xu
Organization	Asia pacific technical center, the coca-cola company
E-mail Address	Yxu3@coca-cola.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:36:50
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	No
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	Yes

If Yes, please note any recommended changes to the AOAC First Action Method as written. Please note that this information can be uploaded as a file attachment.	sample preparation is time consuming, i.e., rotatory evaporation.
Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Ying Xu
Organization	Asia pacific technical center, the coca-cola company
E-mail Address	Yxu3@coca-cola.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:40:52
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	No
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	No
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Fang Yang
Organization	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau
E-mail Address	yffjciq@gmail.com

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-10 01:42:42
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	AOAC Official Method 2014.09
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	Yes
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	No
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Qiong Wu
Organization	Huangshan Tea Quality And Safety Research Center of Huangshan Entry-Exit Inspection & Quarantine Bureau
E-mail Address	wuqiong@ahciq.gov.cn

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-30 09:32:07
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	Chinese Academy of Inspection and Quarantine
Method Name or Manuscript Title	Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in TeaGC-MS, GC-MS/MS, and LC-MS/MS First Action 2014
Please include the manuscript publication reference, if available. (OMA methods have manuscripts unless otherwise noted in the OMA)	Guo-Fang Pang, Chun-Lin Fan, Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang. Journal of AOAC International Vol. 98, No. 5, 2015: 1428-1454.
1. In your experience using the method, does the method perform according to the method"s applicability as written?	Yes
2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?	Yes
3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?	Yes
4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?	Yes
5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.	Yes
6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?	No

Would you like to provide comment(s) or feedback on additional methods? This form contains feedback for three (3) methods. You may now provide feedback on two (2) additional methods.	No, thank you!
Name	Chunyan Song
Organization	ICAS Testing Center (Shanghai)
E-mail Address	2075523968@qq.com

RESIDUES AND TRACE ELEMENTS

High-Throughput Analytical Techniques for Determination of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea, Part VI: Study of the Degradation of 271 Pesticide Residues in Aged Oolong Tea by Gas Chromatography-Tandem Mass Spectrometry and Its Application in Predicting the Residue Concentrations of Target Pesticides

QIAO-YING CHANG
Yanshan University, College of Environmental and Chemical Engineering, Qinhuangdao, Hebei 066004, People's Republic of China
GUO-FANG PANG[1]
Yanshan University, College of Environmental and Chemical Engineering, Qinhuangdao, Hebei 066004, People's Republic of China; Chinese Academy of Inspection and Quarantine, Beijing 100176, People's Republic of China
CHUN-LIN FAN and HUI CHEN
Chinese Academy of Inspection and Quarantine, Beijing 100176, People's Republic of China
ZHI-BIN WANG
Yanshan University, College of Environmental and Chemical Engineering, Qinhuangdao, Hebei 066004, People's Republic of China

The degradation rate of 271 pesticide residues in aged Oolong tea at two spray concentrations, named *a* and *b* (*a* < *b*), were monitored for 120 days using GC–tandem MS (GC-MS/MS). To research the degradation trends and establish regression equations, determination days were plotted as horizontal ordinates and the residue concentrations of pesticide were plotted as vertical ordinates. Here, we consider the degradation equations of 271 pesticides over 40 and 120 days, summarize the degradation rates in six aspects (A–F), and discuss the degradation trends of the 271 pesticides in aged Oolong tea in detail. The results indicate that >70% of the determined pesticides coincide with the degradation regularity of trends A, B, and E, i.e., the concentration of pesticide will decrease within 4 months. Next, 20 representative pesticides were selected for further study at higher spray concentrations, named *c* and *d* (*d* > *c* > *b* > *a*), in aged Oolong tea over another 90 days. The determination days were plotted on the *x*-axis, and the differences between each determined result and first-time-determined value of target pesticides were plotted on the *y*-axis. The logarithmic function was obtained by fitting the 90-day determination results, allowing the degradation value of a target pesticide on a specific day to be calculated. These logarithmic functions at *d* concentration were applied to predict the residue concentrations of pesticides at *c* concentration. Results revealed that 70% of the 20 pesticides had the lower deviation ratios of predicted and measured results.

As one of the world's three major health drinks, tea makes up the majority of exported traditional commodities of China. The tea plant is prone to be attacked by various pests and diseases during its growth because it is mostly planted in warm temperate zones and subtropical areas. Different kinds of pesticides have been widely used to control pests and plant diseases of tea in an effort to increase harvest productivity. However, pesticide residues in tea may cause damage to human health (1–4). With the strengthening of food safety policies in different countries and regions of the world, the awareness of pesticide residues in tea has generated great public concern (5–8).

The study of degradation regularity of pesticides, together with a model to simulate the dynamics in a tea sample, can be applied to analyze and predict pesticide residues in tea. This research is significant for guiding farmers to spray pesticides on tea plants in a reasonable way and is helpful for predicting the risk of pesticide residues in the tea trade. The degradation of pesticides is a complex process affected by many factors, including temperature, humidity, sunshine, metals, etc. (9, 10). Many studies have been carried out on the degradation of pesticides in agricultural products using different kinetic models (11–13). Ozbey and Uygun (14) investigated the behavior of some organophosphorus pesticide residues in peppermint tea during the infusion process. Manikandan et al. (15) studied the leaching of certain pesticides, such as ethion, endosulfan, dicofol, chlorpyrifos, deltamethrin, hexaconazole, fenpropathrin, propargite, quinalphos, and λ-cyhalothrin, from powdered black tea into the brew. Lin et al. (16) studied the

Received March 03, 2016. Accepted by AK March 24, 2016.
[1] Corresponding author's e-mail: ciqpang@163.com
Supplemental information is available on the *J. AOAC Int*. website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
DOI: 10-5740/jaoacint.16-0063

natural degradation dynamics of bifenthrin, fenpropathrin, cypermethrin, and buprofezin on new shoots of the Oolong tea plant using GC. Chen et al. (17) developed a GC-MS method for the analysis of bifenthrin, cyhalothrin, teflubenzuron, flufenoxuron, and chlorfluazuron in dried Oolong tea leaf samples, and then studied the natural degradation of these pesticides in the leaves of Oolong tea trees and the effect of processing steps on the residue.

Hitherto, less attention has been given to the degradation of pesticides in aged tea. To research the degradation regularity of pesticides in aged tea samples, on the basis of our previous studies (18–20), the developed GC-MS/MS method was used to determine the multiresidue of 271 pesticides, including organonitrogen, organophosphorus, organochlorine, organosulfur, carbamates, and pyrethroids, in aged Oolong tea over 3 to 4 months. Meanwhile, the regularity of 271 pesticides in aged Oolong tea determined over 40 and 120 days was discussed in different aspects according to fitting curves. Subsequently, 20 representative pesticides from different classes were optimized for further study. At a higher spray concentration, the residues of the selected 20 pesticides in aged Oolong tea were studied over 90 days to investigate the degradation regularity at different concentrations. The degradation values of target pesticides on a specific day could be predicted by the logarithmic function obtained from plotting the determination time (day) on the *x*-axis and the difference between each determined value and the first-time-determined value of target pesticides on the *y*-axis, according to the degradation results of the 20 pesticides at the higher concentration over 90 days.

Lastly, the proposed procedure was validated by predicting the pesticide residue at one of the Youden pair concentrations according to the logarithmic function from another concentration. The predicted values were compared to the measured results, and they were evaluated by their deviation ratios.

Experimental

Reagents

(a) *Solvents*.—Acetonitrile, dichloromethane, isooctane, and methanol (HPLC grade) were purchased from Dikma Co. (Beijing, China).

(b) *Anhydrous sodium sulfate*.—Analytically pure. Baked at 650°C for 4 h and stored in a desiccator.

(c) *Pesticide standards and internal standard (ISTD; heptachlor epoxide)*.—Purity ≥95% (LGC Promochem, Wesel, Germany).

(d) *Stock standard solutions*.—Weigh 5–10 mg individual pesticide and chemical pollutant standards (accurate to 0.1 mg) into a 10 mL volumetric flask. Dissolve and dilute to volume with methanol, toluene, acetone, acetonitrile, isooctane, etc., depending on each individual compound's solubility. Store all standard stock solutions in the dark at 0–4°C.

(e) *Mixed standard solutions*.—Depending on properties and retention time of each pesticide, all 271 pesticides for GC-MS/MS analysis are divided into three groups. The concentration of each mixed standard solution depends on the sensitivity of each compound for the instrument used for analysis. Mixed standard solutions should be stored in the dark below 4°C.

Material

(a) *SPE cartridge*.—Cleanert® Triple Phase of Tea SPE (Cleanert TPT; 10 mL, 2000 mg; Agela, Tianjin, China).

(b) *Homogenizer*.—Rotational speed higher than 13 500 rpm (report also in *g*-force units; T-25B; IKA-Labortechnik, Staufen, Germany), or equivalent.

(c) *Rotary evaporator*.—Buchi EL131 (Flawil, Switzerland), or equivalent.

(d) *Centrifuge*.—Centrifugal force higher than 2879 × *g* (Z320; B. HermLe AG, Gosheim, Germany), or equivalent.

(e) *Nitrogen evaporator*.—EVAP 112 (Organomation Associates, Inc., New Berlin, MA), or equivalent.

Apparatus and Conditions

(a) *GC-MS/MS system*.—Model 7890A gas chromatograph connected to a Model 7000B triple quadrupole mass spectrometer with electron ionization (EI) source, and equipped with a Model 7693 autosampler with tMass Hunter data processing software system (Agilent Technologies, Wilmington, DE). GC separation was achieved on a DB-1701 capillary column (30 m × 0.25 mm × 0.25 μm; Agilent J&W Scientific, Folsom, CA).

(b) *Conditions*—The oven temperature was programmed as follows: 40°C hold for 1 min, increase to 130°C at 30°C/min, increase to 250°C at 5°C/min, increase to 300°C at 10°C/min, and hold for 5 min. The carrier gas was helium, purity ≥99.999%; the flow rate, 1.2 mL/min; the injection port temperature, 290°C; the injection volume, 1 μL; the injection mode, splitless, purge on after 1.5 min; the ionization voltage, 70 eV; the ion source temperature, 230°C; the GC/MS interface temperature, 280°C; and the ion monitoring mode was multireaction monitor mode. Each compound is monitored by one quantifying precursor/product ion transition and one qualifying precursor/product ion transition.

Preparation Procedures for Aged Tea Samples

Pass Oolong tea leaves (free from the target pesticide after testing) through 10-mesh and then 16-mesh sieves after initial blending in a blender. Spread 500 g sieved Oolong tea leaves uniformly over the bottom of a stainless steel vessel 40 cm in diameter to await spraying. Accurately transfer a certain amount of pesticide mixed standard solution into the full-glass sprayer and spray the tea leaves. Spray while stirring the tea leaves with a glass rod for uniform coverage. After spraying, continue to stir the tea leaves for 30 min to dissipate the volatile solvents from the tea leaves. Place the sprayed tea leaves in a 4 L brown bottle to avoid exposure to light. Store at room temperature and continue oscillation blending for 12 h.

Spread the aged tea on the bottom of a flat-bottomed vessel, draw an X and weigh a total of five portions of aged tea samples collected from the symmetrical four points of the X and from the central area. Submit the samples for GC-MS/MS determination, and calculate the average value of the pesticide content of the aged tea samples and RSD. When the RSD is <4% for GC-MS/MS, it can be judged that tea samples have been sprayed and mixed homogeneously.

Extraction

Weigh 5 g dry tea powder (accurate to 0.01 g) into a 80 mL centrifuge tube, add 15 mL acetonitrile, and homogenize at 13 500 rpm for 1 min. Centrifuge the mixture at 2879 × *g* for 5 min, and transfer the supernatant into a pear-shaped flask. Re-extract the residue with 15 mL acetonitrile and centrifuge the mixture. Combine the two extracts, and rotary evaporate in a water bath at 40°C to about 1 mL for cleanup.

Cleanup

Place a pear-shaped flask under the five-port flask vacuum manifold, and mount a Cleanert TPT cartridge onto the manifold. Add about 2 cm anhydrous sodium sulfate onto the Cleanert TPT cartridge packing material, prewash with 10 mL acetonitrile–toluene (3 + 1, v/v) and discard the effluents to activate the cartridge. Stop the flow through the cartridge when the liquid level in the cartridge barrel has just reached the top of the sodium sulfate packing. Discard the waste solution collected in the pear-shaped flask and replace with a clean pear-shaped flask.

Transfer the concentrated sample extract (*see Extraction* section) into the SPE cartridge, rinse the sample solution bottle with 2 mL acetonitrile–toluene (3 + 1, v/v), and repeat this step thrice, transferring the rinsing liquids to the cartridge. Attach a 50 mL storage device onto the cartridge, and then elute with 25 mL acetonitrile–toluene (3 + 1, v/v), collecting the effluent into the pear-shaped flask by gravity feed. Rotary evaporate the effluent in a water bath at 40°C to about 0.5 mL. Add 40 µL heptachlor epoxide ISTD to the sample. Evaporate to dryness under a stream of nitrogen in a 35°C water bath. Dissolve the dried residue in 1.5 mL hexane, ultrasonicate the sample to mix, and filter through a 0.2 µm membrane filter. The sample is ready for GC-MS/MS analysis.

Results and Discussion

Degradation of 271 Pesticides in Aged Oolong Tea

The residues of 271 pesticides in aged Oolong tea were determined 25 times by GC-MS/MS over 120 days (every 5 days) to monitor their degradation behavior. To study the degradation regularity of the 271 pesticides in aged Oolong tea, scatter diagrams at *a* and *b* spray concentrations (*a* and *b* concns) over 40 and 120 days were prepared, using determination days as horizontal ordinates and concentrations of pesticide residues as vertical ordinates. The degradation equations are summarized in supplemental Table 1 (at *a* concn) and supplemental Table 2 (at *b* concn).

By comparing the degradation equations of pesticides at *a* and *b* concns over 40 and 120 days, it was found that their degradation trends were different. The trends included the following aspects: A—the residues of pesticide dropped exponentially over both 40 and 120 days; B—the residues of pesticide dropped exponentially over 40 days and logarithmically over 120 days; C—the residues of pesticide dropped logarithmically over 40 days and logarithmically/polynomially over 120 days; D—no trend over 40 days, showing only as scatter points (R^2 <0.4), although a dropping trend was seen over 120 days; E—some dropping trend over 40 days, whereas no trend over 120 days, showing only as scatter points (R^2 <0.4); and F—no trend over either 40 or 120 days, showing only as scatter points (R^2 <0.4). In addition, five pesticides were not detected at either *a* or *b* concn.

The degradation regularity of 271 pesticides was studied according to the A–F trends. The ratios of pesticides associated with degradation trends A–F in the 271 pesticides are shown in Figure 1. It was observed that most of the pesticides followed the A, B, or E degradation trends. At *a* concn, the ratios for A, B, and E were 21.8, 35.1, and 24.4%, respectively, and their total number was 220, accounting for 81.2% of the 271 pesticides. At *b* concn, the ratios for A, B, and E were 25.5, 33.9, and 14.8%, respectively, and their total number was 201, accounting for 74.2% of the 271 pesticides. These results demonstrate that A, B, and E degradation trends could represent the main aspects of the 271 pesticides. That is, most of the pesticides dropped exponentially over 40 days, and they presented dropping trends exponentially or logarithmically over 120 days. Although the ratios of pesticides with other degradation trends were small, they did represent a certain degree of degradation regularity. Therefore, all trends (A–F) are discussed below.

Degradation trend A.—Taking propachlor as an example, trend A degradation rates over 40 and 120 days are shown in Figure 2a and b, respectively. It is clearly observed that the concentration of propachlor in aged Oolong tea exponentially decreased with the increase of intervals. It is indicated that the degradation kinetics of trend A is a first-order reaction by Equation 1:

$$C = C_0 e^{-kt} \quad (1)$$

where C is the concentration of each pesticide in aged Oolong tea, C_0 is the initial concentration of each pesticide in aged Oolong tea, k is degradation rate constant, and t is the determination time (day).

It can be concluded that without the influence of other factors, the degradation rate of pesticides in trend A has a direct ratio to the initial concentration of pesticides in aged Oolong tea. Based on the first-order reaction model, the half-life of pesticides in trend A could be calculated by Equation 2:

$$t_{1/2} = \ln 2 / k \quad (2)$$

where $t_{1/2}$ is the half-life of the determined pesticide.

Half-lives were calculated according to the degradation equations of pesticides in trend A listed in supplemental Table 1

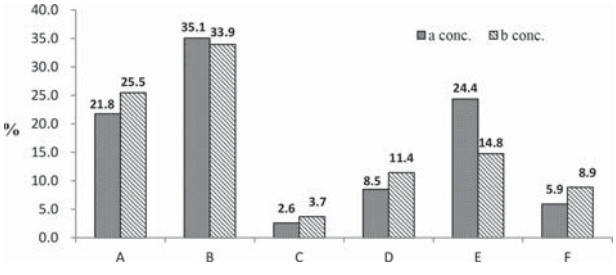


Figure 1. Percentage of pesticides in accordance with degradation trends A–F in 271 pesticides at *a* and *b* concentrations. Values represent the number of pesticides in accordance with each trend multiplied by 100 and divided by the total number (271) of pesticides (% = *n* × 100/*N*).

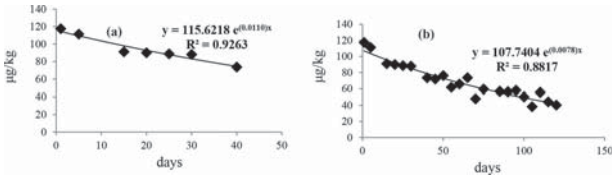


Figure 2. Degradation profiles of degradation trend A (the profiles of propachlor are shown as an example) over (a) 40 days and (b) 120 days. Determination days were plotted as horizontal ordinates; residue concentrations of pesticide were plotted as vertical ordinates.

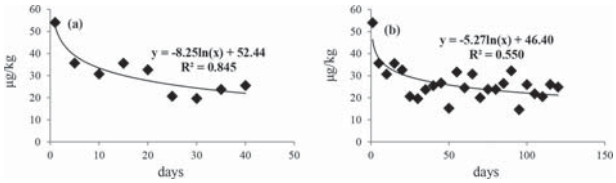


Figure 4. Degradation profiles of degradation trend C (e.g., dichlorofop-methyl) over (a) 40 days and (b) 120 days. Determination days were plotted as horizontal ordinates; residue concentrations of pesticide were plotted as vertical ordinates.

(at *a* concn) and supplemental Table 2 (at *b* concn). Degradation rate constants and their half-lives are also shown in supplemental Tables 1 and 2. By comparing the *k* values of *a* and *b* concns over 40 and 120 days, it can be found that most of the pesticides in trend A had higher *k* values over 40 days than over 120 days, except 12 and 19 pesticides showed the opposite at *a* and *b* concns, respectively. This finding indicated that the concentration of most of the pesticides in type A dropped fast in first 40 days, whereas they decreased slowly in the remaining days. However, chlorfenapyr, bupirimate, fonofos, and furalaxyl had similar degradation equations over both 40 and 120 days. For these four pesticides, therefore, the degradation trend could be expressed by the degradation equations over 40 days. At *a* concn, the half-life of pesticides in degradation trend A ranged from 24.4–223.6 and 44.4–203.9 days according to the degradation equations over 40 and 120 days, respectively. Except for the previously mentioned four pesticides, the half-life of the other pesticides varied over 40 and 120 days, with the biggest difference being156.9 days. At *b* concn, the half-life of pesticides in degradation trend A ranged from 40.8 to 315.1 and 46.8 to 330.1 days, according to the degradation equations over 40 and 120 days, respectively. On the whole, either at *a* or *b* concn, there were great differences in half-lives over 40 and 120 days. Based on actual conditions, the half-life calculated by degradation equations over 120 days was considered to be reasonable. There were 28 pesticides in accordance with degradation trend A at both *a* and *b* concns. By comparing *k* values from their degradation equations over 120 days, 10 pesticides had higher *k* values at *b* concn than at *a* concn, whereas the remaining 18 pesticides showed the opposite.

Degradation trend B—For degradation trend B, the concentration of pesticide in aged Oolong tea dropped faster over 40 days than over the remaining days. From the data in supplemental Tables 1 and 2, it was clear that the exponential equation was suitable for the first 40 days, whereas the logarithmical equation was suitable for 120 days. Taking chlorfenvinphos as an example, the degradation profiles of trend B are shown in Figure 3a and b for 40 and 120 days, respectively.

There were 95 pesticides at *a* concn and 92 pesticides at *b* concn in accordance with degradation trend B, accounting for 35.1 and 33.9% of the 271 pesticides, respectively. Among them, 50 pesticides were in accordance with degradation trend B at both *a* and *b* concns. The *k* values from the degradation equations over 40 days were compared, showing that most of the 36 pesticides had higher *k* values at *a* concn versus *b* concn.

Degradation trend C.—Degradation trend C was similar to degradation trend B; however, the difference was that the concentration of pesticides decreased logarithmically over 40 days (Figure 4). There were 7 and 10 pesticides in accordance with degradation trend C at *a* and *b* concns, respectively. It can be seen from the raw data that the concentrations of pesticides at day 5 or 10 day greatly differed from those on the first day. This logarithmic decrease may be considered the explanation for degradation trend C.

Degradation trend D.—For degradation trend D, the concentration of pesticides presented as scatter points with R^2 values of <0.4 over 40 days, and most of the dropped trend could be fitted by exponential and logarithmical curves and a few fitted by polynomial curves over 120 days (Figure 5). At *a* concn, there were 23 pesticides in accordance with degradation trend D; among them, 15 pesticides decreased exponentially and 8 decreased logarithmically or polynomially over 120 days. At *b* concn, there were 31 pesticides in accordance with degradation trend D, and 23 pesticides decreased exponentially over 120 days. By comparison, it was found that 10 pesticides were in accordance with degradation trend D at both *a* and *b* concns.

Degradation trend E.—Of the 271 pesticides, the ratios of pesticides in accordance with degradation trend E were third-ranked among the A–F aspects. The degradation profiles of 4,4′-dibromobenzophenone over 40 and 120 days are shown as an example of degradation trend E in Figure 6a and b, respectively. For degradation trend E, the concentrations of pesticides decreased exponentially or logarithmically over 40 days, whereas no suitable equations could be used to fit the scatter points over 120 days. It can be seen from supplemental Tables 1 and 2 that there were 66 pesticides in accordance

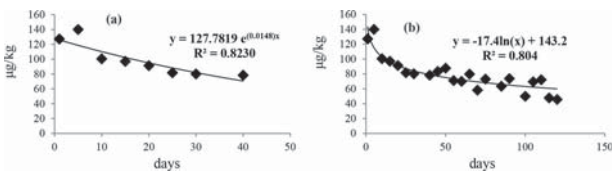


Figure 3. Degradation profiles of degradation trend B (e.g., chlorfenvinphos) over (a) 40 days and (b) 120 days. Determination days were plotted as horizontal ordinates; residue concentrations of pesticide were plotted as vertical ordinates.

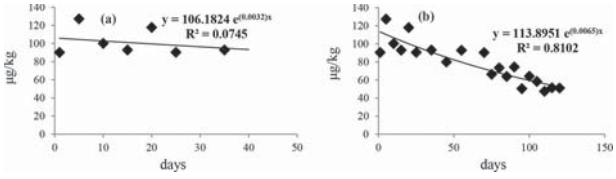


Figure 5. Degradation profiles of degradation trend D (e.g., cycluron) over (a) 40 days and (b) 120 days. Determination days were plotted as horizontal ordinates; residue concentrations of pesticide were plotted as vertical ordinates.

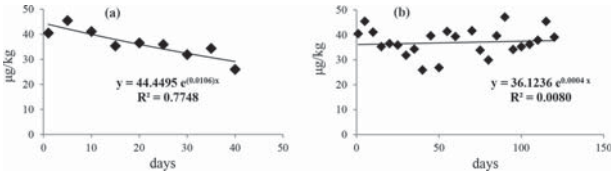


Figure 6. Degradation profiles of degradation trend E (e.g., 4, 4′-dibromobenzophenone) over (a) 40 days and (b) 120 days. Determination days were plotted as horizontal ordinates; residue concentrations of pesticide were plotted as vertical ordinates.

with degradation trend E at *a* concn, and among them, 9 and 21 pesticides were also in accordance with degradation trends A and B, respectively, at *b* concn, accounting for 45.5% of the 66 pesticides. Meanwhile, there were 40 pesticides in accordance with degradation trend E at *b* concn, but only 3 and 5 of these pesticides corresponded with degradation trends A and B, respectively, at *a* concn. At the same time, there were 24 pesticides in accordance with degradation trend E at both concentrations, accounting for 36.4 and 60.0% of the pesticides that were in accordance with degradation trend E at *a* and *b* concns, respectively.

Degradation trend F.—Taking kresoxim-methyl as an example, the profiles of degradation trend F are shown in Figure 7. The concentrations of the degraded pesticides presented as scatter points, and no degradation trend can be found by any of the above-mentioned fitting curves with R^2 values ≥0.4. The unsteadiness properties of these pesticides in aged Oolong tea during storage might be the reason. There were 16 pesticides at *a* concn and 24 pesticides at *b* concn in accordance with degradation trend F, accounting for 5.9 and 8.9% of 271 pesticides, respectively. In addition, nine pesticides were in accordance with degradation trend F at both concentrations.

This discussion of degradation trends A–F indicates that the 271 pesticides studied here have relatively complex degradation trends in aged Oolong tea, with various fitting curves at different concentrations. Among the degradation trends A–F, degradation trends A, B, and F had higher ratios than the others. All the pesticides in accordance with degradation trends A, B, and E decreased exponentially over 40 days and decreased mainly exponentially or logarithmically over 120 days. In addition, although no degradation trend was seen for the pesticides of degradation trend D over 40 days, they decreased mainly exponentially over 120 days. The conclusion is that pesticides in tea degrade slowly, with concentrations of pesticides decreasing within 4 months: at concentration *a*, the deviation for each pesticide from day 1 to day 120 falls within the range 0.2–85.6% (mean, 36.4%), whereas at concentration *b*, the deviation for each pesticide from day 1 to day 120 falls within the range 4.0–92.7% (mean, 50.8%).

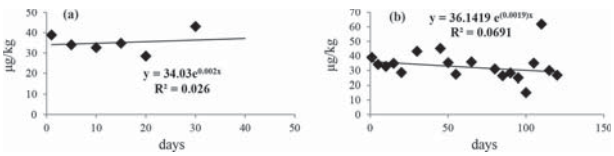


Figure 7. Degradation profiles of degradation trend F (e.g., kresoxim-methyl) over (a) 40 days and (b) 120 days. Determination days were plotted as horizontal ordinates; residue concentrations of pesticide were plotted as vertical ordinates.

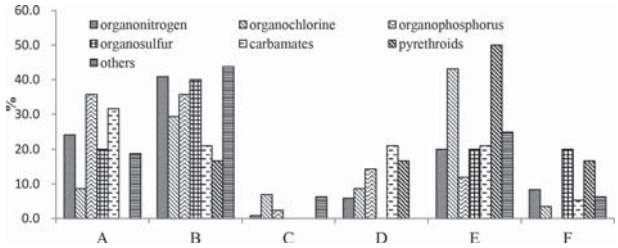


Figure 8. Distributions of pesticides according to different classes within A–F aspects at *a* concn. For each degradation trend (*x*-axis), percentages (*y*-axis) were calculated as the number of pesticides in each class in accordance with the degradation trend multiplied by 100 and divided by the total number of pesticides in the class.

Pesticides in Different Classes

To further investigate the degradation trends, pesticides in A–F aspects were divided into different classes according to organonitrogen, organophosphorus, organochlorine, organosulfur, carbamates, and pyrethroids, and "others." Their distributions can be found in Figures 8 and 9.

It is clear that most of the 271 pesticides are in the organonitrogen, organophosphorus, and organochlorine classes; therefore, the pesticides are discussed according to these three classes. For the pesticides in degradation trend A, most were organonitrogen and organophosphorus. For degradation trend B, the number of organophosphorus and organochlorine pesticides was equal but far below the number of organonitrogen pesticides. The number of organochlorine pesticides for degradation trend E was far higher than for organonitrogen and organophosphorus pesticides, and the number of organophosphorus pesticides was 5 and 3 at *a* and *b* concns, respectively. The organophosphorus and organochlorine pesticides can be further discussed for degradation trends A and B combined (A/B) and E. At *a* concn, 37.9 and 71.4% pesticides were organochlorine and organophosphorus, respectively, for degradation trend A/B. At the same time, 43.1 and 11.9% pesticides were organochlorine and organophosphorus, respectively, for degradation trend E. At *b* concn, the respective percentages of organochlorine and organophosphorus pesticides for degradation trend A/B were 41.8 and 71.8%. For degradation trend E, the respective percentages for organochlorine and organophosphorus pesticides were 34.5 and 7.7%. These results suggest that most of the organophosphorus pesticides degraded in accordance with degradation trends A and B in aged Oolong tea. In contrast, most of the organochlorine pesticides decreased according to degradation trend E in aged Oolong tea.

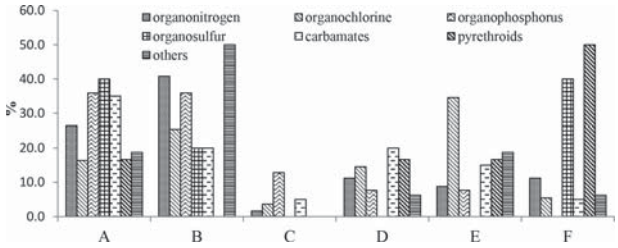


Figure 9. Distributions of pesticides according to different classes within A–F aspects at *b* concn. For each degradation trend (*x*-axis), percentages (*y*-axis) were calculated as the number of pesticides in each class in accordance with the degradation trend multiplied by 100 and divided by the total number of pesticides in the class.

Practical Application of Degradation Regularity

Degradation regularity of 20 representative pesticides.—The single-laboratory validation results of AOAC INTERNATIONAL priority research project, "High-Throughput Analytical Techniques for the Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea by GC/MS, GC/MS/MS and LC/MS/MS: Collaborative Study, First Action **2014.09**," showed that the method could be used for determination of as many as 653 target pesticides.[21] To reduce the workload and guarantee a smooth AOAC interlaboratory study, an alternative "shrunken" protocol was proposed by AOAC. Here, using the shrunken protocol, the pesticides commonly used in growing tea, as well as those necessary to be determined for the international tea trade, were selected from the 271 pesticides determined by GC-MS/MS. It should be noted that these pesticides, after being sprayed onto tea, are all of relatively good stability and their polarities are widely representative. On the basis of the degradation equations in supplemental Tables 1 and 2 and discussions regarding them, 20 representative pesticides were optimized (*see* Table 1).

The degradation regularity of the 20 representative pesticides in aged Oolong tea was studied at *c* and *d* concns ($d > c > b > a$) over another 90 days by GC-MS/MS. Similarly, their degradation equations were obtained by plotting the determination time (every 5 days) on the *x*-axis and the concentration on the *y*-axis (*see* Table 2). It can be seen from Table 2 that the degradation of the 20 representative pesticides agrees well with the logarithmic equation over 90 days, i.e., the pesticides degraded slowly in aged Oolong tea over 3 months.

Table 1. Retention time and monitored ion transitions for the 20 pesticides by GC-MS/MS

No.	Pesticide	Retention time, min	Quantifying precursor/product ion transition	Qualifying precursor/product ion transition
ISTD	Heptachlor epoxide	22.15	353/263	353/282
1	Trifluralin	15.41	306/264	306/206
2	Tefluthrin	17.4	177/127	177/101
3	Pyrimethanil	17.42	200/199	183/102
4	Propyzamide	18.91	173/145	173/109
5	Pirimicarb	19.02	238/166	238/96
6	Dimethenamid	19.73	230/154	230/111
7	Fenchlorphos	19.83	287/272	287/242
8	Tolclofos-methyl	19.87	267/252	267/93
9	Pirimiphos-methyl	20.36	290/233	290/125
10	2,4′-DDE	22.79	318/248	318/246
11	Bromophos-ethyl	23.16	359/303	359/331
12	4,4′-DDE	23.9	318/248	318/246
13	Procymidone	24.7	283/96	283/255
14	Picoxystrobin	24.75	335/173	335/303
15	Quinoxyfen	27.18	237/208	237/182
16	Chlorfenapyr	27.37	408/59	408/363
17	Benalaxyl	27.66	148/105	148/79
18	Bifenthrin	28.63	181/166	181/165
19	Diflufenican	28.73	266/218	266/246
20	Bromopropylate	29.46	341/185	341/183

From supplemental Tables 1 and 2, it can been seen that, except for pirimicarb, fenchlorphos, and 4,4′-dichlorodiphenyldichloroethylene (DDE), the other 17 optimized pesticides dropped exponentially or logarithmically over 120 days at *a* and *b* concns. At the higher *c* and *d* concns, however, all 20 optimized pesticides dropped logarithmically over 90 days. Therefore, it can be concluded that their degradation regularity is in accordance with a logarithmic equation with spray concentration increasing.

Prediction of pesticide residues in aged Oolong tea.—The discussion above (*see Degradation Regularity of 20 Representative Pesticides* section) indicates that the degradation behavior of the pesticides in aged Oolong tea has certain regularity. However, it should be noted that this process is time consuming for multiple determinations. Therefore, it is necessary to propose a method for predicting the residue of pesticides in aged Oolong tea. Here, we develop and validate a prediction method by taking the raw degradation data of *c* and *d* concns.

Based on the results of pesticides in aged Oolong tea determined by GC-MS/MS over 90 days (from the raw data of *d* concn), trend charts (eg, dimethenamid, *see* Figure 10) were plotted, with determination time (day) on the *x*-axis and the difference between each measured value and the first-time-measured value (degradation value) of target pesticides on the *y*-axis. The logarithmic equations were obtained by fitting the 90-day determination results. From these equations, the degradation value of any of the 20 target pesticides at any specific day could be calculated and applied to the raw data generated for that pesticide in a particular laboratory.

The logarithmic functions of the 20 pesticides at *d* concn (listed in Table 3) were applied to predict the residue concentrations of pesticides in aged Oolong tea at *c* concn. The predicted residue of each pesticide on a particular day could be obtained by subtracting the degradation value of this day from the concentration of the first day.

Accordingly, the residue concentrations of 20 pesticides in aged Oolong tea at different 5-day degradation intervals (days 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, and 90) after spraying at *c* concn were predicted, and they were compared with the measured results determined by GC-MS/MS (*see* Tables 4 and 5). It can be seen from Tables 4 and 5 that the deviation ratios of trifluralin, tefluthrin, and dimethenamid were higher as compared to other pesticides at different intervals, with deviation ratio ranges of −23.1 to 21.5, 12.2–26.0, and 6.6–24.0%, respectively. They were followed by pirimiphos-methyl, tolclofos-methyl, and fenchlorphos, with deviation ratio ranges of −2.8 to 21.8, 5.8 to 23.1, and −2.0 to 24.4%, respectively. The remaining 14 pesticides had relatively lower deviation ratios, except for part of the intervals. It can be also seen that the lowest deviation ratios of the 20 pesticides were different. The numbers of pesticides that had the lowest deviation ratios at the 20-, 30-, and 45-day intervals were 4, 9, and 3, respectively. In addition, the highest deviation ratio of 2,4′-DDE, 4,4′-DDE, and bromopropylate was found at days 85, 85 (which were close to the deviation ratios at day 80), and 35, respectively, whereas for all the other pesticides, the highest deviation ratio was found at day 80. This finding could be due to the results at day 80 being abnormal. To evaluate

Table 2. Degradation equations of 20 representative pesticides in aged Oolong tea at *c* and *d* concns over 90 days by GC-MS/MS[a]

		c Concn			*d* Concn		
No.	Pesticide	Initial value	Equation over 90 days	R^2	Initial value	Equation over 90 days	R^2
1	Trifluralin	214.4	y = −6.93ln(x) + 222.0	0.548	248.6	y = −14.1ln(x) + 249.5	0.787
2	Tefluthrin	106.1	y = −4.52ln(x) + 105.4	0.742	122.6	y = −7.94ln(x) + 117.8	0.832
3	Pyrimethanil	116.9	y = −5.96ln(x) + 120.1	0.693	124.7	y = −8.06ln(x) + 126.1	0.77
4	Propyzamide	119.6	y = −4.06ln(x) + 125.0	0.512	126.6	y = −5.73ln(x) + 130.7	0.598
5	Pirimicarb	114.7	y = −6.65ln(x) + 119.5	0.822	126.2	y = −9.11ln(x) + 128.6	0.873
6	Dimethenamid	46.2	y = −2.23ln(x) + 47.66	0.702	51.9	y = −3.52ln(x) + 52.19	0.86
7	Fenchlorphos	240.3	y = −10.1ln(x) + 245.1	0.533	267.4	y = −15.5ln(x) + 263.7	0.785
8	Tolclofos-methyl	116.7	y = −4.00ln(x) + 118.1	0.543	130.5	y = −7.08ln(x) + 129.6	0.78
9	Pirimiphos-methyl	113.0	y = −5.43ln(x) + 117.0	0.74	126.3	y = −8.31ln(x) + 127.9	0.867
10	2,4′-DDE	451.7	y = −21.7ln(x) + 463.0	0.794	466.9	y = −26.4ln(x) + 476.8	0.824
11	Bromophos-ethyl	116.0	y = −5.74ln(x) + 119.4	0.686	124.2	y = −7.74ln(x) + 126.7	0.784
12	4,4′-DDE	451.7	y = −20.2ln(x) + 460	0.768	466.9	y = −24.6ln(x) + 473.3	0.805
13	Procymidone	117.9	y = −4.22ln(x) + 120.5	0.587	120.3	y = −5.34ln(x) + 123.3	0.733
14	Picoxystrobin	234.0	y = −10.7ln(x) + 240.3	0.717	236.0	y = −12.3ln(x) + 244.3	0.774
15	Quinoxyfen	115.9	y = −6.64ln(x) + 122.1	0.667	116.4	y = −6.57ln(x) + 119.3	0.653
16	Chlorfenapyr	965.0	y = −36.0ln(x) + 984.2	0.579	966.0	y = −42.4ln(x) + 995.3	0.65
17	Benalaxyl	116.8	y = −6.24ln(x) + 121.7	0.763	118.9	y = −7.50ln(x) + 124.2	0.746
18	Bifenthrin	112.9	y = −5.87ln(x) + 116.2	0.653	114.8	y = −7.09ln(x) + 119.1	0.728
19	Diflufenican	118.6	y = −5.93ln(x) + 122.9	0.571	119.4	y = −5.86ln(x) + 122.3	0.576
20	Bromopropylate	237.4	y = −11.3ln(x) + 249.2	0.524	240.0	y = −12.1ln(x) + 248.9	0.647

[a] The degradation equations were obtained by plotting determination time (every 5 days) on the *x*-axis and concentration on the *y*-axis.

the deviation ratio accurately, the deviation ratio ranges of the pesticides were recalculated without the interval data of day 80 (*see* Table 5).

Among the above-mentioned remaining 14 pesticides, the deviation ratios were <15%, with most <10%, except for pirimicarb and bromophos-ethyl, which had the highest deviation ratios of 17.1 and 16.8%, respectively, and quinoxyfen and benalaxyl, which had the lowest deviation ratios of −24.3 and −16.0%, respectively. It is evident that the proposed method for predicting the residue concentrations of pesticides in aged Oolong tea in this study is accurate.

The initial values of pesticides at *c* and *d* concns, together with their deviation ratios, are also listed in Table 5. It can be seen that the six pesticides with relatively higher deviation

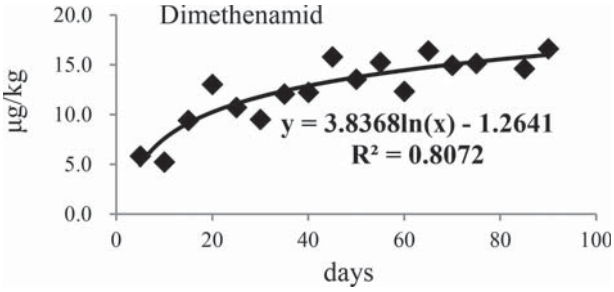


Figure 10. Logarithmic chart of dimethenamid in aged Oolong tea. The determination times (days) were plotted on the *x*-axis, and the differences between each measured value and the first-time-measured value (degradation value) of target pesticides were plotted on the *y*-axis.

Table 3. Logarithmic functions of 20 pesticides in aged Oolong tea at *d* concn[a]

No.	Pesticide	Logarithmic function	R^2
1	Trifluralin	y = 12.21ln(x) + 3.401	0.705
2	Tefluthrin	y = 6.336ln(x) + 10.94	0.641
3	Pyrimethanil	y = 8.822ln(x) − 5.055	0.758
4	Propyzamide	y = 6.399ln(x) − 4.981	0.620
5	Pirimicarb	y = 9.929ln(x) − 5.560	0.800
6	Dimethenamid	y = 3.836ln(x) − 1.264	0.807
7	Fenchlorphos	y = 18.07ln(x) − 9.564	0.768
8	Tolclofos-methyl	y = 7.461ln(x) + 0.113	0.698
9	Pirimiphos-methyl	y = 8.866ln(x) − 3.763	0.781
10	2,4′-DDE	y = 29.77ln(x) − 22.61	0.748
11	Bromophos-ethyl	y = 9.26ln(x) − 7.603	0.757
12	4,4′-DDE	y = 29.07ln(x) − 23.00	0.705
13	Procymidone	y = 6.414ln(x) − 7.533	0.600
14	Picoxystrobin	y = 15.28ln(x) − 19.24	0.740
15	Quinoxyfen	y = 6.511ln(x) − 3.341	0.744
16	Chlorfenapyr	y = 45.47ln(x) − 38.61	0.630
17	Benalaxyl	y = 9.157ln(x) − 12.91	0.722
18	Bifenthrin	y = 8.334ln(x) − 9.895	0.761
19	Diflufenican	y = 7.221ln(x) − 8.177	0.730
20	Bromopropylate	y = 15.97ln(x) − 23.75	0.596

[a] Determination time (day) was plotted on the *x*-axis and the difference between each measured value and the first-time-measured value (degradation value) of the target pesticides was plotted on the *y*-axis, and then the logarithmic functions were fitted.

Table 4. Deviation ratios of predicted and determined results of *c* concn at different time intervals[a]

		Time interval, days																	
No.	Pesticide	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
1	Trifluralin	13.3	14.1	11.6	−22.9	21.0	15.4	14.1	18.0	11.6	16.0	−23.1	17.8	10.0	19.2	11.4	21.5	20.3	20.2
2	Tefluthrin	15.2	17.4	16.0	14.1	15.1	25.5	16.1	19.7	12.2	23.5	20.3	20.7	17.8	20.6	21.2	26.0	25.3	21.4
3	Pyrimethanil	5.4	11.2	9.7	3.6	3.5	7.7	6.2	9.0	0.3	11.1	6.6	8.8	6.3	11.7	10.4	20.0	13.7	14.1
4	Propyzamide	6.9	11.4	9.9	3.6	4.9	7.6	9.2	7.1	0.0	11.4	3.4	8.6	8.9	8.6	7.8	15.4	12.4	10.4
5	Pirimicarb	7.4	11.9	10.7	5.7	9.5	13.7	11.6	11.2	8.1	15.7	8.5	12.6	11.5	13.2	13.4	20.6	17.1	14.5
6	Dimethenamid	9.4	13.0	11.4	6.6	15.3	18.0	17.6	16.3	7.9	17.5	12.3	17.2	13.7	19.9	17.0	24.0	21.4	18.9
7	Fenchlorphos	6.5	12.8	11.3	−2.0	9.2	12.8	4.1	12.0	8.0	13.9	3.8	15.6	16.0	17.7	12.7	24.4	19.3	18.5
8	Tolclofos-methyl	9.5	13.7	11.8	5.8	11.9	9.4	8.2	13.6	9.2	16.1	9.7	17.0	15.8	17.4	14.5	23.1	19.9	17.6
9	Pirimiphos-methyl	9.2	13.9	13.0	6.1	11.2	10.1	9.2	13.1	−2.8	13.1	11.3	13.5	12.9	16.0	14.9	21.8	20.6	17.6
10	2,4′-DDE	0.8	5.6	7.0	4.5	4.1	−0.1	5.3	2.6	0.2	7.3	5.7	4.1	0.4	4.3	6.7	10.8	11.3	4.6
11	Bromophos-ethyl	3.8	10.5	10.8	2.4	8.0	1.2	5.6	6.0	5.7	10.5	3.4	11.6	9.2	13.2	10.7	17.8	16.8	12.4
12	4,4′-DDE	0.4	5.2	6.4	3.9	3.5	−0.9	4.5	1.8	−0.7	6.4	5.4	4.5	0.4	5.2	7.9	10.8	11.4	5.6
13	Procymidone	−2.2	4.0	5.7	4.4	3.7	−3.9	6.3	−0.5	−2.3	4.8	2.0	3.9	−2.6	1.9	6.2	9.8	8.6	2.9
14	Picoxystrobin	−3.6	2.7	5.5	4.0	2.5	−3.2	8.4	−2.9	−0.7	3.2	0.4	3.2	−4.9	2.1	6.2	8.9	7.1	0.1
15	Quinoxyfen	2.3	11.2	10.1	4.1	1.2	−24.3	8.1	−6.0	−8.9	0.7	−1.2	2.1	−2.4	4.5	5.6	14.7	6.1	3.2
16	Chlorfenapyr	−1.9	4.6	6.2	2.0	2.9	−5.6	6.7	−4.7	−1.8	3.1	−2.7	3.7	−1.0	2.3	4.0	8.4	6.0	−1.6
17	Benalaxyl	−4.2	4.2	4.6	4.0	3.0	−16.0	6.8	−4.2	−9.7	4.6	1.7	4.3	−3.4	3.0	7.4	8.7	6.5	−0.2
18	Bifenthrin	−4.6	4.3	5.4	7.8	3.1	−0.1	10.0	−4.2	−9.5	3.5	4.2	4.7	−3.0	3.7	8.9	11.1	7.8	−0.3
19	Diflufenican	−2.2	9.1	7.3	2.0	1.8	−11.8	9.5	−5.6	−7.9	0.5	−2.6	2.3	−3.1	4.7	6.6	13.5	8.2	2.5
20	Bromopropylate	−0.8	9.1	7.3	−0.5	6.0	−8.6	10.5	−6.0	−2.3	2.1	−1.7	4.5	−3.8	10.0	6.9	10.2	7.4	0.3

[a] Values are deviation ratios (%) calculated as (determined result − predicted value) × 100 ÷ determined result.

ratios of predicted and measured results also have the relatively higher deviation ratios of their initial values (10.1–13.7%). However, the deviation ratios of initial values of the other 14 pesticides were <10%, with a range of 0.1–9.1%. With the decrease of the deviation ratio of initial values, the deviation ratio of predicted and measured results gets smaller. It can be concluded that the closer their initial values, the better the results obtained.

Table 5. Initial values of *c* and *d* concns and the range of deviation ratios of predicted and determined results

		Initial value			Range of deviation ratio, %[b]	Revised range of deviation ratio, %[c]
No.	Pesticide	*c* Concn, μg/kg	*d* Concn, μg/kg	Deviation ratio, %[a]		
1	Trifluralin	214.4	248.6	13.7	−23.1 to 21.5	−23.1 to 21.0
2	Tefluthrin	106.1	122.6	13.5	12.2–26.0	12.2–25.5
3	Pyrimethanil	116.9	124.7	6.3	0.3–20.0	0.3–14.1
4	Propyzamide	119.6	126.6	5.5	0–15.4	0–12.4
5	Pirimicarb	114.7	126.2	9.1	5.7–20.6	5.7–17.1
6	Dimethenamid	46.2	51.9	11.1	6.6–24.0	6.6–21.4
7	Fenchlorphos	240.3	267.4	10.1	−2.0 to 24.4	−2.0 to 19.3
8	Tolclofos-methyl	116.7	130.5	10.5	5.8–23.1	5.8–19.9
9	Pirimiphos-methyl	113.0	126.3	10.6	−2.8 to 21.8	−2.8 to 20.6
10	2,4′-DDE	451.7	466.9	3.3	−0.1 to 11.3	−0.1 to 11.3
11	Bromophos-ethyl	116.0	124.2	6.6	1.2–17.8	1.2–16.8
12	4,4′-DDE	451.7	466.9	3.3	−0.9 to 11.4	−0.9 to 11.4
13	Procymidone	117.9	120.3	2.0	−3.9 to 9.8	−3.9 to 8.6
14	Picoxystrobin	234.0	236.0	0.9	−4.9 to 8.9	−4.9 to 8.4
15	Quinoxyfen	115.9	116.4	0.4	−24.3 to 14.7	−24.3 to 11.2
16	Chlorfenapyr	965.0	966.0	0.1	−5.6 to 8.4	−5.6 to 6.7
17	Benalaxyl	116.8	118.9	1.7	−16.0 to 8.7	−16.0 to 7.4
18	Bifenthrin	112.9	114.8	1.7	−9.5 to 11.1	−9.5 to 10.0
19	Diflufenican	118.6	119.4	0.7	−11.8 to 13.5	−11.8 to 9.5
20	Bromopropylate	237.4	240.0	1.1	−8.6 to 10.5	−8.6 to 10.5

[a] Deviation ratios are calculated as (*d* Concn − *c* concn) × 100 ÷ *d* concn.

[b] Between predicted and determined results.

[c] Between predicted and determined results without the 80-day interval results.

Conclusions

In summary, the degradation regularity of 271 pesticides in aged Oolong tea over 120 days was studied by the developed GC-MS/MS method. The results indicate that >70% of the 271 pesticides decreased exponentially or logarithmically in aged Oolong tea, confirming that the pesticides in aged Oolong tea degrade slowly and the concentrations of pesticides decrease with the increase of time intervals over 4 months. Further discussion of the different classes of pesticides suggests that most of the organophosphorus pesticides degraded in accordance with degradation trends A and B in aged Oolong tea and that most of the organochlorine pesticides decreased according to degradation trend E in aged Oolong tea.

The pesticide residues in aged Oolong tea were predicted accurately by subtracting the degradation value of target pesticides on a specific day from the logarithmical curves, generated by plotting determination time (day) and the difference between each measured value and first-time-measured value of target pesticides on the *x*- and *y*-axes, respectively. The predicted results of 14 pesticides were satisfactory by comparing them with measured results at one of the concentrations of the Youden pair.

It is our hope that the obtained degradation regularity of 271 pesticides in aged Oolong tea will be helpful for studying the stability of standard material of multipesticide residues in tea. In addition, we propose that the prediction procedure of pesticides in aged Oolong tea in the present study may offer a new method for the error analysis of multiresidue of pesticides in other complex matrixes of international, interlaboratory study.

Acknowledgments

This work was financially supported by the General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ) special public welfare project of China (Project No. 201010080).

Reference

(1) Ballesteros, E., & Parrado, M.J. (2004) *J. Chromatogr. A* **1029**, 267–273. doi:10.1016/j.chroma.2003.12.009

(2) Kamel, F., & Hoppin, J.A. (2004) *Environ. Health Perspect.* **112**, 950–958. doi:10.1289/ehp.7135

(3) Gros, M., Petrović, M., & Barceló, D. (2006) *Talanta* **70**, 678–690. doi:10.1016/j.talanta.2006.05.024

(4) Park, J.Y., Choi, J.H., Abd El-Aty, A.M., Kim, B.M., Oh, J.H., Do, J.A., Kwon, K.S., Shim, K.H., Choi, O.J., Shin, S.C., & Shim, J.H. (2011) *Food Chem.* **128**, 241–253. doi:10.1016/j.foodchem.2011.02.065

(5) Lu, C.H., Liu, X.G., & Dong, F.S. (2010) *Anal. Chim. Acta* **678**, 56–62. doi:10.1016/j.aca.2010.08.015

(6) Kanrar, B., Mandal, S., & Bhattacharyya, A. (2010) *J. Chromatogr. A* **1217**, 1926–1933. doi:10.1016/j.chroma.2010.01.062

(7) Chen, G.Q., Cao, P.Y., & Liu, R.J. (2011) *Food Chem.* **125**, 1406–1411. doi:10.1016/j.foodchem.2010.10.017

(8) European Union, Informal coordination of MRLs established in Directives 76/895/EEC, 86/362/EEC, 86/363/EEC, and 90/642/EEC, http://europa.eu.int/comm/food/plant/protection/pesticides/index.en.htm

(9) Pehkonen, S.O., & Zhang, Q. (2002) *Environ. Sci. Technol.* **32**, 17–72. doi:10.1080/10643380290813444

(10) Liu, T. F., Sun, C., Ta, N., Hong, J., Yang, S.G., & Chen, C.X. (2007) *J. Environ. Sci. (China)* **19**, 1235–1238. doi:10.1016/S1001-0742(07)60201-0

(11) Juraske, R., Antón, A., & Castells, F. (2008) *Chemosphere* **70**, 1748–1755. doi:10.1016/j.chemosphere.2007.08.047

(12) Sniegowski, K., Mertens, J., Diels, J., Smolders, E., & Springael, D. (2009) *Chemosphere* **75**, 726–731. doi:10.1016/j.chemosphere.2009.01.050

(13) Uygun, U., Senoz, B., Öztürk, S., & Koksel, H. (2009) *Food Chem.* **117**, 261–264. doi:10.1016/j.foodchem.2009.03.111

(14) Ozbey, A., & Uygun, U. (2007) *Food Chem.* **104**, 237–241. doi:10.1016/j.foodchem.2006.11.034

(15) Manikandan, N., Seenivasan, S., Ganapathy, M.N.K., Muraleedharan, N.N., & Selvasundaram, R. (2009) *Food Chem.* **113**, 522–525. doi:10.1016/j.foodchem.2008.07.094

(16) Lin, J.K., Li, X.F., Lin, X.D., & Tu, L.J. (2008) *Chin. Agric. Sci. Bull.* **24**, 104–111

(17) Chen, L., ShangGuan, L.M., Wu, Y.N., Xu, L.J., & Fu, F.F. (2012) *Food Contr.* **25**, 433–440. doi:10.1016/j.foodcont.2011.11.027

(18) Pang, G.F., Fan, C.L., Zhang, F., Li, Y., Chang, Q.Y., Cao, Y.Z., Liu, Y.M., Li, Z.Y., Wang, Q.J., Hu, X.Y., & Liang, P. (2011) *J. AOAC Int.* **94**, 1253–1296

(19) Fan, C.L., Chang, Q.Y., Pang, G.F., Li, Z.Y., Kang, J., Pan, G.Q., Zheng, S.Z., Wang, W.W., Yao, C.C., & Ji, X.X. (2013) *J. AOAC Int.* **96**, 432–440. doi:10.5740/jaoacint.10-215

(20) Pang, G.F., Fan, C.L., Chang, Q.Y., Li, Y., Kang, J., Wang, W.W., Cao, J., Zhao, Y.B., Li, N., & Li, Z.Y. (2013) *J. AOAC Int.* **96**, 887–896. doi:10.5740/jaoacint.10-251

(21) Pang, G.F., Fan, C.L., Cao, Y.Z., Yan, F., Li, Y., Kang, J., Chen, H., & Chang, Q.Y. (2015) *J. AOAC Int.* **98**, 1428–1454. doi: 10.5740/jaoacint.15021

Expert Review Panel on Pesticide Residues
OFFICIAL CHAIR'S EXPERT REVIEW PANEL REPORT

ACKNOWLEDGMENT

The undersigned chair hereby confirms that the following document has been reviewed and constitutes the final version of the Official Chair's Report for the Expert Review Panel on Pesticide Residues held on Monday, September 8, 2014 during the AOAC Annual Meeting and Exposition at the Boca Raton Resort and Club, 501 East Camino Reel, Boca Raton, Florida 33432.

DR. JOE BOISON, CANADIAN FOOD INSPECTION AGENCY (CFIA)

Expert Review Panel Chair

October 14, 2014

Date

AOAC RESEARCH INSTITUTE
2275 Research Blvd, Suite 300
Rockville, Maryland 20850
UNITED STATES

Contact:
La'Kia Phillips, Conformity Assessment Coordinator at lphillips@aoac.org
Deborah McKenzie, Sr. Director, DMcKenzie@aoac.org

EXPERT REVIEW PANEL MEETING ATTENDEES

Expert Review Panel Chair (s)
Joe Boison, Canadian Food Inspection Agency (CFIA)

Expert Review Panel Members
Amy Brown, Florida Department of Agriculture
Jo Marie Cook (Alternate), Florida Department of Agriculture
Julie Kowalski, Restek Corporation
John Reuther, Eurofins
Marina Torres, LATU
Jian Wang, Canadian Food Inspection Agency (CFIA)
Xiaoyan Wang, United Chemical Technologies, Inc. (UCT)

Method Authors
Dr. Guo-Fang Pang, Chinese Academy of Inspection and Quarantine

AOAC Staff
Deborah McKenzie
Tien Milor
La'Kia Phillips

Observers
Muna Aljabir, Central Food Lab
Tyson Friday, Frontier Coop
Jana Huckrabolt, Institute of Chemical Technology
Tom Phillips, MD Department of Agriculture
Jana Pugerhbovn, Institute of Chemical Technology
Nancy Thiex, AOAC RI Consultant
David Whitman, 3M Food Safety
David Woollard, Hill Laboratories (NZ)

EXPERT REVIEW PANEL, METHOD BACKGROUND, AND CONCLUSIONS

Criteria for Vetting Methods to be considered:
AOAC convened the *Official Methods of Analysis*^{SM} (OMA) Expert Review Panel for Pesticide Residues on Monday, September 8, 2014 from 4:30pm to 7:30pm during the AOAC Annual Meeting and Exposition in Boca Raton, Florida. The purpose of the meeting was to 1) Review the Collaborative Study Manuscript/ OMAMAN-14: High-Throughput Analytical Techniques For The Determination And Confirmation Of Residues Of 653 Multi-Class Pesticides And Chemical Pollutants In Tea By GC-MS, GC-MS/MS And LC-MS/MS (Study Director: Guo-Fang Pang, Chun-Lin Fan,Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, Qiao-Ying Chang, Chinese Academy of Inspection and Quarantine, No. 3 Gaobeidian North Rd 100123, Chaoyang District, Beijing, People's Republic of China) and to 2) discuss First to Final Action requirements and feedback mechanisms. The candidate method was reviewed against the approved collaborative study protocol. Supplemental information was also provided to the reviewers which included the collaborative study manuscript, Appendix 1.1-1.5 Analytical Instrumentation used in the Collaborative Study, Appendix 2 Determination Results of Collaborative Study, Appendix 3: Results of Practice Samples, Appendix 4: The Statistical Results, and an Journal Article: High-Throughput GC-MS and HPLC-MS/MS Techniques for the Multiclass, multiresidue Determination of 653 Pesticides and Chemical Pollutants in Tea.

Criteria for Vetting Experts and Selection Process:
The following seven (7) candidates and one (1) alternate member were submitted for consideration by the Official Methods Board to evaluate candidate methods for Pesticide Residues methods as per the Expert Review Panel (ERP) Policies and Procedures. The candidates were highly recommended by the Chemical Contaminants and Residues in Foods Community, have participated in various AOAC activities, including but limited to, Method Centric Committees that were formed under the legacy OMA pathway, and were vetted by the Official Methods Board. The experts are Amy Brown, Jo Marie Cook (Alternate), Julie Kowalski, John Reuther, Marina Torres, Jian Wang, and Xiaoyan Wang.

ERP Orientation:
The ERP members have completed the mandatory AOAC Expert Review Panel Orientation Webinar on Wednesday, July 16, 2014.

Expert Review Panel Meeting Quorum
The meeting of the Expert Review Panel was held in person. A quorum is the presence of seven (7) members or 2/3 of the total vetted ERP, whichever is greater. Seven (7) out of the seven (7) voting members were present and therefore met a quorum to conduct the meeting.

Standard Method Performance Requirements (SMPRs): N/A

Conclusion:
The Expert Review Panel reviewed OMAMAN-14: High-Throughput Analytical Techniques For The Determination And Confirmation Of Residues Of 653 Multi-Class Pesticides And Chemical Pollutants In Tea By GC-MS, GC-MS/MS And LC-MS/MS and adopted this method for First Action Official Method status by a unanimous decision with additional revisions as noted in the meeting minutes.

Subsequent ERP Activities:
ERP members will continue to evaluate the method for 2 years.

MEETING MINUTES

I. Welcome and Introductions

The Expert Review Panel Chair, Dr. Joe Boison welcomed Expert Review Panel members and initiated introductions. The Chair discussed with the panel the goal of the meeting.

II. Review of AOAC Volunteer Policies

Deborah McKenzie presented an overview of AOAC Volunteer Policies, Volunteer Acceptance Agreement and and Expert Review Panel Policies and Procedures which included Volunteer Conflicts of Interest, Policy on the Use of the Association, Name, Initials, Identifying Insignia, Letterhead, and Business Cards, Antitrust Policy Statement and Guidelines, and the Volunteer Acceptance Form (VAF). All members of the ERP were required to submit and sign the Volunteer Acceptance Form.

III. Expert Review Panel Process Overview and Guidelines

Deborah McKenzie presented an overview of the Expert Review panel process. The presentation included information regarding method submission, recruitment of ERP members, composition and vetting expertise, method assignments, meeting logistics, consensus, First Action to Final Action requirements, method modifications, publications, and documentation.

IV. Review of Methods

All ERP members presented a review and discussed the proposed collaborative study manuscript for High-Throughput Analytical Techniques for the Determination and Confirmation of Residues of 653 Multi-Class Pesticides and Chemical Pollutants in Tea by GC-MS, GC-MS/MS and LC-MS/MS. The method author, Dr. Guo-Fang Pang of the Chinese Academy of Inspection and Quarantine, was present and able to address questions and concerns of the ERP members. A summary of comments was provided to the ERP members.[1]

MOTION:
Motion by Wang, J.; Second by Reuther that this method be recommended for First Action Official Method Status.
Consensus demonstrated by: 7 in favor, 0 opposed, and 0 abstentions (Unanimous). ***Motion Passed.***

MOTION:
Motion by Wang, J.; Second by Brown that the revisions requested by the ERP be provided for review.

- **Provide data on the parameters of the method for all of the 653 Analytes**
- **Provide clarity to the text**
- **Include the data on hydration**

Consensus demonstrated by: 7 in favor, 0 opposed, and 0 abstentions (Unanimous). ***Motion Passed.***

V. Discuss Final Action Requirements for First Action Official Methods (if applicable)

No further action was discussed at this time.

VI. Adjournment

[1] Attachment 1: Summary of Expert Reviewer Comments for OMAMAN-14

130th AOAC INTERNATIONAL Annual Meeting And Exposition
AOAC Expert Review Panel for Pesticide Residues
September 21, 2016 Dallas, TX

Official Methods of Analysis[SM] (OMA)

AOAC EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES

AOAC Official Method 2014.09

Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea GC-MS, GC-MS/MS, and LC-MS/MS First Action 2014

2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION
WEDNESDAY, SEPTEMBER 21, 2016
1:00PM – 2:00PM

SHERATON DALLAS HOTEL
400 N. OLIVE STREET
DALLAS, TEXAS 75201 USA

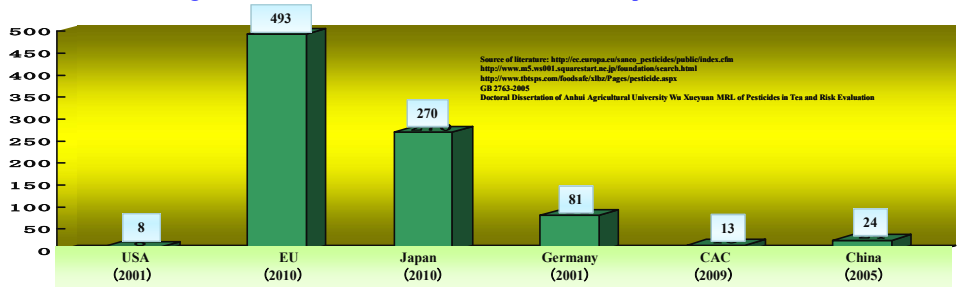
TECHNICAL INFORMATION

- Background and History

Research background: development of the analytical technique for multi-residues in tea becomes imperative.

The threshold of limit for pesticide residues in tea is high for international trade. At present, there are 17 countries and international organizations that have stipulated MRL levels for over 800 pesticide residues in tea*.

The high threshold of international trade from limit of pesticide residue in tea



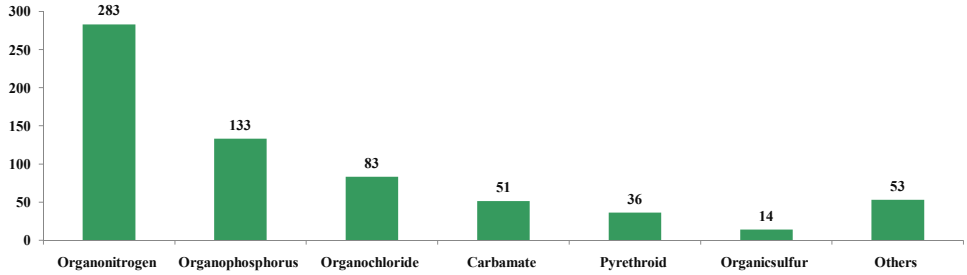
*EU, Germany, Holland, Switzerland, Hungry, Israel, CAC, China, Japan, Chinese Taiwan, Korea, USA, Australia, India, Kenya and South Africa (till 2006).

2

- **Chemical Structure of 653 multi-residue pesticides in tea for AOAC Official Method 2014.19 First action**

Pesticide quantities and ratios (%)

Pesticides	Organonitrogen	Organophosphorus	Organochloride	Carbamate	Pyrethroid	Organicsulfur	Others
Quantities	283	133	83	51	36	14	53
Ratios	43.3%	20.4%	12.7%	7.8%	5.5%	2.1%	8.1%



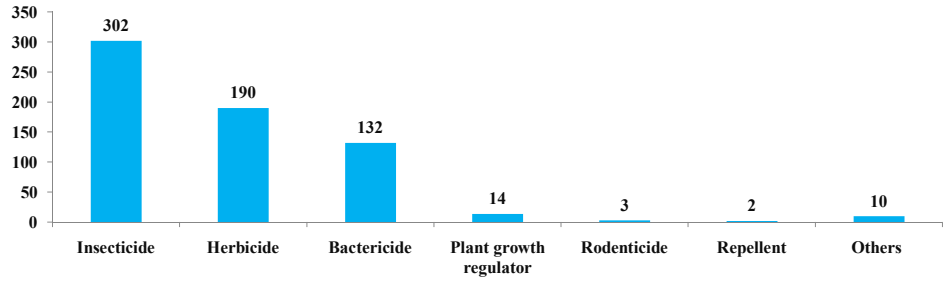
3

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

- **Physiology (biological function) of 653 multi-residue pesticides in tea for AOAC Official Method 2014.19 First action**

Pesticide quantities and ratios (%)

Pesticides	Insecticide	Herbicide	Bactericide	Plant growth regulator	Rodenticide	Repellent	Others
Quantities	302	190	132	14	3	2	10
Ratios	46.2%	29.1%	20.2%	2.1%	0.5%	0.3%	1.5%



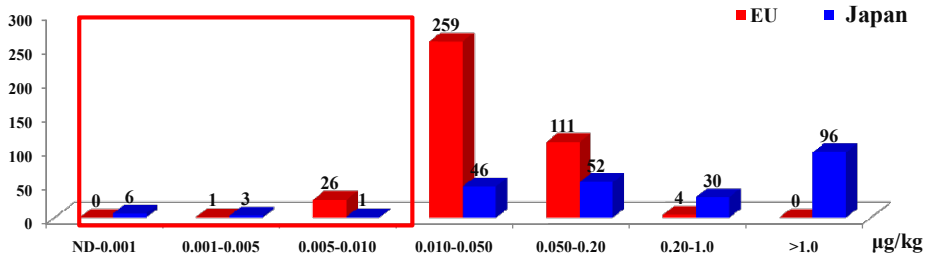
4

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

REGULATORY INFORMATION

- Regulatory Organizations
  - Safe level; tolerances; maximum levels (EU and Japan)

MRL	ND-0.001	0.001-0.005	0.005-0.010	0.010-0.050	0.050-0.20	0.20-1.0	>1.0	MRLs.
EU	0	1	26	259	111	4	0	401
Japan	6	3	1	46	52	30	96	234

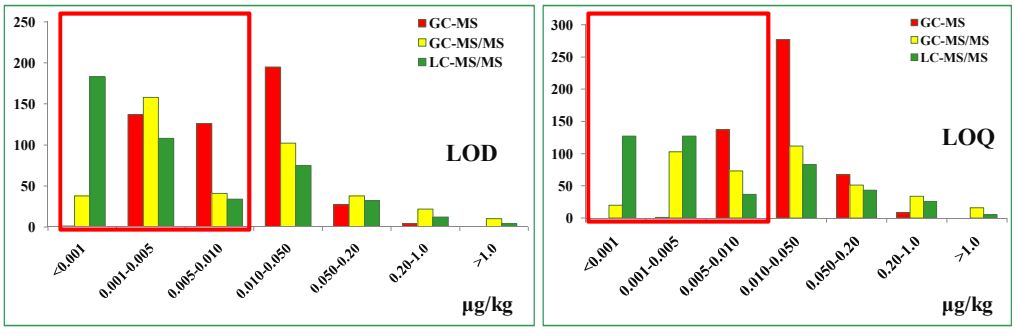


5

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

REGULATORY INFORMATION

- Regulatory Organizations
  - Expected concentration for identity methods (AOAC Official Method 2014.09)



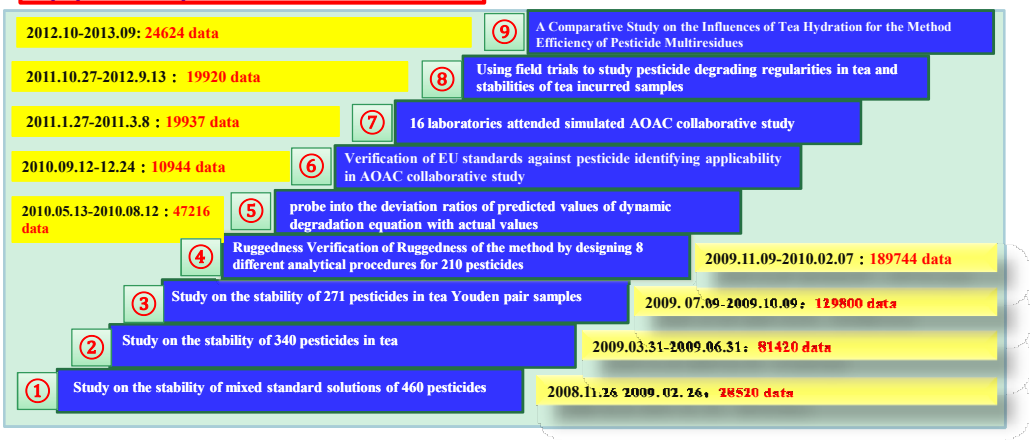
6

SUMMARY OF METHOD INCLUDING METHOD SCOPE/APPLICABILITY

- **Supporting Data including Method Optimization, Analytical Ranges, Accuracy and Recovery, Precision, Reproducibility and System Suitability**

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

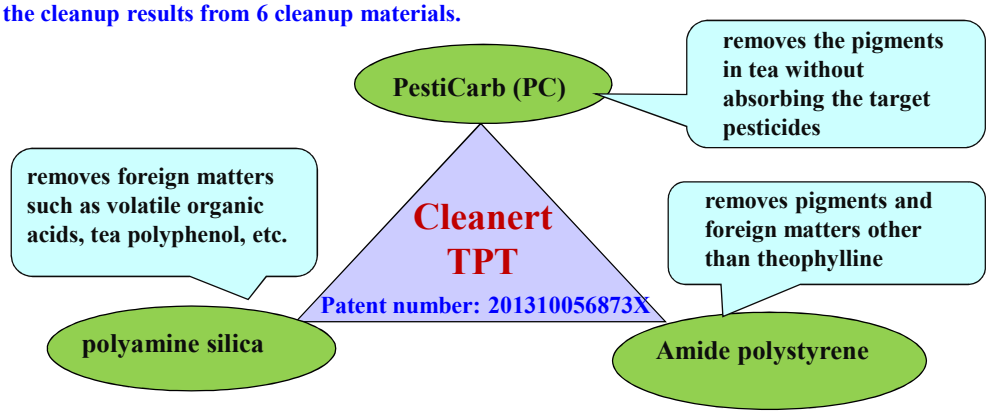
■**Single-laboratory validation:** the methodology study of this high-throughput technique experienced 9 stages for 4 years from 2009-2013, with 511929 data obtained, and 7 papers were published in J. AOAC Int.



EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

■a three-group Cleanert TPT Cartridge has been developed, which has a unique cleanup effect on 653 chemical compounds in tea.

An exclusive cleanup column for tea has been optimized and selected by a comparative study of the cleanup results from 6 cleanup materials.



EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

■Special study on the effect of tea hydration on the efficiency of the analytical method

Correlation comparison of fortified recoveries of 456 pesticides with pesticide logKow values with the three methods of M1, M2 and M3

329 out of 456 pesticides have been found to have logKow values (logKow range: -0.75～+8.20). A scatter diagram is drawn with logKow as horizontal ordinate and recoveries as vertical ordinate, and a chart of correlations of recoveries for the three methods at three fortification levels with pesticide polarities is derived.

The pesticide number within 70-120% recovery at three fortification levels

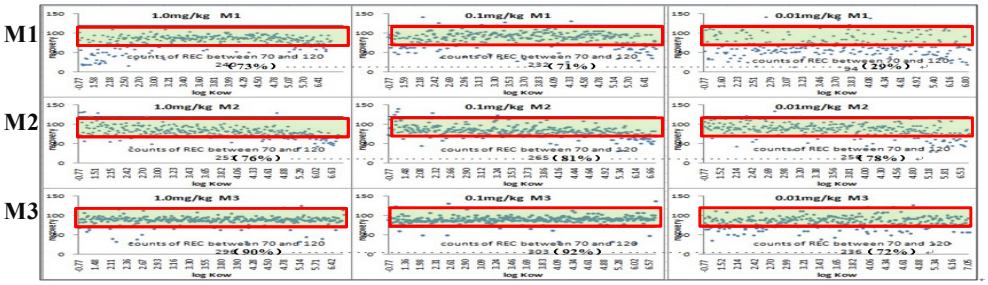
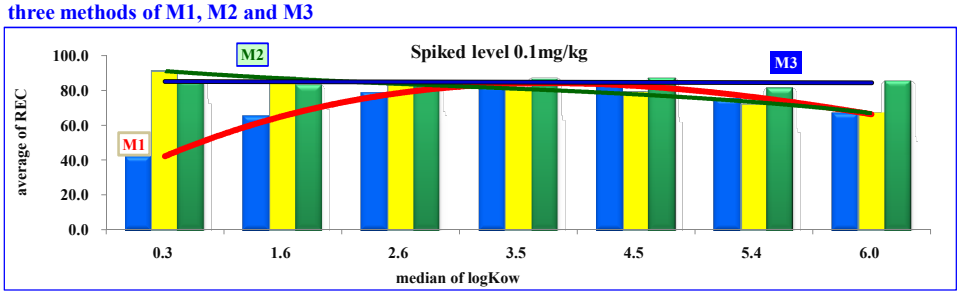


Diagram of correlation distribution of pesticide recoveries with logKow within the range of -0.77～8.20 at three fortification levels of M1, M2 and M3

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

■Special study on the effect of tea hydration on the efficiency of the analytical method

Correlation comparison of fortified recoveries of 456 pesticides with pesticide logKow values with the three methods of M1, M2 and M3



The recovery change diagram for 7 polarity sectors by the three methods

Conclusion: M3's pure acetonitirle extraction has achieved relatively good results for pesticides of different polarities, which takes a balanced, stable and similar to straight trend line. While M1's hydration increases the extraction efficiency of certain polarity water-soluble pesticides; but the adoption of hexane liquid/liquid partitioning further causes certain loss of extracted polarity pesticides, leading to an arc trend line high in the middle and low at both ends; Overall speaking, M3's extraction efficiency is superior to M1's. For M2, hydration procedures are likewise adopted, which increases the extraction efficiency of the pesticides of strong polarities and lowers the extraction efficiency of the pesticides of weak polarity. Because of the application of SPE, the line of M2 is similar to downtrend line with the front rise and the back-end fall.

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

■ Organization and implementation of AOAC collaborative study

Collaborators for AOAC collaborative study: 30 labs from 11 countries and regions participated in the collaborative study by GC-MS, GC-MS/MS and LC-MS/MS

USA	Canada	Germany	Spain
Italy	Belgium	Japan	Korea
India	China	Taiwan	

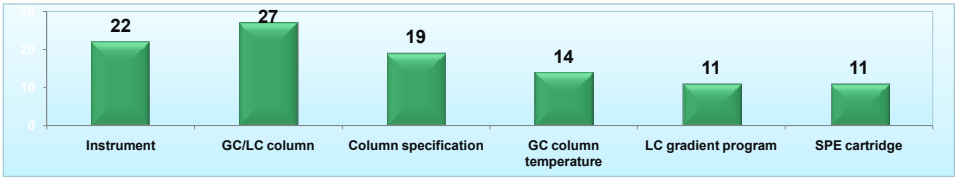
America: USA, Canada; Europe: Germany, Spain, Italy, Belgium; Asia: Japan, Korea, India, China and Taiwan

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

■Comprehensive technical conditions were used by 30 Labs

The comprehensive instrument, analytical parameters and SPE cartridge of AOAC collaborative study

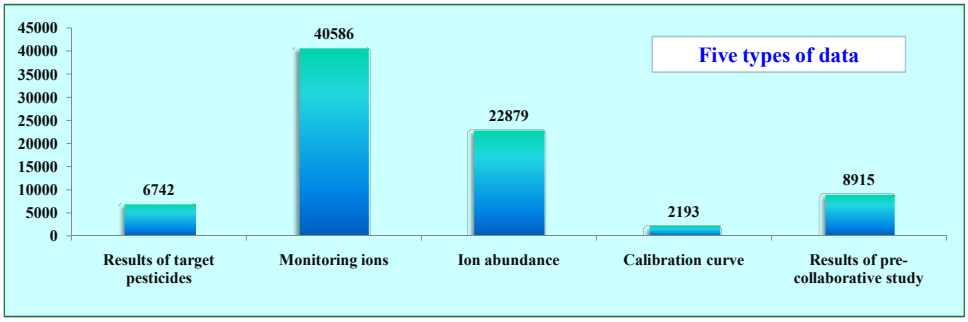
Technical conditions	GC-MS (16 Labs)	GC-MS/MS (15 Labs)	LC-MS/MS (24 Labs)
Instrument （22）	5 types: Agilent; Varian	5 types: Agilent; Waters; Thermo Fisher and Bruker	12 types: Agilent; SHIMADZU; Waters and Thermo Fisher
Chromatographic column （27）	5 brands：DB-1701; Restek Rtx 1701; MR2; HP-5 ms; HP-5MSUI	8 brands：DB-1701; HP-5 ms; HP-5MSUI; HP-5MSI; DB-5MS UI; DB5-MS; BR-5ms; Rxi 5 Sil	14 brands：ZORBAX SB-C18; phenomenex Luna C8; Waters Atlantis C18; phenomenex Luna C18
Specification （19）	30m×0.25mm×0.25μm	3 Specification：30m×0.25mm×0.25μm; 15m×0.25mm×0.25μm; length 20m/Diam. 0.180mm/Film 0.18μm	15 Specification：3.5 μm×100mm ×2.1mm; 3.5μm×150mm×2.1;150mm×2.00mm×3μm
①GC column temperature (14) ②LC gradient program(11)	5 GC column temperature ：40°C hold 1 min , at 30°C/min to 130°C, at 5°C/min to 250°C, at 10°C/min to 300 °C, hold 5 min	9 GC column temperature ：40°C hold 1 min , at 30°C/min to 130°C, at 5°C/min to 250°C, at 10°C/min to 300 °C, hold 5 min	11 GC column temperature：0min-99% A, 3 min-70% A, 6 min-60% A, 9 min-60% A, 15 min -40% A, 19 min -1% A, 23 min -1% A, 23.01 min -99% A
SPE cartridge(12)	3 SPE cartridges ：Cleanert TPT; UCT (ECPSACB506); Envicarb/PSA	4 SPE cartridges ：Cleanert TPT; InertSep GC/PSA; Agilent Technolongies BE Carbon 500mg/PSA; Envi Carb/PSA	5 SPE cartridges ：Cleanert TPT; InertSep GC/PSA; Agilent Technolongies BE Carbon 500mg/PSA; Envi-Carb; Envi Carb/PSA



EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

■30 laboratories have submitted to SD clusters of data totaling 81315

the method efficiency can be evaluated comprehensively with these five classes of data, and errors can be analyzed and traced. In addition, it also includes a 5000-page of raw data reports and chromatographs.



A lot of outliers obtained by one out of 30 laboratories were eliminated because of its digression from the operation protocol, so this laboratory's data was excluded from the evaluation of the method that ensued.

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

■Method efficiencies of GC-MS, GC-MS/MS and LC-MS/MS

■ Method efficiency of GC-MS determination of 20 pesticides (16 Labs)

Samples	Avg, Rec, %	RSD_r, %	RSD_R, %	HorRat
Green tea fortified samples	87.0-96.0	2.1-4.9	6.5-9.9	0.3-0.5
Oolong tea fortified samples	81.0-91.1	2.8-7.8	12.5-25.0	0.5-1.3
Oolong tea aged samples	-	2.1-6.1	9.1-20.0	0.4-1.0
Green tea incurred	-	3.3-4.0	12.2-23.0	0.7-1.0

■ Method efficiency of GC-MS/MS determination of 20 pesticides (14 Labs)

Samples	Avg, Rec, %	RSD_r, %	RSD_R, %	HorRat
Green tea fortified samples	87.0-97.1	3.1-6.0	6.6-14.8	0.3-0.7
Oolong tea fortified samples	77.1-90.8	1.4-5.4	7.0-32.7	0.4-1.3
Oolong tea aged samples	-	4.6-9.6	21.7-34.7	1.1-1.8
Green tea incurred	-	5.6-5.6	14.1-22.2	0.8-0.9

■Method efficiency of LC-MS/MS determination of 20 pesticides (23 Labs)

Samples	Avg, Rec, %	RSD_r, %	RSD_R, %	HorRat
Green tea fortified samples	91.7-97.2	4.9-8.6	8.0-15.8	0.3-0.6
Oolong tea fortified samples	81.7-93.8	3.6-10.5	13.8-30.4	0.4-1.3
Oolong tea aged samples	-	5.1-9.3	16.8-35.1	0.7-1.6
Green tea incurred	-	8.9-10.8	21.1-24.5	0.8-1.1

Based on the results from the statistical analysis of AOAC method efficiency software, all the parameters derived from the experiment have fulfilled the technical requirements of AOAC standards.

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

An overview on how your organization can obtain feedback for your method performance?

Date: July 14-Aug 20, 2016. Feedback requested from: 30 collaborative laboratories and 45 laboratories using Cleanert TPT. The feedback information has been received from a total of 15 laboratories (excluding the information submitted online), including three overseas laboratories: Japan, Spain and Taiwan of China, and see the table below.

Lab	Organization	Question 1	Question 2	Question 3	Question 4	Question 5	Question 6
Lab-1	Asia pacific technical center, the coca-cola company	Y	N	Y	N	N	Y
Lab-2	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau	Y	N	N	Y	N	N
Lab-3	Huangshan Tea Quality And Safety Research Center of Huangshan Entry-Exit Inspection & Quarantine Bureau	Y	Y	Y	Y	N	N
Lab-4	Food inspection central of CIQ ShenZhen	Y	N	N	Y	N	N
Lab-5	Qinhuangdao Entry-exit Inspection and Quarantine Bureau	Y	N	N	Y	N	N
Lab-6	Japan grain inspection association central research laboratory	Y	N	N	N	N	N
*Lab-7	*Tunding substation, tea research and extension station, executive yuan,(Taiwan)*	N	N	N	N	N	N
Lab-8	Tea research institute Chinese Academy of agricultural sciences	Y	Y	Y	Y	Y	N
Lab-9	EURL-FV, University of Almería	Y	N	Y	N	N	N
Lab-10	Dalian entry-exit inspection and quarantine bureau	Y	N	N	Y	N	N
Lab-11	ICAS Testing Center (Shanghai)	Y	Y	Y	Y	Y	N
Lab-12	BENGBU Center for Disease Control and prevention	Y	Y	Y	N	N	N
Lab-13	Shanghai Bino Testing Service Co., Ltd	Y	Y	Y	Y	Y	N
Lab-14	DENO TESTING Service (Shanghai)	Y	Y	Y	Y	Y	Y
Lab-15	Tea Research Institute, Chinese Academy of Agricultural Sciences	Y	Y	Y	Y	N	Y

*** This lab didn't use the method, so its feedback is not used for analysis.**

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

15 laboratories that have furnished us with the feedback information

Laboratory-type	Lab	Location
1. Overseas laboratories (3 Labs)	①Lab -6	Japan
	②Lab-7	Taiwan of China
	③Lab-9	Spain
2. International laboratories in China (4 labs)	①Lab-1	Shanghai, China
	②Lab-11	Shanghai, China
	③Lab-13	Shanghai, China
	⑤Lab-14	Shanghai, China
3. Domestic laboratories (8 labs)	①Lab-2	Fujian, China
	②Lab-3	Huangshan, China
	③Lab-4	Shenzhen, China
	④Lab-5	Qinhuangdao, China
	⑤Lab-8	Zhejiang, China
	⑥Lab-10	Dalian, China
	⑦Lab-12	Anhui, China
	⑧Lab-15	Zhejiang, China

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

METHOD APPLICABILITY

1. In your experience using the method, does the method perform according to the method's applicability as written?

Lab	Organization	Question 1
Lab-1	Asia pacific technical center, the coca-cola company	Y
Lab-2	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau	Y
Lab-3	Huangshan Tea Quality And Safety Research Center of Huangshan Entry-Exit Inspection & Quarantine Bureau	Y
Lab-4	Food inspection central of CIQ ShenZhen	Y
Lab-5	Qinhuangdao Entry-exit Inspection and Quarantine Bureau	Y
Lab-6	Japan grain inspection association central research laboratory	Y
Lab-8	Tea research institute Chinese Academy of agricultural sciences	Y
Lab-9	EURL-FV, University of Almería	Y
Lab-10	Dalian entry-exit inspection and quarantine bureau	Y
Lab-11	ICAS Testing Center (Shanghai)	Y
Lab-12	BENGBU Center for Disease Control and prevention	Y
Lab-13	Shanghai Bino Testing Service Co., Ltd	Y
Lab-14	DENO TESTING Service (Shanghai)	Y
Lab-15	Tea Research Institute, Chinese Academy of Agricultural Sciences	Y

✓ Yes: 14 Labs

✓ Lab-2: Comment: *The method is used in routine analysis for multi-pesticides in tea and* ***handling disputed results in our laboratory.***

✓ *Lab-15:* Comment*: Being capable of using exclusive Cleanert-TPT column to conduct inspection in the aspect of environmental pollutions..*

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

SAFETY CONCERNS

2. In your experience with the method, are there any safety concerns identified while using or regarding use of the method?

Lab	Organization	Question 2
Lab-1	Asia pacific technical center, the coca-cola company	N
Lab-2	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau	N
Lab-3	Huangshan Tea Quality And Safety Research Center of Huangshan Entry-Exit Inspection & Quarantine Bureau	Y
Lab-4	Food inspection central of CIQ ShenZhen	N
Lab-5	Qinhuangdao Entry-exit Inspection and Quarantine Bureau	N
Lab-6	Japan grain inspection association central research laboratory	N
Lab-8	Tea research institute Chinese Academy of agricultural sciences	Y
Lab-9	EURL-FV, University of Almería	N
Lab-10	Dalian entry-exit inspection and quarantine bureau	N
Lab-11	ICAS Testing Center (Shanghai)	Y
Lab-12	BENGBU Center for Disease Control and prevention	Y
Lab-13	Shanghai Bino Testing Service Co., Ltd	Y
Lab-14	DENO TESTING Service (Shanghai)	Y
Lab-15	Tea Research Institute, Chinese Academy of Agricultural Sciences	Y

✓Yes: 7 Labs No: 7 Labs

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

REFERENCE MATERIALS

3. In your experience with the method, are there available reference materials? Were there any concerns identified while using or regarding use of the method?

Lab	Organization	Question 3
Lab-1	Asia pacific technical center, the coca-cola company	Y
Lab-2	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau	N
Lab-3	Huangshan Tea Quality And Safety Research Center of Huangshan Entry-Exit Inspection & Quarantine Bureau	Y
Lab-4	Food inspection central of CIQ ShenZhen	N
Lab-5	Qinhuangdao Entry-exit Inspection and Quarantine Bureau	N
Lab-6	Japan grain inspection association central research laboratory	N
Lab-8	Tea research institute Chinese Academy of agricultural sciences	Y
Lab-9	EURL-FV, University of Almería	Y
Lab-10	Dalian entry-exit inspection and quarantine bureau	N
Lab-11	ICAS Testing Center (Shanghai)	Y
Lab-12	BENGBU Center for Disease Control and prevention	Y
Lab-13	Shanghai Bino Testing Service Co., Ltd	Y
Lab-14	DENO TESTING Service (Shanghai)	Y
Lab-15	Tea Research Institute, Chinese Academy of Agricultural Sciences	Y

✓Yes: 9 Labs No: 5 Labs

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

SINGLE LABORATORY VALIDATION

4. In your experience with the method, do you have data demonstrating linearity, accuracy, repeatability, LOD/LOQ?

Lab	Organization	Question 4
Lab-1	Asia pacific technical center, the coca-cola company	N
Lab-2	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau	Y
Lab-3	Huangshan Tea Quality And Safety Research Center of Huangshan Entry-Exit Inspection & Quarantine Bureau	Y
Lab-4	Food inspection central of CIQ ShenZhen	Y
Lab-5	Qinhuangdao Entry-exit Inspection and Quarantine Bureau	Y
Lab-6	Japan grain inspection association central research laboratory	N
Lab-8	Tea research institute Chinese Academy of agricultural sciences	Y
Lab-9	EURL-FV, University of Almería	N
Lab-10	Dalian entry-exit inspection and quarantine bureau	Y
Lab-11	ICAS Testing Center (Shanghai)	Y
Lab-12	BENGBU Center for Disease Control and prevention	N
Lab-13	Shanghai Bino Testing Service Co., Ltd	Y
Lab-14	DENO TESTING Service (Shanghai)	Y
Lab-15	Tea Research Institute, Chinese Academy of Agricultural Sciences	Y

✓Yes: 10 Labs No: 4 Labs

✓Lab -2: *The data obtained using AOAC Official Method 2014.09 to demonstrate the linearity, accuracy and repeatability attached in Appendix (see Table below).*

✓Lab-13: *Recoveries for 41 pesticides in green tea and black tea determined by GC-MS (see Table below).*

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

Lab-2:

Determination results of multi-residues in tea by GC-MS

No.	Pesticides	Retention time/min	Green tea										
			Calibration curve			Spike levels (μg/kg)	Recovery/%						RSD/ %
			R^2	Slope	Intercept		No.1	No.2	No.3	No.4	No.5	AVE	
IS	heptachlor	18.787											
1	trifluralin	9.940	0.9997	1.0858	-0.0155	200	82.2	85.5	83.7	84.7	94.2	86.1	5.47
2	tefluthrin	12.609	0.9982	1.8509	0.3906	100	88.1	89.7	87.6	87.3	97.3	90.0	4.66
3	pyrimethanil	14.156	0.9977	2.6666	0.5547	100	81.3	83.3	80.9	80.8	94.5	84.2	6.96
4	propyzamide	13.246	0.9984	0.9642	0.2457	100	83.8	85.5	80.3	81.5	87.2	83.7	3.37
5	pirimicarb	15.174	0.9981	1.5951	0.4131	100	91.6	94.2	83.1	91.0	103.7	92.7	7.99
6	dimethenamid	15.305	0.9963	0.4662	0.1679	40	86.7	95.2	90.8	89.8	102.3	93.0	6.52
7	tolclofos-methyl	16.264	0.9971	1.3229	0.3183	100	88.3	90.9	88.5	87.9	97.4	90.6	4.40
8	fenchlorphos	16.017	0.9965	1.2458	0.3545	200	95.1	99.2	95.4	96.7	103.6	98.0	3.62
9	pirimiphos-methyl	16.337	0.9977	0.5001	0.1095	100	90.1	92.0	89.7	89.1	98.1	91.8	4.01
10	2,4-DDE	20.000	0.9977	4.0213	0.7936	400	113.4	101.0	86.4	84.7	94.6	96.0	12.18
11	bromophos-ethyl	19.611	0.9973	0.3133	0.0691	100	90.5	92.3	89.6	89.4	99.1	92.2	4.36
12	4,4-DDE	21.820	0.9978	3.1875	0.6239	400	83.9	86.1	83.9	83.8	92.9	86.1	4.56
13	procymidone	19.907	0.9975	0.3892	0.0890	100	87.7	92.6	90.8	87.6	98.0	91.3	4.68
14	picoxystrobin	21.198	0.9979	0.7657	0.1335	200	88.1	91.4	87.6	87.7	95.2	90.0	3.68
15	chlorfenapyr	23.250	0.9976	0.4209	0.0998	800	97.2	99.1	95.9	95.4	102.6	98.0	3.01
16	quinoxyfen	27.183	0.9980	1.1099	0.2239	100	81.3	84.3	82.1	81.7	91.9	84.2	5.27
17	benalaxyl	26.357	0.9976	1.5987	0.3213	100	85.9	90.7	87.0	86.2	93.9	88.7	3.91
18	bifenthrin	28.293	0.9978	3.0963	0.8757	100	85.0	88.4	85.7	84.2	92.7	87.2	3.97
19	diflufenican	27.751	0.9982	2.0066	0.3759	100	86.3	89.8	86.5	85.2	94.3	88.4	4.20
20	bromopropylate	29.781	0.9978	1.8023	0.3328	200	87.0	91.5	88.5	87.3	95.5	90.0	3.99

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

Lab-2:

Determination results of multi-residues in tea by LC-MS/MS

No.	Pesticides	Retention time/min	Green tea										
			Calibration curve			Spike levels (μg/kg)	Recovery/%						RSD/ %
			R2	Slope	Intercept		No.1	No.2	No.3	No.4	No.5	AVE	
IS	chlorpyrifos-methyl	17.618											
1	imidacloprid	5.663	0.9987	0.0850	0.0856	45	115.4	103.8	114.8	111.4	105.3	110.1	4.87
2	propoxur	8.264	0.9989	1.8593	31.8969	50	108.6	93.3	114.0	108.4	108.1	106.5	7.30
3	monolinuron	9.409	0.9980	0.0116	-0.0314	20	87.3	91.6	93.1	86.3	91.1	89.9	3.28
4	clomazone	11.107	0.9991	0.7681	0.9358	20	93.8	94.0	100.2	96.6	105.5	98.0	5.00
5	ethoprophos	13.899	0.9991	0.3678	1.0466	20	101.1	92.7	103.0	97.1	104.0	99.6	4.65
6	triadimefon	14.096	0.9985	0.2186	-1.0063	20	103.6	94.2	102.9	98.2	104.2	100.6	4.28
7	acetochlor	15.138	0.9990	0.2468	-0.3841	40	99.4	94.0	102.2	99.4	105.2	100.0	4.14
8	flutolanil	15.671	0.9969	0.4262	-0.8631	20	105.4	96.1	105.9	101.6	105.0	102.8	4.01
9	benalaxyl	16.24	0.9990	2.5914	18.3183	20	99.9	92.5	102.5	98.9	104.0	99.6	4.44
10	kresoxim-methyl	16.571	0.9946	0.1833	5.8772	200	107.1	96.0	92.0	103.6	103.7	100.5	6.21
11	picoxystrobin	17.119	0.9965	1.4234	1.7725	20	133.5	97.5	97.4	102.5	110.3	108.2	13.93
12	pirimiphos-methyl	16.453	0.9999	1.2458	5.7368	20	93.1	90.0	94.3	91.7	98.5	93.5	3.45
13	diazinon	17.09	0.9997	2.9582	-2.6557	20	105.9	95.5	97.4	97.1	108.8	101.0	5.93
14	bensulide	17.405	0.9952	0.2903	0.2172	60	116.7	93.3	99.7	103.2	106.0	103.8	8.30
15	quinoxyfen	17.491	0.9961	0.6027	4.4482	100	81.6	95.6	89.3	93.8	99.6	92.0	7.50
16	tebufenpyrad	18.016	0.9941	0.0441	-0.3803	20	114.5	99.8	97.9	105.3	106.4	104.8	6.22
17	indoxacarb	18.262	0.9992	0.0263	-0.0882	20	100.6	94.3	110.6	104.2	104.9	102.9	5.84
18	trifloxystrobin	18.311	0.9978	0.4936	-1.0594	20	101.7	98.6	101.7	100.2	108.9	102.2	3.85
19	chlorpyrifos	18.884	0.9928	0.0072	-0.0321	200	111.4	126.6	92.8	87.2	87.1	101.0	17.26
20	butralin	19.292	0.9956	0.0022	-0.0035	20	72.1	84.2	82.1	99.2	128.0	93.1	23.37

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

Lab-13:

Recoveries for 41 pesticides in green tea and black tea determined by GC-MS: 70%-94%

Pesticides	Recovery	Pesticides	Recovery	Pesticides	Recovery
Phorate	84%	Fenthion	82%	Cypermethrin	88%
Quintozene	81%	Malathion	82%	Fenvalerate	90%
Diazinon	85%	Fenitrothion	84%	Deltamethrin	87%
Fonofos	80%	Triadimefon	78%	Trifluralin	84%
Etrimfos	79%	Pendimethalin	81%	Sulfotep	81%
Dimethoate	70%	Quinalphos	88%	A-HCH	84%
Aldrin	81%	Phenthoate	81%	Δ-HCH	88%
Vinclozolin	79%	Procymidone	92%	B-HCH	83%
Buprofezin	82%	Dieldrin	80%	Dicofol	85%
Metalaxyl	81%	Methidathion	80%	p,p'-DDD	79%
Methyl-parathion	85%	Fenamiphos	80%	o,p'-DDD	80%
Chlorpyrifos	90%	Terbufos	81%	o,p'-DDT	80%
Chlorpyrifosmethyl	93%	Bifenthrin	94%	p,p'-DDE	80%
Triazophos	84%	Fenpropathrin	89%		

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

REPRODUCIBILITY/UNCERTAINTY AND PROBABILITY OF DETECTION

5. Do you have any information that supports the reproducibility of this method as written? If so, please specify and submit information.

Lab	Organization	Question 5
Lab-1	Asia pacific technical center, the coca-cola company	N
Lab-2	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau	N
Lab-3	Huangshan Tea Quality And Safety Research Center of Huangshan Entry-Exit Inspection & Quarantine Bureau	N
Lab-4	Food inspection central of CIQ ShenZhen	N
Lab-5	Qinhuangdao Entry-exit Inspection and Quarantine Bureau	N
Lab-6	Japan grain inspection association central research laboratory	N
Lab-8	Tea research institute Chinese Academy of agricultural sciences	Y
Lab-9	EURL-FV, University of Almería	N
Lab-10	Dalian entry-exit inspection and quarantine bureau	N
Lab-11	ICAS Testing Center (Shanghai)	Y
Lab-12	BENGBU Center for Disease Control and prevention	N
Lab-13	Shanghai Bino Testing Service Co., Ltd	Y
Lab-14	DENO TESTING Service (Shanghai)	Y
Lab-15	Tea Research Institute, Chinese Academy of Agricultural Sciences	N

✓Yes: 4 Labs No:10 Labs

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

ADDITIONAL FEEDBACK

6. Based on your experience with the method, are there any recommended changes to the AOAC First Action method as written?

Lab	Organization	Question 6
Lab-1	Asia pacific technical center, the coca-cola company	Y
Lab-2	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau	N
Lab-3	Huangshan Tea Quality And Safety Research Center of Huangshan Entry-Exit Inspection & Quarantine Bureau	N
Lab-4	Food inspection central of CIQ ShenZhen	N
Lab-5	Qinhuangdao Entry-exit Inspection and Quarantine Bureau	N
Lab-6	Japan grain inspection association central research laboratory	N
Lab-8	Tea research institute Chinese Academy of agricultural sciences	N
Lab-9	EURL-FV, University of Almería	N
Lab-10	Dalian entry-exit inspection and quarantine bureau	N
Lab-11	ICAS Testing Center (Shanghai)	N
Lab-12	BENGBU Center for Disease Control and prevention	N
Lab-13	Shanghai Bino Testing Service Co., Ltd	N
Lab-14	DENO TESTING Service (Shanghai)	Y
Lab-15	Tea Research Institute, Chinese Academy of Agricultural Sciences	Y

■Yes: 3 Labs No: 11 Labs

■Lab-1: sample preparation is time consuming, *i.e.*, rotatory evaporation.
Reply: This problem is solved with the second technique called Tuber Vap introduced in the method.

■Lab-14: hoping to use QuEChERs method
Reply: the efficiency of QuEChERs method cannot meet the requirement.

■Lab-15: Concrete opinion is not available.

The overall evaluation of the method by Lab-8:

- This comprehensive method provides an effective extraction of diverse pesticides in tea and cleanup of the co-extracted interference during the sample preparation.
- Compared with former routine strategy, it is simple, convenient and time-saving.
- With the sensitive LOD and LOQ, it is a satisfactory choice in our lab to analyze the pesticide residues in tea as a routine method.
- In 2015, residue levels in 600 samples had been collected by applying the method in First Action 2014 and the former version, which supplied the reliable basic data for our research.

EXPERT REVIEW PANEL FOR PESTICIDE RESIDUES
2016 AOAC INTERNATIONAL ANNUAL MEETING & EXPOSITION, DALLAS , TEXAS
WEDNESDAY, SEPTEMBER 21, 2016

Chinese Academy Inspection and Quarantine
ciqpang@163.com

Feedback (the sequence is based on the "AOAC-RI ERP Book", different with the order of the feedback in Pang's presentation PPT)

No.	Submission Date	Perspective	Name	Organization	Email	Question 1	Question 2	Question 3	Question 4	Question 5	Question 6	Note
1.	2016.7.8	Expert review panel member	Xiaoyan Wang	UCT	xwang@unitedchem.com	Y	N	N	Y	Y	N	Https://sampleprep.unitedchem.com/media/at_assets/tech_doc_info/pesticides_in_tea_AOAC
2.	2016.7.29	Expert review panel member	Joe boison	CFIA	Joe.boison@inspection.gc.ca							I，nor my agency laboratories have not had the opportunity to use the described method, so I don't really have any experimental feedback to provide. I will wait until I hear feedback from other actual users to make any comments I may have.
3.	2016.8.30	Method End-user	Chen Wang	Tea research institute, Chinese Academy of Agricultural Sciences	wangchen@tricaas.com	Y	Y	Y	Y	N	Y	
4.	2016.8.30	Method End-user	Jia Ren	DENO TESTING Service(Shanghai)	renjia@shdenuo.com	Y	Y	Y	Y	Y	Y	Hoping to use QuEchERs method
5.	2016.8.30	Method End-user	Jianjun Wang	Shanghai Bino Testing Service Co., Ltd	Bino_wjj@126.com	Y	Y	Y	Y	Y	N	Annex 1
6.	2016.8.30	Method End-user	Hong Chang	BENGBU Center for Disease Control and Prevention	494283390@qq.com	Y	Y	Y	N	N	N	
7.	2016.8.10	Method End-user	Daliang Huang	Dalian entry-exit inspection and quarantine bureau	ciqzhhdl@163.com	Y	N	N	Y	N	N	
8.	2016.8.10	Method End-user	Amadeo R. Fernandez-Alba	EURL-RV, University of Almeria	amadeo@ual.es	Y	N	Y	N	N	N	
9.	2016.8.10	Method End-user	Li Zhou	Tea research institute Chinese Academy of agricultural Sciences	lizhou@tricas.com	Y	Y	Y	Y	Y	N	
10.	2016.8.10	Method End-user	YU-Ju Huang	Tunding substation, tea research and extension station, executive yuan (Taiwan)	Tds511@ttes.gov.tw	N	N	N	N	N	N	
11.	2016.8.10	Method End-user	Naoki Kanamaru	Japan grain inspection association central research laboratory	n-kanamaru@kokken.or.jp	Y	N	N	N	N	N	
12.	2016.8.10	Method End-user	Yanzhong Cao	Qinhuangdao Entry-exit Inspection and Quarantine Bureau	qhdciq@aliyun.com	Y	N	N	Y	N	N	

No.	Submission Date	Perspective	Name	Organization	Email	Question 1	Question 2	Question 3	Question 4	Question 5	Question 6	Note
13.	2016.8.10	Method End-user	Liqi Xie	Food inspection central of CIQ ShenZhen	Ms64@163.com	Y	N	N	Y	N	N	
14.	2016.8.10	Method End-user	Fang Yang	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau	yffjciq@gmail.com	Y	N	N	Y	N	N	Annex 2
15.	2016.7.18	Method End-user	Yanzhong Cao	Qinhuangdao Entry-exit Inspection and Quarantine Bureau	qhdciq@aliyun.com	Y	N	N	Y	N	N	***Same to No.12***
16.	2016.7.18	Method End-user	Liqi Xie	Food inspection central of CIQ ShenZhen	Ms64@163.com	Y	N	N	Y	N	N	***Same to No.13***
17.	2016.7.18	Method End-user	Naoki Kanamaru	Japan grain inspection association central research laboratory	n-kanamaru@kokken.or.jp	Y	N	N	N	N	N	***Same to No.11***
18.	2016.8.10	Method End-user	Ying Xu	Asia pacific technical center, the coca-cola company	Yxu3@coca-cola.com	Y	N	Y	N	N	Y	sample preparation is time consuming, *i.e.*, rotatory evaporation.
19.	2016.8.10	Method End-user	Ying Xu	Asia pacific technical center, the coca-cola company	Yxu3@coca-cola.com	Y	N	Y	N	N	Y	***Same to No.18***
20.	2016.8.10	Method End-user	Fang Yang	Inspection & Quarantine Technique Centre of Fujian Entry-exit Inspection and Quarantine Bureau	yffjciq@gmail.com	Y	N	N	Y	N	N	***Same to No.14***
21.	2016.8.10	Method End-user	Qiong Wu	Huangshan Tea Quality And Safety Research Center of Huangshan Entry-Exit Inspection & Quarantine Bureau	wuqiong@ahciq.gov.cn	Y	Y	Y	Y	N	N	
22.	2016.8.30	Method End-user	Chunyan Song	ICAS Testing Center (Shanghai)	2075523968@qq.com	Y	Y	Y	Y	Y	N	

Annex 1

Recoveries for 41 pesticides in green tea and black tea determined by GC-MS: 70%-94%

No.	Pesticides	Recovery
1.	Phorate	84%
2.	Quintozene	81%
3.	Diazinon	85%
4.	Fonofos	80%
5.	Etrimfos	79%
6.	Dimethoate	70%
7.	Aldrin	81%
8.	Vinclozolin	79%
9.	Buprofezin	82%
10.	Metalaxyl	81%
11.	Methyl-parathion	85%
12.	Chlorpyrifos	90%
13.	Chlorpyrifosmethyl	93%
14.	Triazophos	84%
15.	Fenthion	82%
16.	Malathion	82%
17.	Fenitrothion	84%
18.	Triadimefon	78%
19.	Pendimethalin	81%
20.	Quinalphos	88%
21.	Phenthoate	81%

No.	Pesticides	Recovery
22.	Procymidone	92%
23.	Dieldrin	80%
24.	Methidathion	80%
25.	Fenamiphos	80%
26.	Terbufos	81%
27.	Bifenthrin	94%
28.	Fenpropathrin	89%
29.	Cypermethrin	88%
30.	Fenvalerate	90%
31.	Deltamethrin	87%
32.	Trifluralin	84%
33.	Sulfotep	81%
34.	A-HCH	84%
35.	Δ-HCH	88%
36.	B-HCH	83%
37.	Dicofol	85%
38.	p,p'-DDD	79%
39.	o,p'-DDD	80%
40.	o,p'-DDT	80%
41.	p,p'-DDE	80%

Annex 2

Determination results of multi-residues in tea by GC-MS

No.	Pesticides	Retention time/min	Green tea										
			Calibration curve			Spike levels (μg/kg)	Recovery/%						RSD/%
			R^2	Slope	Intercept		No.1	No.2	No.3	No.4	No.5	AVE	
IS	heptachlor	18.787											
1	trifluralin	9.940	0.9997	1.0858	-0.0155	200	82.2	85.5	83.7	84.7	94.2	86.1	5.47
2	tefluthrin	12.609	0.9982	1.8509	0.3906	100	88.1	89.7	87.6	87.3	97.3	90.0	4.66
3	pyrimethanil	14.156	0.9977	2.6666	0.5547	100	81.3	83.3	80.9	80.8	94.5	84.2	6.96
4	propyzamide	13.246	0.9984	0.9642	0.2457	100	83.8	85.5	80.3	81.5	87.2	83.7	3.37
5	pirimicarb	15.174	0.9981	1.5951	0.4131	100	91.6	94.2	83.1	91.0	103.7	92.7	7.99
6	dimethenamid	15.305	0.9963	0.4662	0.1679	40	86.7	95.2	90.8	89.8	102.3	93.0	6.52
7	tolclofos-methyl	16.264	0.9971	1.3229	0.3183	100	88.3	90.9	88.5	87.9	97.4	90.6	4.40
8	fenchlorphos	16.017	0.9965	1.2458	0.3545	200	95.1	99.2	95.4	96.7	103.6	98.0	3.62
9	pirimiphos-methyl	16.337	0.9977	0.5001	0.1095	100	90.1	92.0	89.7	89.1	98.1	91.8	4.01
10	2,4-DDE	20.000	0.9977	4.0213	0.7936	400	113.4	101.0	86.4	84.7	94.6	96.0	12.18
11	bromophos-ethyl	19.611	0.9973	0.3133	0.0691	100	90.5	92.3	89.6	89.4	99.1	92.2	4.36
12	4,4-DDE	21.820	0.9978	3.1875	0.6239	400	83.9	86.1	83.9	83.8	92.9	86.1	4.56
13	procymidone	19.907	0.9975	0.3892	0.0890	100	87.7	92.6	90.8	87.6	98.0	91.3	4.68
14	picoxystrobin	21.198	0.9979	0.7657	0.1335	200	88.1	91.4	87.6	87.7	95.2	90.0	3.68
15	chlorfenapyr	23.250	0.9976	0.4209	0.0998	800	97.2	99.1	95.9	95.4	102.6	98.0	3.01
16	quinoxyfen	27.183	0.9980	1.1099	0.2239	100	81.3	84.3	82.1	81.7	91.9	84.2	5.27
17	benalaxyl	26.357	0.9976	1.5987	0.3213	100	85.9	90.7	87.0	86.2	93.9	88.7	3.91
18	bifenthrin	28.293	0.9978	3.0963	0.8757	100	85.0	88.4	85.7	84.2	92.7	87.2	3.97
19	diflufenican	27.751	0.9982	2.0066	0.3759	100	86.3	89.8	86.5	85.2	94.3	88.4	4.20
20	bromopropylate	29.781	0.9978	1.8023	0.3328	200	87.0	91.5	88.5	87.3	95.5	90.0	3.99

Determination results of multi-residues in tea by LC-MS/MS

No.	Pesticides	Retention time/min	Green tea										
			Calibration curve			Spike levels (μg/kg)	Recovery/%						RSD/%
			R2	Slope	Intercept		No.1	No.2	No.3	No.4	No.5	AVE	
IS	chlorpyrifos-methyl	17.618											
1	imidacloprid	5.663	0.9987	0.0850	0.0856	45	115.4	103.8	114.8	111.4	105.3	110.1	4.87
2	propoxur	8.264	0.9989	1.8593	31.8969	50	108.6	93.3	114.0	108.4	108.1	106.5	7.30
3	monolinuron	9.409	0.9980	0.0116	-0.0314	20	87.3	91.6	93.1	86.3	91.1	89.9	3.28
4	clomazone	11.107	0.9991	0.7681	0.9358	20	93.8	94.0	100.2	96.6	105.5	98.0	5.00
5	ethoprophos	13.899	0.9991	0.3678	1.0466	20	101.1	92.7	103.0	97.1	104.0	99.6	4.65
6	triadimefon	14.096	0.9985	0.2186	-1.0063	20	103.6	94.2	102.9	98.2	104.2	100.6	4.28
7	acetochlor	15.138	0.9990	0.2468	-0.3841	40	99.4	94.0	102.2	99.4	105.2	100.0	4.14
8	flutolanil	15.671	0.9969	0.4262	-0.8631	20	105.4	96.1	105.9	101.6	105.0	102.8	4.01
9	benalaxyl	16.24	0.9990	2.5914	18.3183	20	99.9	92.5	102.5	98.9	104.0	99.6	4.44
10	kresoxim-methyl	16.571	0.9946	0.1833	5.8772	200	107.1	96.0	92.0	103.6	103.7	100.5	6.21
11	picoxystrobin	17.119	0.9965	1.4234	1.7725	20	133.5	97.5	97.4	102.5	110.3	108.2	13.93
12	pirimiphos-methyl	16.453	0.9999	1.2458	5.7368	20	93.1	90.0	94.3	91.7	98.5	93.5	3.45
13	diazinon	17.09	0.9997	2.9582	-2.6557	20	105.9	95.5	97.4	97.1	108.8	101.0	5.93
14	bensulide	17.405	0.9952	0.2903	0.2172	60	116.7	93.3	99.7	103.2	106.0	103.8	8.30
15	quinoxyfen	17.491	0.9961	0.6027	4.4482	100	81.6	95.6	89.3	93.8	99.6	92.0	7.50
16	tebufenpyrad	18.016	0.9941	0.0441	-0.3803	20	114.5	99.8	97.9	105.3	106.4	104.8	6.22
17	indoxacarb	18.262	0.9992	0.0263	-0.0882	20	100.6	94.3	110.6	104.2	104.9	102.9	5.84
18	trifloxystrobin	18.311	0.9978	0.4936	-1.0594	20	101.7	98.6	101.7	100.2	108.9	102.2	3.85
19	chlorpyrifos	18.884	0.9928	0.0072	-0.0321	200	111.4	126.6	92.8	87.2	87.1	101.0	17.26
20	butralin	19.292	0.9956	0.0022	-0.0035	20	72.1	84.2	82.1	99.2	128.0	93.1	23.37

AOAC Official Method 2014.10
Dietary Starch in Animal Feeds and Pet Food
Enzymatic-Colorimetric Method
First Action 2014

(Applicable for the determination of dietary starch in forages, grains, grain by-products, dry, semi-moist, and moist pet food products, and mixed feeds that range in concentration from 1 to 100%.)

Caution: Acetic acid is flammable in both liquid and vapor forms. It can cause severe skin burns and eye damage and is toxic if inhaled. Avoid breathing fumes. Wear protective gloves, clothing, and eye and face protection.

α-Amylase and glucose oxidase are respiratory sensitizers, which may cause allergy or asthma symptoms. Avoid breathing dust. Amylase preparations can cause allergic reactions in hypersensitive individuals. Avoid inhaling aerosols or dusts.

Benzoic acid causes serious eye damage and respiratory irritation. Avoid breathing dust and mist. Wear eye protection.

Phenol can be toxic and cause severe burns and eye damage. It is suspected of causing genetic defects and may cause damage to organs. Do not breathe dust or fumes. Wear protective gloves, clothing, eye protection, and face protection.

See Table **2014.10** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Ground or homogenized animal feed and pet food test portions are mixed with acetic acid buffer and heat-stable α-amylase and incubated at 100°C for 1 h with periodic mixing to gelatinize and partially hydrolyze the starch. After cooling, amyloglucosidase is added and the test mixture is incubated for 2 h at 50°C. The digested mixture is clarified, diluted as needed, and glucose detected in the resulting test solution using a colorimetric glucose oxidase-peroxidase (GOPOD) method that is sensitive and specific to glucose. Free glucose is measured simultaneously or in a separate analytical run for each test sample by carrying a second test portion of each test sample through the procedure omitting enzymes and incubating at 100°C for 1 h with periodic mixing. Dietary starch is determined as 0.9 times the difference of glucose in the digested test portion minus free glucose in the undigested test portion.

B. Apparatus

(**a**) *Grinding mill*.—Mills such as an abrasion mill equipped with a 1.0 to 0.5 mm screen, or a cutting mill with 0.5 mm screen, or other appropriate device to grind test samples to pass a 40 mesh screen.

(**b**) *Homogenizer, blender, or mixer*.—To provide homogenous suspension of canned pet food, liquid animal feed, semi-moist pet food, and other materials containing less than 85% dry matter.

(**c**) *Bench centrifuge or microcentrifuge.*—Capable of centrifuging at 1000 × *g* to 10 000 × *g*.

(**d**) *Water bath.*—Capable of maintaining 50 ± 1°C.

(**e**) *Vortex mixer.*

(**f**) *pH meter.*

(**g**) *Stop clock timer (digital).*

(**h**) *Top-loading balance*.—Capable of weighing accurately to ±0.01 g.

(**i**) *Analytical balance.*—Capable of weighing accurately to ±0.0001 g.

(**j**) *Laboratory ovens.*—With forced-convection; capable of maintaining 100 ± 1°C for carrying out incubations.

(**k**) *Spectrophotometer*.—Capable of operating at absorbances of 505 nm.

(**l**) *Pipets.*—Capable of delivering 0.1 and 1.0 mL; with disposable tips.

(**m**) *Positive-displacement repeating pipet.*—Capable of accurately delivering 0.1, 1.0, and 3.0 mL.

(**n**) *Dispenser.*—1000 mL or greater capacity; capable of accurately delivering 20 and 30 mL.

(**o**) *Glass test tubes*.—16 × 100 mm.

(**p**) *Glass tubes.*—25 × 200 mm, with polytetrafluoroethylene (PTFE)-lined screw caps or comparable tubes to hold 51.1 mL and allow for adequate mixing when sealed.

(**q**) *Plastic film.*—Or similarly nonreactive material.

(**r**) *Magnetic stir plate.*

(**s**) *Glass fiber filter.*—With 1.6 µm retention.

Table 2014.10. Method performance for determination of dietary starch in feeds

Material	No. of labs	Mean, %	s_r	s_R	RSD_r %	RSD_R %	r[a]	R[b]
Moist canned dog food	11	1.54	0.03	0.09	2.21	5.99	0.10	0.26
Low starch horse feed	13	7.02	0.23	0.36	3.32	5.19	0.65	1.02
Dry ground corn	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54
Complete dairy feed	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48
Soybean meal	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31
Distillers grains	13	4.11	0.11	0.20	2.67	4.94	0.31	0.57
Pelleted poultry feed	13	28.24	0.73	1.34	2.58	4.76	2.04	3.76
Corn silage	13	39.04	0.80	1.88	2.05	4.82	2.24	5.27
Dog kibble, dry	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46
Alfalfa pellets	13	1.38	0.12	0.13	8.61	9.69	0.33	0.38

[a] $r = 2.8 \times s_r$.

[b] $R = 2.8 \times s_R$.

(**t**) *Hardened filter paper.*—With 22 µm retention.

C. Reagents

Note: Use high-quality distilled or deionized water for all water additions.

(**a**) *Acetate buffer (100 mM, pH 5.0).*—Weigh 6.0 g or pipet 5.71 mL glacial acetic acid and transfer immediately to a flask; quantitatively transfer weighed acid with H_2O rinses. Bring volume to ca 850 mL. While stirring solution on a magnetic stir plate, adjust pH to 5.0 ± 0.1 with 1 M NaOH solution. Dilute to 1 L with H_2O. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**b**) *Heat-stable α-amylase solution.*—Liquid, heat-stable, α-amylase (examples: Product Termamyl 120 L, Novozymes North America, Franklinton, NC, USA; Product Multifect AA 21L, Genencor International, Rochester, NY, USA; origin: *Bacillus licheniformis*, or equivalent). Should not contain greater than 0.5% glucose. pH optima must include 5.5–5.8.

Based on Bacterial Amylase Unit (BAU) method.—Approximately 83000 BAU/mL of concentrated enzyme (1 BAU is defined as the amount of enzyme that will dextrinize starch at the rate of 1 mg/min at pH 6.6 and 30 ± 0.1°C; 1). If modifications of volume delivered are necessary due to enzymatic activity of the enzyme used, the volume used per test portion should deliver approximately 8300 ± 20 BAU (1).

The enzymes should be of a purity meeting the specifications listed in **991.43** (*see* 32.1.17), but as modified below for application in the assay for dietary starch. The enzyme preparation used must be validated within laboratory to verify efficacy, as well as lack of interference. Recommended validation: analyze 0.1 g test portions of purified glucose, sucrose, and purified corn starch with the enzymatic portion of the dietary starch assay and using a free glucose value of zero in calculations. Analyses with candidate enzyme should give values of [mean ± standard deviation (SD)] glucose: 90 ± 2%, starch: 100±2%, and sucrose: 0.7 ± 0.3% on a dry matter basis. To test for interference from release of glucose from fiber carbohydrates, analyze 0.1 g test portions of α-cellulose and barley β-glucan that are not contaminated with free glucose with the enzymatic portion of the dietary starch assay. Recovery of these substrates should be less than 0.5% on a dry matter basis [*see* **991.43** (*see* 32.1.17)]. Use AOAC approved methods for determination of dry matters of the samples. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%), or background absorbance readings will interfere with test sample measurements.

(**c**) *Diluted amyloglucosidase solution.*—Dilute concentrated amyloglucosidase with 100 mM sodium acetate buffer, **C**(**a**), to give 1 mL of solution per test portion with 2 to 5 mL excess. Add 1/3 of needed buffer to an appropriately sized graduated cylinder. Pipet concentrated amyloglucosidase into buffer, rinsing tip by taking up and expelling buffer in the graduated cylinder. Bring to desired volume with additional buffer. Cap cylinder with plastic film and invert cylinder repeatedly to mix. The concentrated amyloglucosidase used should not contain greater than 0.5% glucose, and should have a pH optimum of 4.0 and pH stability between 4.0–5.5 (example of concentrated amyloglucosidase: Product E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland; origin: *Aspergillus niger*, or equivalent).

(*1*) *Based on release of glucose from soluble starch or glycogen.*—200 U/mL (1 unit of enzyme activity is defined as the amount of enzyme required to release 1 µmole glucose/min at pH 4.5 and 40°C; 21).

(*2*) *Based on p-nitrophenyl-β-maltoside method.*—13 units/mL (1 unit is defined as the amount of enzyme required to release 1 µmole *p*-nitrophenol from *p*-nitrophenyl-β-maltoside/min at pH 4.5 and 40°C; 2). Follow the protocol described in **C**(**b**) for standards and procedure for testing adequacy of enzyme activity and lack of side activity.

The enzyme used must be validated within laboratory to verify efficacy as well as lack of interference. Use the same validation procedure as described for heat-stable α-amylase, **C**(**b**).

(**d**) *Benzoic acid solution (0.2%).*—Weigh 2.0 g benzoic acid (solid, ACS reagent, >99.5% purity) and add to a flask. Bring flask to 1 L volume with H_2O. Add magnetic stir bar, stopper flask, and allow to stir overnight to dissolve benzoic acid. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**e**) *GOPOD reagent.*—(*1*) *Mixture of glucose oxidase, 7000 U/L, free from catalase activity; peroxidase, 7000 U/L; and 4-aminoantipyrine, 0.74 mM.*—Prepare by dissolving 9.1 g Na_2HPO_4 (dibasic, anhydrous) and 5.0 g KH_2PO_4 in ca 300 mL H_2O in a 1 L volumetric flask. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add 1.0 g phenol (ACS grade) and 0.15 g 4-aminoantipyrine. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add glucose oxidase (7000 U) and peroxidase (7000 U), rinse enzymes into flask with H_2O, and swirl gently to dissolve without causing excessive foaming. Bring to 1 L volume with H_2O. Seal and invert repeatedly to mix. Filter solution through a glass fiber filter with 1.6 µm retention, **B**(**s**). Store in a sealed amber bottle at ca 4°C. Reagent life: 1 month. Before use in test sample determinations, determine a standard curve for the reagent using a 5-point standard curve using **C**(**e**) and **C**(**f**) according to **D**(**b**).

(*2*) Alternatively, use another AOAC-approved glucose-specific assay that has passed in laboratory validation to accurately determine glucose concentrations of glucose standard solutions and give values equivalent to the values listed for determination of efficacy of enzymes. Recommended validation: analyze all five glucose working standard solutions and 100 mg test portions of purified glucose, purified sucrose, and purified corn starch that have been processed through the enzymatic hydrolysis portion of the dietary starch procedure and using a free glucose value of zero in calculations. The glucose values of the working standard solutions should be predicted ±6 µg glucose/mL. On a dry matter basis, the control sample glucose should give a dietary starch value (mean ± SD) of 90 ± 2%, corn starch at 100 ± 2%, and sucrose 0.7 ± 0.3%.

(**f**) *Glucose working standard solutions.*—0, 250, 500, 750, and 1000 µg/mL. Determine the dry matter of powdered crystalline glucose (purity ≥99.5%) by an AOAC-approved method. Weigh approximately 62.5, 125, 187.5, and 250 mg portions of glucose and record weight to 0.0001 g. Rinse each portion of glucose from weigh paper into a separate 250 mL volumetric flask with 0.2% benzoic acid solution, **C**(**d**), and swirl to dissolve. Bring each standard to 250 mL volume with 0.2% benzoic acid solution, **C**(**d**), to give four independent glucose standard solutions. The 0.2% benzoic acid solution, **C**(**d**), serves as the 0 µg/mL standard solution. Multiply weight of glucose by dry matter percentage and percentage purity as provided by the manufacturer in the certificate of analysis and divide by 250 mL to calculate actual glucose concentrations of the solutions. Prepare solutions at least one day before use to allow

equilibration of α- and β- forms of the glucose. Standard solutions may be stored at room temperature for 6 months.

(**g**) *Internal quality control samples*.—Powdered crystalline glucose (purity ≥99.5%) and isolated corn starch. For the corn starch sample, crude protein as nitrogen content × 6.25 and ash should be determined to determine the nonprotein organic matter content of the sample. For use in recovery calculations, actual starch content of the corn starch control sample is estimated as 100% minus ash% and minus crude protein%, all on a dry matter basis. Analyze 100 mg of each sample with each batch of test samples. Glucose will allow evaluation of quantitative recovery, and starch will allow evaluation of quantitative recovery and efficacy of the assay.

(**h**) *Determination of accuracy of volume additions for use of summative volume approach*.—The method as described relies on accurate volumetric additions in order to use the sum of volumes to describe test solution volume. Accuracy of volume additions can be evaluated before the assay by the following procedure: Using 1–2 L distilled water at ambient temperature, determine the g/mL density of the water by recording the weight of three empty volumetric flasks (volumes between 50 and 100 mL), add the water to bring to volume, and weigh the flasks + water. Calculate water density g/mL as:

$$\text{Water density, g/mL} = [(\text{flask + water, g}) - (\text{flask, g})]/\text{water volume mL}$$

Record the weights of five empty tubes used for the dietary starch assay. Using the ambient temperature water and the devices used to deliver the liquid volumes for the enzymatic hydrolysis portion of the assay, deliver the 30, 0.1, 1, and 20 mL volumes to each tube (total of 51.1 mL in each tube). Record the weight of each tube + water. Calculate the grams of water in each tube as:

$$\text{Water in each tube, g} = (\text{tube + water, g}) - (\text{tube, g})$$

Divide the weight of water in each tube by the determined average density of water to give the volume of water in each tube. The deviation should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. If the deviations are greater than these, after the addition of 20 mL water during the dietary starch assay, individual samples should be quantitatively transferred with filtration through hardened filter paper with a 22 µm retention, **B**(**t**), into a 100 mL volumetric flask and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

D. Preparation of Reagent Blanks, Standard Curves, and Test Samples

(**a**) *Reagent blank*.—For each assay, two reaction tubes containing only the reagents added for each method are carried through the entire procedure. Reagent blanks diluted to the same degree as samples (no dilution or diluted to the same degree as control and test samples) are analyzed. Absorbance values for the reagent blanks are subtracted from absorbance values of the test solutions prepared from test and control samples.

(**b**) *Standard curves*.—Pipet 0.1 mL of 0.2% benzoic acid solution, **C**(**d**), and nominal 250, 500, 750, and 1000 µg/mL working standard glucose solutions, **C**(**f**), in duplicate into the bottoms of 16 × 100 mm glass culture tubes. Add 3.0 mL GOPOD reagent, **C**(**e**), to each tube using a positive displacement repeating pipet aimed against wall of tube, so it will mix well with the sample. Vortex tubes. Cover tops of tubes with plastic film. Incubate in a 50°C water bath for 20 min. Read absorbance at 505 nm using the 0 µg glucose/mL standard to zero the spectrophotometer. All readings should be completed within 30 min of the end of incubation; avoid subjecting solutions to sunlight as this degrades the chromogen. Calculate the quadratic equation describing the relationship of glucose µg/mL (response variable) and absorbance (abs) at 505 nm (independent variable) using all individual absorbances (do not average within standard). The equation will have the form:

$$\text{Glucose, µg/mL} = \text{abs} \times \text{quadratic coefficient} + \text{abs} \times \text{linear coefficient} + \text{intercept}$$

Use this standard curve to calculate glucose µg/mL in test solutions. A new standard curve should be run with each glucose determination run.

(**c**) *Test samples*.—Feed and pet food amenable to drying should be dried at 55°C in a forced-air oven. Dried materials are then ground to pass the 0.5 or 1.0 mm screen of an abrasion mill or the 0.5 mm screen of a cutting mill or other mill to give an equivalent fineness of grind (to pass a 40 mesh screen). Ground, dried materials are transferred into a wide mouthed jar and mixed well by inversion and tumbling before subsampling. Semi-moist, moist, or liquid products may be homogenized, blended, or mixed to ensure homogeneity and reduced particle size (3).

E. Determination of Dietary Starch

The analyses for free glucose and enzymatically released glucose + free glucose may be performed in separate analytical runs. For flow of assay, *see* Figure **2014.10**.

(*1*) Accurately weigh two test portions (W_E, W_F) of 100 to 500 mg each of dried test samples or 500 mg semi-moist, moist, or liquid samples (for all samples, use ≤500 mg, containing

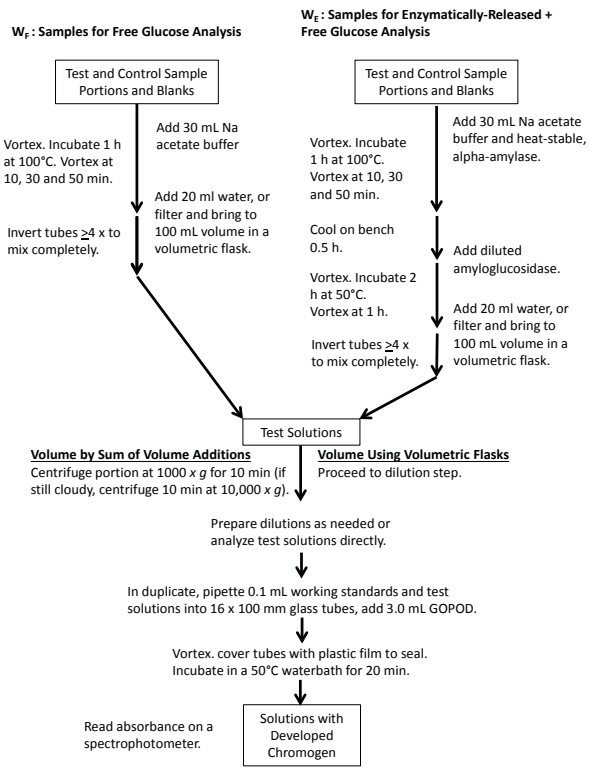


Figure 2014.10. Flow chart of the dietary starch assay.

≤100 mg dietary starch; use 500 mg for samples containing <2% dietary starch) into screw-cap glass tubes. Test portion W_E is for the analysis of enzymatically released glucose and W_F is for the determination of free glucose. In addition to unknowns, weigh test portions (W_E, W_F) of D-glucose and purified corn starch, which serve as quality control samples **C**(**g**). Also include two tubes with no test portion to serve as reagent blanks per each analytical run for free glucose or enzymatically released glucose + free glucose.

(*2*) Dispense 30 mL of 0.1 M sodium acetate buffer, **C**(**a**), into each tube.

(*3*) To tubes with test portions designated W_E and to each of the reagent blanks to be used with analysis of enzymatically released glucose + free glucose, add a volume of heat-stable, α-amylase, **C**(**b**), to deliver ca 1800 to 2100 liquefon units or 8200 to 8300 BAU of enzyme activity (typically 0.1 mL enzyme as purchased); do not add the amylase to W_F and to the reagent blanks to be used with free glucose determinations. Cap tubes and vortex to mix.

Note: Vortex tube so that the solution column extends to the cap, washing the entire interior of the tube and dispersing the test portion.

(*4*) Incubate all tubes for 1 h at 100°C in a forced-air oven, vortexing tubes at 10, 30, and 50 min of incubation.

(*5*) Cool tubes at ambient temperature on bench for 0.5 h. At this point, separate tubes designated for free glucose analysis (tubes containing W_F test portions and reagent blanks with no enzyme) from the rest of the run. Those designated for free glucose should skip steps (*6*) and (*7*) and continue with steps (*8*)–(*13*).

(*6*) Add 1 mL of diluted amyloglucosidase solution, **C**(**c**), to W_E test and quality control samples and reagent blanks. Vortex tubes.

(*7*) Incubate tubes for 2 h in a water bath at 50°C, vortexing at 1 h of incubation.

(*8*) Add 20 mL water to each tube. Cap and invert at least four times to mix completely. Proceed immediately through steps (*9*)–(*13*).

(*9*) (*a*) *Volume by sum of volume additions*.—Transfer ca 1.5 mL test sample solutions to microcentrifuge tubes, and centrifuge at 1000 × *g* for 10 min. If the sample remains cloudy after centrifugation, centrifuge an additional 10 min at 10 000 × *g* to clarify the solution before proceeding. Solutions may increase in temperature during centrifugation; allow centrifuged solutions to come to room temperature before preparing dilution.

(*b*) *Volume using volumetric flasks*.—Quantitatively transfer test sample solutions with filtration through a hardened paper filter with 22 µm retention and rinses with water to 100 mL volumetric flasks.

(*10*) Prepare dilutions as needed with distilled or deionized water. Solutions from control samples and test samples estimated to give greater than 1000 µg glucose/mL concentrations of free and released glucose should be diluted 1 in 10 if processed as in (*9*)(*a*) or 1 in 5 if processed as in (*9*)(*b*). Reagent blanks should be diluted to provide solutions with the same dilutions as used with the test solutions, so that the diluted reagent blank solutions can be used to make corrections for similarly diluted test solutions. Dilutions may be prepared using volumetric flasks or by accurate pipetting. If done by pipetting, use a minimum of 0.5 mL test sample or control solution to minimize the impact of variation in pipetting small volumes.

(*11*) Pipet 0.1 mL in duplicate of glucose working standard solutions (0, 250, 500, 750, and 1000 µg/mL glucose), **C**(**f**), and reagent blank, quality control sample, and test sample solutions into the bottoms of 16 × 100 mm glass test tubes using two tubes/solution. Add 3.0 mL GOPOD reagent, **C**(**e**)(*1*), to each tube. Vortex tubes. Place tubes in a rack and cover with plastic film to seal.

Note: Alternative to the use of the GOPOD method, proceed with alternate glucose determination method, **C**(**e**)(*2*), for measurement of glucose in working standards, reagent blank, control sample, and test sample solutions.

(*12*) Incubate in a 50°C water bath for 20 min.

(*13*) Set spectrophotometer to measure absorbance at 505 nm. After the incubation is complete, zero the spectrophotometer with the GOPOD-reacted 0 µg/mL working standard solution. Read absorbances of remaining GOPOD-reacted working standard solutions, and reagent blank, control sample, and test sample solutions. All reacted solutions must be read within 30 min of the end of the GOPOD incubation. The duplicate absorbance values are averaged for each reagent blank, test sample, and control sample solution and used in *Calculations*.

F. Calculations

Determine the quadratic equation that fits the absorbances of the working standard solutions. The absorbance values, A_{CF} or A_{CE}, are the independent variables (X), and actual glucose concentrations are the dependent variables (Y). Individual absorbance values of the working standard solutions, not averages, are used. The equation has the form:

$$\mu g\ Glucose/mL = (A_{CF\ or\ CE}^{\ 2} \times Q + A_{CF\ or\ CE} \times S + I)$$

Calculate dietary starch content in test sample as received as follows:

$$\text{Free glucose, \%} = (A_{CF}^{\ 2} \times Q + A_{CF} \times S + I) \times V_F \times DF_F \times 1/1\,000\,000 \times 1/W_F \times 162/180 \times 100$$

$$\text{Dietary starch, \%} = [(A_{CE}^{\ 2} \times Q + A_{CE} \times S + I) \times V_E \times DF_E \times 1/1\,000\,000 \times 1/W_E \times 162/180 \times 100] - \text{free glucose \%}$$

where subscript F represents values for samples analyzed for free glucose and subscript E represents values for samples treated with amylase and amyloglucosidase; A_{CF}, A_{CE} = absorbance of reaction solutions minus the absorbance of the appropriately diluted reagent blank, values are averages of the two replicates for each test solution; Q = quadratic slope term, S = linear slope term, and I = intercept of the standard curve to convert absorbance values to µg glucose/mL; V_F, V_E = final sample solution volume, ca 50.0 mL for V_F and 51.1 mL for V_E if done by summation of volumetric additions, otherwise, by size of volumetric flask used; DF = dilution factor, e.g., 0.5 mL sample solution diluted into 5.0 mL = 5.0/0.5 = 10; 1 g/1 000 000 µg = conversion from µg to g; W_E, W_F = test portion weight, as received; 162/180 = factor to convert from measured glucose as determined, to anhydroglucose, as occurs in starch.

If test samples are run in duplicate portions, the free glucose % in the dietary starch equation is the average free glucose % value determined for the test sample.

References: (1) Food Chemicals Codex (2014) 9th Ed., The United States Pharmacopeial Convention, Rockville, MD, USA, Appendix V, Enzyme Assays, α-Amylase Activity (Bacterial), pp 1392–1393

(2) McCleary, B.V., Bouhet, G., & Driguez, H. (1991) *Biotechnol. Tech.* **5**, 255–258

(3) Thiex, N., Novotny, L., Ramsey, C., Latimer, G., Torma, L., & Beine, R. (2000) *Guidelines for Preparing Laboratory Samples*, Association of American Feed Control Officials, Inc., Champaign, IL, USA

J. AOAC Int. **98**, 397(2015)
DOI: 10.5740/jaoacint.15-012

Posted: April 30, 2015

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-29 13:22:31
Please indicate your perspective	Method Developer
AOAC Official Methods Number (XXXX.XX)	2014.10
Method Name or Manuscript Title	Dietary Starch in Animal Feeds and Pet Food
Please include the manuscript publication reference1if available. (OMA methods have manuscripts unless otherl ise noted in the OMA)	Hall, M. B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method. Collaborative study. J. AOAC Int. 98:397-409.
x pload documentation to support the Method Applicability gnformation	2015 Hall JAOACI Starch Collab Study.pdf
Please attach any documentation to support your feedbac, 1if available. (The ma"imum file siwe is ?2M3 . Multiple files may be attached.)	2009 JAOACI GOP method eval.pdf 2009 JAOACI starch method comp.pdf 2015 Higgs CNCPS library.pdf
Would you li, e to provide comment(s) or feedbac, on additional methods4 This form contains feedbac, for three (L) methods. / ou may nol provide feedbac, on tl o (?) additional methods.	No, thank you!
Name	Mary Beth Hall
OrQaniwation	USDA - Agricultural Research Service
56mail Address	marybeth.hall@ars.usda.gov

FOOD COMPOSITION AND ADDITIVES

Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method: Collaborative Study

MARY BETH HALL
U. S. Department of Agriculture–Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Dr, Madison, WI 53706

Collaborators: J. Arbaugh; K. Binkerd; A. Carlson; T. Doan; T. Grant; C. Heuer; H. D. Inerowicz; B. Jean-Louis; R. Johnson; J. Jordan; D. Kondratko; E. Maciel; K. McCallum; D. Meyer; C. A. Odijk; A. Parganlija-Ramic; T. Potts; L. Ruiz; S. Snodgrass; D. Taysom; S. Trupia; B. Steinlicht; D. Welch

Starch, glycogen, maltooligosaccharides, and other α-1,4- and α-1,6-linked glucose carbohydrates, exclusive of resistant starch, are collectively termed "dietary starch". This nutritionally important fraction is increasingly measured for use in diet formulation for animals as it can have positive or negative effects on animal performance and health by affecting energy supply, glycemic index, and formation of fermentation products by gut microbes. AOAC Method 920.40 that was used for measuring dietary starch in animal feeds was invalidated due to discontinued production of a required enzyme. As a replacement, an enzymatic-colorimetric starch assay developed in 1997 that had advantages in ease of sample handling and accuracy compared to other methods was considered. The assay was further modified to improve utilization of laboratory resources and reduce time required for the assay. The assay is quasi-empirical: glucose is the analyte detected, but its release is determined by run conditions and specification of enzymes. The modified assay was tested in an AOAC collaborative study to evaluate its accuracy and reliability for determination of dietary starch in animal feedstuffs and pet foods. In the assay, samples are incubated in screw cap tubes with thermostable α-amylase in pH 5.0 sodium acetate buffer for 1 h at 100°C with periodic mixing to gelatinize and partially hydrolyze α-glucan. Amyloglucosidase is added, and the reaction mixture is incubated at 50°C for 2 h and mixed once. After subsequent addition of water, mixing, clarification, and dilution as needed, free + enzymatically released glucose are measured. Values from a separate determination of free glucose are subtracted to give values for enzymatically released glucose. Dietary starch equals enzymatically released glucose multiplied by 162/180 (or 0.9) divided by the weight of the as received sample. Fifteen laboratories that represented feed company, regulatory, research, and commercial feed testing laboratories analyzed 10 homogenous test materials representing animal feedstuffs and pet foods in duplicate using the dietary starch assay. The test samples ranged from 1 to 70% in dietary starch content and included moist canned dog food, alfalfa pellets, distillers grains, ground corn grain, poultry feed, low starch horse feed, dry dog kibbles, complete dairy cattle feed, soybean meal, and corn silage. The average within-laboratory repeatability SD (s_r) for percentage dietary starch in the test samples was 0.49 with a range of 0.03 to 1.56, and among-laboratory repeatability SDs (s_R) averaged 0.96 with a range of 0.09 to 2.69. The HorRat averaged 2.0 for all test samples and 1.9 for test samples containing greater than 2% dietary starch. The HorRat results are comparable to those found for AOAC Method 996.11, which measures starch in cereal products. It is recommended that the dietary starch method be accepted for Official First Action status.

Received August 5, 2014.
The method was approved by the Expert Review Panel for Dietary Starch.

The Expert Review Panel for Dietary Starch invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

Mention of any trademark or proprietary product in this paper does not constitute a guarantee or warranty of the product by the USDA or the Agricultural Research Service and does not imply its approval to the exclusion of other products that also may be suitable.

Corresponding author's e-mail: marybeth.hall@ars.usda.gov
DOI: 10.5740/jaoacint.15-012

Starch is an important, frequently analyzed component of animal feedstuffs. It can have substantial positive effects on animal performance and potential undesirable effects on glycemic response and animal health (1). AOAC *Official Method*SM **920.40** for starch in animal feeds (2) is no longer valid because of discontinued production of the enzyme "Rhozyme-S" (Rohm and Haas, Philadelphia, PA) specified in the procedure. Accordingly, another approved method for starch in animal feeds is needed. Additionally, new terminology is needed to define "starch" to more accurately describe the nutritionally relevant fraction of interest, and the definition can be used to specify the analysis.

Starch has long been defined as a natural vegetable polymer consisting of long linear unbranched chains of α-1,4-linked D-glucose units (amylose) and/or long α-1,6-branched chains of α-1,4-linked glucose units (amylopectin; 3). However, the amylases and amyloglucosidases that specifically hydrolyze the linkages in plant starch also hydrolyze those same linkages in glycogen from animal (4) or microbial (5) sources and in maltooligosaccharides that are breakdown products of starch

but are not polysaccharides. Accordingly, enzymatic starch methods do not measure plant starch alone (6), unless animal and microbial ingredients and the feedstuffs that contain them are excluded from analysis. From a nutritional standpoint, inclusion of glycogen, starch, and maltooligosaccharides more completely describes the pool of carbohydrate that is potentially available to digestion by salivary or small intestinal amylases or amyloglucosidases (7), but the pool can not be called "starch" because that term is well established as referring to a plant polysaccharide.

Recognizing the aim of nutritional characterization, the Laboratory Methods & Services Committee of the Association of American Feed Control Officials with involvement of researchers and industry arrived at a definition for "Dietary Starch": An alpha-linked-glucose carbohydrate of or derived from plants, animals, or microbes from which glucose is released through the hydrolytic actions of purified α-amylases and amyloglucosidases that are specifically active only on α-(1-4) and α-(1-6) linkages in feed materials that have been gelatinized in heated, mildly acidic buffer. Its concentration in feed is determined by enzymatically converting the α-linked glucose carbohydrate to glucose and then measuring the liberated glucose. This definition encompasses plant starch, glycogen, maltooligosaccharides, and maltose/isomaltose. The use of mildly acidic buffer for the gelatinization excludes the use of alkali or dimethyl sulfoxide and, thus, excludes resistant starch from inclusion in the dietary starch fraction.

The proposed dietary starch method avoids known analytical defects and allows handling of diverse physical forms of samples. It is based on an assay published by Bach Knudsen (8) that was slightly modified to improve use of laboratory resources, reduce run time, and maintain starch recovery (9). It is similar in chemistry to AOAC Method **996.11** (10), but differs in the buffer used and in sample handling procedures and gave a greater recovery of starch (9). Specific to the dietary starch assay, all enzymatic reactions are carried out in an acidic buffer that improves recovery by limiting the production of maltulose, an isomerization product produced at more neutral pH (11). Maltulose is resistant to enzymatic hydrolysis and reduces starch recovery. The use of a screw cap tube as a reaction vessel allows for more vigorous mixing, which is useful for all types of feed materials but may be essential for those that clump, are moist, or do not behave like dry, ground powders. Although enzymes used in development of the method will be listed, learning from the loss of AOAC Method **920.40** (2), this assay will not be set to use specific commercial enzymes but rather enzymes with specific activity that give desired results under the conditions of the method. The detection method specified is a colorimetric glucose oxidase-peroxidase method based on an assay developed by Karkalas (12), but recommendations are made to use other approved chromatographic analyses if interferences such as antioxidants are present.

Collaborative Study

Method Performance Parameters and Optimization

The performance parameters of the dietary starch procedure were investigated by the Study Director, who developed the method evaluated in this study. The following factors were evaluated:

(*1*) *Repeatability.*—As tested previously in a single laboratory, the SDs of within laboratory replicates for dietary starch analysis of food and feed substrates were low (dietary starch mean = 46.9%, s_r = 0.48%; dry matter basis; 9).

(*2*) *LOD.*—LOD for the dietary starch assay was calculated from absorbance values as the mean reagent blank value + 3 × SD (13). The means and SD were calculated for the absorbances of duplicate readings for seven undiluted with-enzyme reagent blanks from six separate assay runs. For each reagent blank, the value of the mean absorbance + 3 SD was used in the glucose standard curve determined for that run to calculate the detected glucose value. This value was multiplied by the final reaction volume (51.1 mL), by 162/180 to convert glucose to a starch basis, and converted to g. The calculated dietary starch LOD are 0.3% of sample weight based on analysis of a 100 mg test portion.

(*3*) *Accuracy/recovery.*—Recovery of pure corn starch was determined on samples analyzed singly in five separate analytical runs and in duplicate in an additional run. The average recovery ± SD was 99.3 ± 0.8% on a dry matter basis. In the collaborative study, the average dietary starch value for the control corn starch sample was 89.9 ± 3.7% on an as received basis with an estimated actual value of 89.4%.

(*4*) *Linearity.*—Linearity of the dietary starch assay was evaluated on a dry matter basis using purified corn starch samples weighing 25, 50, 75, and 100 mg analyzed on 3 separate days. The effect of starch amount tended to have a linear effect on recovery (P = 0.07), but the difference was small at a maximum of 2 percentage units between the highest and lowest recoveries. The least squares means ± SD for recovery were 101.9 ± 1.7, 99.9 ± 0.2, 100.3 ± 0.4, and 100.0 ± 0.7% for 25, 50, 75, and 100 mg of corn starch, respectively.

(*5*) *Specificity.*—The dietary starch method gave very low values (mean ± SD) for sucrose (0.17 ± 0.00% of sample dry matter), α-cellulose (0.03 ± .02% of air dried sample), and isolated oat beta-glucan (0.31 ± 0.09% of air dried sample), indicating that run conditions and enzyme preparations used did not appreciably hydrolyze these feed components. Sucrose, in particular, has been shown to interfere with starch analysis (14), likely due to side activity of the enzyme preparations used. Use of separate free glucose determinations allows correction for free glucose and background absorbance associated with each sample. The final detection method, the glucose oxidase – peroxidase (GOPOD) method, is specific for glucose, which limits interference from other carbohydrates.

(*6*) *Interference.*—Antioxidants can depress glucose detection in the GOPOD assay. Addition of ascorbic acid as a model antioxidant gave a linear decrease in absorbance at additions of greater than 10 μmoles of ascorbic acid (15). The effect was relatively small up to 10 μmol of ascorbic acid. Investigations into the antioxidant content of foodstuffs (16) showed that most of the high starch or leafy vegetable foods had hydrophilic antioxidant values that would be equivalent to less than 10 μmoles of ascorbic acid/0.1 g of dry matter. Exceptions included foods high in phenolic compounds (e.g., beets and red sorghum grain with antioxidant content approximately equivalent to 23 and 14 μmol ascorbic acid, respectively). Because of the interference in the GOPOD assay, another method for measuring glucose should be considered for feeds or foods exceeding 10 to 20 μmol of hydrophilic antioxidant/0.1 g of test sample dry matter.

(*7*) *Use of quadratic standard curves.*—The standard curves in the GOPOD assay are slightly nonlinear, and this is normal for this assay within the glucose concentrations commonly used (15). The linear equations describing glucose standard curves had R^2 of nearly 1.0 (0.9998 to 1.0) suggesting a very good fit to the linear form but the intercepts were not 0. Thus, when the standard curves were used to predict glucose concentrations of the standard solutions used to produce them, the predicted values frequently differed slightly from the expected values. It was determined that a quadratic form fit the standard curves better than a linear form based on significance of the quadratic term in the regression equation, the reduction in the root mean squared error of the standard curve and the relative decrease in residual sums of squares (residual = observed minus predicted) between the linear and quadratic equations, and evaluation of the residual versus predicted value plots (15). Other nonlinear forms were not explored.

(*8*) *Determination of final volume by summation of liquid additions.*—The method uses summing of added reagent volumes or use of volumetric flasks to give the final volume of test solutions before dilution. Total volumes of test solutions and dilutions can be determined by summing of added volumes if accurately quantitative volumetric pipets and dispensers are used to add reagents. An evaluation of summation of volumes and determination of final volume by weight and density showed no difference in recovery of glucose ($P = 0.21$) or of corn starch ($P = 0.62$) analyzed with the dietary starch assay. The density of test sample solutions and reagent blanks appears to be quite consistent (0.999 g/mL, SD = 0.002, $n = 120$ from 16 analysis runs over 16 months). Accuracy of reagent additions can be determined by the final weight of total added liquid [(weight of tube + test sample + liquid) minus (weight of tube + test sample)]. The weights of total added liquid are 49.9 and 51.0 g for the portions of the assay run without or with enzyme additions, respectively. The deviations from these values should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. Alternatively, after the addition of water, test solutions can be quantitatively transferred with filtration through Whatman (Florham Park, NJ) 54 or equivalent paper into 100 mL volumetric flasks and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

(*9*) *Ease of use/efficiency.*—The method has the advantage that all reagent additions are made to samples in tubes that can be handled in racks. It does not require transfer of sample until the final dilution and measurement of glucose. Vortexing of the sealed tubes rinses the entire interior of the tube with solution, thus minimizing the possibility that test samples will escape contact with reagents. Studies verified the acceptability of using the same temperature for the amyloglucosidase digestion and glucose analysis incubations (15), which allowed more economic use of laboratory resources.

(*10*) *Use of control samples.*—The use of glucose and corn starch as control samples allows evaluation of quantitative recovery, and starch allows evaluation of quantitative recovery and efficacy of the assay.

(*11*) *Evaluation of enzymes for suitability.*—It is essential that the enzymes and run conditions used release only glucose bound by α-1,6- and α-1,4-linkages and give close to 100% recovery of corn starch. Sucrose is the most common interfering carbohydrate encountered in feedstuffs (14) typically due to its hydrolysis through side activity of the enzyme preparations used. Though the run conditions used will not hydrolyze sucrose, commonly available enzyme preparations have activity that can and are thus unsuitable for this assay. Analysis of glucose, corn starch, and sucrose with candidate enzymes should give values (mean ± SD) of glucose 90 ± 2%, starch 100 ± 2%, and sucrose 0.7 ± 0.3% on a dry matter basis. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%) or background absorbance readings will interfere with test sample measurements.

(*12*) *Method of glucose detection.*—The dietary starch protocol specifies use of an enzymatic-colorimetric assay that has been found to be very precise (15). However, it also allows use of other AOAC-approved glucose-specific assays that have been proven in laboratory validation to be appropriate for the dietary starch assay. On this basis, qualifying assays that are devoid of interference and are, thus, more suitable for use on specific matrixes, or are preferred in a given laboratory may be used.

Collaborating Laboratories

The 15 laboratories that participated in the study represented eight regulatory laboratories, three commercial feed testing laboratories, two feed company laboratories, and two research laboratories. One each of research, commercial feed testing, and regulatory laboratories that expressed interest in participating did not complete the study. Participating laboratories received no compensation. Collaborators were provided with blind test samples, control glucose and corn starch, thermostable α-amylase (Multifect AA 21L, Genencor International, Rochester, NY), amyloglucosidase (E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland), glucose standards, electronic data sheets, and larger reaction tubes if needed. They were required to prepare the GOPOD reagent, perform the dietary starch assay as written, analyze test samples in duplicate, and provide comments and detailed result forms containing both raw and calculated data describing their analyses of three blind familiarization test materials, 10 blind collaborative study test materials, and control samples for dietary starch.

Materials

Test materials selected for the collaborative study covered a wide range of dietary starch contents, ranging from 1 to 69% on an as-received basis and derived from single batches of manufactured and commodity feedstuffs used with different animal species. The test sample grinding and homogenizing methods used were designed to produce materials that would pass a 40 mesh screen. By virtue of their diverse handling characteristics, a number of different methods were used to prepare the samples for analysis. Corn silage, poultry feed, low starch horse feed, and alfalfa pellets were ground through the 6 mm screen of a cutting mill (Pulverisette 19, Fritsch GmbH, Idar-Oberstein, Germany) and then processed through the 0.5 mm screen of a centrifugal mill (ZM200 with 12 blade knife, Retsch GmbH, Haan, Germany). Dry corn, soybean meal, and distillers grains were ground to pass the 0.5 mm screen of a centrifugal mill (ZM200 with 12 blade knife), as

Table 1. Homogeneity of dietary starch for four sample sets of each test material[a]

Material	*n*	Mean, %	s_r	s_R	RSD_r, %	RSD_R,%	2.8 × s_r	2.8 × s_R	HorRat
Moist canned dog food	4	1.58	0.01	0.02	0.86	1.06	0.04	0.05	0.29
Low starch horse feed	4	7.17	0.06	0.11	0.85	1.56	0.17	0.31	0.53
Dry ground corn	4	72.70	0.34	0.34	0.46	0.46	0.95	0.95	0.22
Complete dairy feed	4	28.38	0.10	0.40	0.34	1.40	0.27	1.11	0.58
Soybean meal	4	1.17	0.05	0.05	3.94	3.94	0.13	0.13	1.01
Distillers grains	4	4.23	0.06	0.06	1.37	1.37	0.16	0.16	0.43
Pelleted poultry feed	4	28.50	0.32	0.32	1.14	1.14	0.91	0.91	0.47
Corn silage	4	41.15	1.06	1.06	2.58	2.58	2.98	2.98	1.13
Dog kibble, dry	4	27.82	0.95	1.01	3.43	3.64	2.67	2.83	1.50
Alfalfa pellets	4	1.46	0.04	0.05	3.06	3.18	0.12	0.13	0.84

[a] s_r = SD of repeatability within sample; s_R = SD within and among sample sets; RSD_r = repeatability SD; RSD_R = reproducibility SD.

was the textured dairy complete feed but with dry ice used in the grinding of this sample. Dog kibble was ground with a kitchen processing mill (Assistent, MagicMill, Upper Saddle River, NJ) and further processed through a blending mill (1095 Knifetec sample mill, Foss Tecator, Höganäs, Sweden). The moist, canned dog food was homogenized with a commercial blender (Waring laboratory blender, 14-509-66, Fisher Scientific, Pittsburgh, PA). Dry ground test samples were subsampled using a rotary splitter (Laborette 27, Fritsch GmbH) and stored at –20°C in vacuum sealed bags (3.5 mil nylon polyethylene standard barrier vacuum bag, DCE, Inc., Springville, CA) until shipment. Homogenized moist dog food was transferred to individual sealed plastic bags (Whirl-Pak 58 mL, B01009WA, Nasco, Fort Atkinson, WI) and stored at –20°C. Test sample weights/bag were approximately 20 g for dried ground samples and 25 g of homogenized moist dog food.

For the collaborative study, individual test samples were labeled with a letter. Dry test samples and control samples were packed together in a sealed plastic bag. The homogenized moist dog food test sample and enzymes were packaged in an insulated container with a frozen ice pack. Materials were shipped overnight to the laboratories with directions to place the homogenized moist dog food test sample in the freezer until analysis. That sample was to be thawed overnight at 4°C, and all analyses in the dietary starch procedure were to be performed on it on the following day; no such limitations were placed on analyses of the dry test samples.

As per the example of Mertens (17), dietary starch analyses in duplicate of four randomly selected samples of each test material were used to evaluate random variation within and among samples. In this application, the SD of repeatability within sample (s_r) and SD of reproducibility among laboratories (s_R) calculated using the AOAC spreadsheet designed for evaluating collaborative studies represent the variation within and between separate samples of test materials as tested in the Study Director's laboratory. The s_r and s_R were similar within each sample, indicating that the prepared test samples were homogenous (Table 1). The HorRat values for corn silage and dog kibble were greater than 1.1. As concluded in a similar evaluation (17), these results suggest that these samples were less homogenous or for some reason more difficult to analyze for dietary starch than the other samples. For the dog kibble test sample, small dark particles that did not dissolve or degrade and had the coloration of one form of kibble present in the original unground material were visible in the acetate buffer during incubations.

Statistical Analyses

Data from all laboratories were reviewed for data entry and calculation errors before statistical evaluation, and results were reverified if values were identified as outliers. Ranking scores (18) were used to identify laboratories that were outliers across all materials. Data from the one such identified laboratory were excluded from further data analysis.

The AOAC INTERNATIONAL Interlaboratory Study Workbook for Evaluation of Blind Duplicates (Version 2.0, 2006) spreadsheet was used to evaluate data from the collaborative study and from the homogeneity test performed in the Study Director's laboratory.

AOAC Official Method 2014.10
Dietary Starch in Animal Feeds and Pet Food
Enzymatic-Colorimetric Method
First Action 2014

(Applicable for the determination of dietary starch in forages, grains, grain by-products, dry, semi-moist, and moist pet food products, and mixed feeds that range in concentration from 1 to 100%.)

Caution: Acetic acid is flammable in both liquid and vapor forms. It can cause severe skin burns and eye damage and is toxic if inhaled. Avoid breathing fumes. Wear protective gloves, clothing, and eye and face protection.

α-Amylase and glucose oxidase are respiratory sensitizers, which may cause allergy or asthma symptoms. Avoid breathing dust. Amylase preparations can cause allergic reactions in hypersensitive individuals. Avoid inhaling aerosols or dusts.

Benzoic acid causes serious eye damage and respiratory irritation. Avoid breathing dust and mist. Wear eye protection.

Phenol can be toxic and cause severe burns and eye damage. It is suspected of causing genetic defects and may cause damage to organs. Do not breathe dust or fumes. Wear protective gloves, clothing, eye protection, and face protection.

See Table **2014.10** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Ground or homogenized animal feed and pet food test portions are mixed with acetic acid buffer and heat-stable α-amylase and incubated at 100°C for 1 h with periodic mixing to gelatinize and partially hydrolyze the starch. After cooling, amyloglucosidase is added and the test mixture is incubated for 2 h at 50°C. The digested mixture is clarified, diluted as needed, and glucose detected in the resulting test solution using a colorimetric glucose oxidase-peroxidase (GOPOD) method that is sensitive and specific to glucose. Free glucose is measured simultaneously or in a separate analytical run for each test sample by carrying a second test portion of each test sample through the procedure omitting enzymes and incubating at 100°C for 1 h with periodic mixing. Dietary starch is determined as 0.9 times the difference of glucose in the digested test portion minus free glucose in the undigested test portion.

B. Apparatus

(**a**) *Grinding mill*.—Mills such as an abrasion mill equipped with a 1.0 to 0.5 mm screen, or a cutting mill with 0.5 mm screen, or other appropriate device to grind test samples to pass a 40 mesh screen.

(**b**) *Homogenizer, blender, or mixer*.—To provide homogenous suspension of canned pet food, liquid animal feed, semi-moist pet food, and other materials containing less than 85% dry matter.

(**c**) *Bench centrifuge or microcentrifuge*.—Capable of centrifuging at 1000 × *g* to 10 000 × *g*.

(**d**) *Water bath*.—Capable of maintaining 50 ± 1°C.

(**e**) *Vortex mixer*.

(**f**) *pH meter*.

(**g**) *Stop clock timer (digital)*.

(**h**) *Top-loading balance*.—Capable of weighing accurately to ±0.01 g.

(**i**) *Analytical balance*.—Capable of weighing accurately to ±0.0001 g.

(**j**) *Laboratory ovens*.—With forced-convection; capable of maintaining 100 ± 1°C for carrying out incubations.

(**k**) *Spectrophotometer*.—Capable of operating at absorbances of 505 nm.

(**l**) *Pipets*.—Capable of delivering 0.1 and 1.0 mL; with disposable tips.

(**m**) *Positive-displacement repeating pipet*.—Capable of accurately delivering 0.1, 1.0, and 3.0 mL.

(**n**) *Dispenser*.—1000 mL or greater capacity; capable of accurately delivering 20 and 30 mL.

(**o**) *Glass test tubes*.—16 × 100 mm.

(**p**) *Glass tubes*.—25 × 200 mm, with polytetrafluoroethylene (PTFE)-lined screw caps or comparable tubes to hold 51.1 mL and allow for adequate mixing when sealed.

(**q**) *Plastic film*.—Or similarly nonreactive material.

(**r**) *Magnetic stir plate*.

(**s**) *Glass fiber filter*.—With 1.6 µm retention.

(**t**) *Hardened filter paper*.—With 22 µm retention.

C. Reagents

Note: Use high-quality distilled or deionized water for all water additions.

(**a**) *Acetate buffer (100 mM, pH 5.0)*.—Weigh 6.0 g or pipet 5.71 mL glacial acetic acid and transfer immediately to a flask; quantitatively transfer weighed acid with H_2O rinses. Bring volume to ca 850 mL. While stirring solution on a magnetic stir plate, adjust pH to 5.0 ± 0.1 with 1 M NaOH solution. Dilute to 1 L with H_2O. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**b**) Heat-stable α-amylase solution.—Liquid, heat-stable, α-amylase (examples: Product Termamyl 120 L, Novozymes North America, Franklinton, NC; Product Multifect AA 21L, Genencor International, Rochester, NY; origin: Bacillus licheniformis, or equivalent). Should not contain greater than 0.5% glucose. pH optima must include 5.5–5.8.

Based on Bacterial Amylase Unit (BAU) method.—Approximately 83000 BAU/mL of concentrated enzyme (1 BAU is defined as the amount of enzyme that will dextrinize starch at the rate of 1 mg/min at pH 6.6 and 30 ± 0.1°C; 19). If modifications of volume delivered are necessary due to enzymatic activity of the enzyme used, the volume used per test portion should deliver approximately 8300 ± 20 BAU (19).

Table 2014.10. Method performance for determination of dietary starch in feeds

Material	No. of labs	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	r[a]	R[b]
Moist canned dog food	11	1.54	0.03	0.09	2.21	5.99	0.10	0.26
Low starch horse feed	13	7.02	0.23	0.36	3.32	5.19	0.65	1.02
Dry ground corn	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54
Complete dairy feed	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48
Soybean meal	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31
Distillers grains	13	4.11	0.11	0.20	2.67	4.94	0.31	0.57
Pelleted poultry feed	13	28.24	0.73	1.34	2.58	4.76	2.04	3.76
Corn silage	13	39.04	0.80	1.88	2.05	4.82	2.24	5.27
Dog kibble, dry	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46
Alfalfa pellets	13	1.38	0.12	0.13	8.61	9.69	0.33	0.38

[a] $r = 2.8 \times s_r$.

[b] $R = 2.8 \times s_R$.

The enzymes should be of a purity meeting the specifications listed in *Official Method*SM **991.43** (20), but as modified below for application in the assay for dietary starch. The enzyme preparation used must be validated within laboratory to verify efficacy, as well as lack of interference. Recommended validation: analyze 0.1 g test portions of purified glucose, sucrose, and purified corn starch with the enzymatic portion of the dietary starch assay and using a free glucose value of zero in calculations. Analyses with candidate enzyme should give values of [mean ± standard deviation (SD)] glucose: 90 ± 2%, starch: 100 ± 2%, and sucrose: 0.7 ± 0.3% on a dry matter basis. To test for interference from release of glucose from fiber carbohydrates, analyze 0.1 g test portions of α-cellulose and barley β-glucan that are not contaminated with free glucose with the enzymatic portion of the dietary starch assay. Recovery of these substrates should be less than 0.5% on a dry matter basis (20). Use AOAC approved methods for determination of dry matters of the samples. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%), or background absorbance readings will interfere with test sample measurements.

(**c**) *Diluted amyloglucosidase solution.*—Dilute concentrated amyloglucosidase with 100 mM sodium acetate buffer, **C**(**a**), to give 1 mL of solution per test portion with 2 to 5 mL excess. Add 1/3 of needed buffer to an appropriately sized graduated cylinder. Pipet concentrated amyloglucosidase into buffer, rinsing tip by taking up and expelling buffer in the graduated cylinder. Bring to desired volume with additional buffer. Cap cylinder with plastic film and invert cylinder repeatedly to mix. The concentrated amyloglucosidase used should not contain greater than 0.5% glucose, and should have a pH optimum of 4.0 and pH stability between 4.0–5.5 (example of concentrated amyloglucosidase: Product E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland; origin: *Aspergillus niger*, or equivalent).

(*1*) *Based on release of glucose from soluble starch or glycogen.*—200 U/mL (1 unit of enzyme activity is defined as the amount of enzyme required to release 1 μmole glucose/min at pH 4.5 and 40°C; 21).

(*2*) *Based on p-nitrophenyl-β-maltoside method.*—13 units/mL (1 unit is defined as the amount of enzyme required to release 1 μmole *p*-nitrophenol from *p*-nitrophenyl-β-maltoside/min at pH 4.5 and 40°C; 22).

The enzyme used must be validated within laboratory to verify efficacy as well as lack of interference. Use the same validation procedure as described for heat-stable α-amylase, **C**(**b**).

(**d**) *Benzoic acid solution (0.2%).*—Weigh 2.0 g benzoic acid (solid, ACS reagent, >99.5% purity) and add to a flask. Bring flask to 1 L volume with H_2O. Add magnetic stir bar, stopper flask, and allow to stir overnight to dissolve benzoic acid. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**e**) *GOPOD reagent.*—(*1*) *Mixture of glucose oxidase, 7000 U/L, free from catalase activity; peroxidase, 7000 U/L; and 4-aminoantipyrine, 0.74 mM.*—Prepare by dissolving 9.1 g Na_2HPO_4 (dibasic, anhydrous) and 5.0 g KH_2PO_4 in ca 300 mL H_2O in a 1 L volumetric flask. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add 1.0 g phenol (ACS grade) and 0.15 g 4-aminoantipyrine. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add glucose oxidase (7000 U) and peroxidase (7000 U), rinse enzymes into flask with H_2O, and swirl gently to dissolve without causing excessive foaming. Bring to 1 L volume with H_2O. Seal and invert repeatedly to mix. Filter solution through a glass fiber filter with 1.6 μm retention, **B**(**s**). Store in a sealed amber bottle at ca 4°C. Reagent life: 1 month. Before use in test sample determinations, determine a standard curve for the reagent using a 5-point standard curve using **C**(**e**) and **C**(**f**) according to **D**(**b**).

(*2*) Alternatively, use another AOAC-approved glucose-specific assay that has passed in laboratory validation to accurately determine glucose concentrations of glucose standard solutions and give values equivalent to the values listed for determination of efficacy of enzymes. Recommended validation: analyze all five glucose working standard solutions and 100 mg test portions of purified glucose, purified sucrose, and purified corn starch that have been processed through the enzymatic hydrolysis portion of the dietary starch procedure and using a free glucose value of zero in calculations. The glucose values of the working standard solutions should be predicted ±6 μg glucose/mL. On a dry matter basis, the control sample glucose should give a dietary starch value (mean ± SD) of 90 ± 2%, corn starch at 100 ± 2%, and sucrose 0.7 ± 0.3%.

(**f**) *Glucose working standard solutions.*—0, 250, 500, 750, and 1000 μg/mL. Determine the dry matter of powdered crystalline glucose (purity ≥99.5%) by an AOAC-approved method. Weigh approximately 62.5, 125, 187.5, and 250 mg portions of glucose and record weight to 0.0001 g. Rinse each portion of glucose from weigh paper into a separate 250 mL volumetric flask with 0.2% benzoic acid solution, **C**(**d**), and swirl to dissolve. Bring each standard to 250 mL volume with 0.2% benzoic acid solution, **C**(**d**), to give four independent glucose standard solutions. The 0.2% benzoic acid solution, **C**(**d**), serves as the 0 μg/mL standard solution. Multiply weight of glucose by dry matter percentage and percentage purity as provided by the manufacturer in the certificate of analysis and divide by 250 mL to calculate actual glucose concentrations of the solutions. Prepare solutions at least one day before use to allow equilibration of α- and β- forms of the glucose. Standard solutions may be stored at room temperature for 6 months.

(**g**) *Internal quality control samples*.—Powdered crystalline glucose (purity ≥99.5%) and isolated corn starch. For the corn starch sample, crude protein as nitrogen content × 6.25 and ash should be determined to determine the nonprotein organic matter content of the sample. For use in recovery calculations, actual starch content of the corn starch control sample is estimated as 100% minus ash% and minus crude protein%, all on a dry matter basis. Analyze 100 mg of each sample with each batch of test samples. Glucose will allow evaluation of quantitative recovery, and starch will allow evaluation of quantitative recovery and efficacy of the assay.

(**h**) *Determination of accuracy of volume additions for use of summative volume approach*.—The method as described relies on accurate volumetric additions in order to use the sum of volumes to describe test solution volume. Accuracy of volume additions can be evaluated before the assay by the following procedure: Using 1–2 L distilled water at ambient temperature, determine the g/mL density of the water by recording the weight of three empty volumetric flasks (volumes between 50 and

100 mL), add the water to bring to volume, and weigh the flasks + water. Calculate water density g/mL as:

$$\text{Water density g/mL} = [(\text{flask + water, g}) - (\text{flask, g})]/\text{water volume mL}$$

Record the weights of five empty tubes used for the dietary starch assay. Using the ambient temperature water and the devices used to deliver the liquid volumes for the enzymatic hydrolysis portion of the assay, deliver the 30, 0.1, 1, and 20 mL volumes to each tube (total of 51.1 mL in each tube). Record the weight of each tube + water. Calculate the grams of water in each tube as:

$$\text{Water in each tube, g} = (\text{tube + water, g}) - (\text{tube, g})$$

Divide the weight of water in each tube by the determined average density of water to give the volume of water in each tube. The deviation should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. If the deviations are greater than these, after the addition of 20 mL water during the dietary starch assay, individual samples should be quantitatively transferred with filtration through hardened filter paper with a 22 µm retention, **B**(**t**), into a 100 mL volumetric flask and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

D. Preparation of Reagent Blanks, Standard Curves, and Test Samples

(**a**) *Reagent blank.*—For each assay, two reaction tubes containing only the reagents added for each method are carried through the entire procedure. Reagent blanks diluted to the same degree as samples (no dilution or diluted to the same degree as control and test samples) are analyzed. Absorbance values for the reagent blanks are subtracted from absorbance values of the test solutions prepared from test and control samples.

(**b**) *Standard curves.*—Pipet 0.1 mL of 0.2% benzoic acid solution, **C**(**d**), and nominal 250, 500, 750, and 1000 µg/mL working standard glucose solutions, **C**(**f**), in duplicate into the bottoms of 16 × 100 mm glass culture tubes. Add 3.0 mL GOPOD reagent, **C**(**e**), to each tube using a positive displacement repeating pipet aimed against wall of tube, so it will mix well with the sample. Vortex tubes. Cover tops of tubes with plastic film. Incubate in a 50°C water bath for 20 min. Read absorbance at 505 nm using the 0 µg glucose/mL standard to zero the spectrophotometer. All readings should be completed within 30 min of the end of incubation; avoid subjecting solutions to sunlight as this degrades the chromogen. Calculate the quadratic equation describing the relationship of glucose µg/mL (response variable) and absorbance (abs) at 505 nm (independent variable) using all individual absorbances (do not average within standard). The equation will have the form:

$$\text{Glucose, µg/mL} = \text{abs x quadratic coefficient} + \text{abs} \times \text{linear coefficient} + \text{intercept}$$

Use this standard curve to calculate glucose µg/mL in test solutions. A new standard curve should be run with each glucose determination run.

(**c**) *Test samples.*—Feed and pet food amenable to drying should be dried at 55°C in a forced-air oven. Dried materials are then ground to pass the 0.5 or 1.0 mm screen of an abrasion mill or the 0.5 mm screen of a cutting mill or other mill to give an equivalent fineness of grind (to pass a 40 mesh screen). Ground, dried materials are transferred into a wide mouthed jar and mixed well by inversion and tumbling before subsampling. Semi-moist, moist, or liquid products may be homogenized, blended, or mixed to ensure homogeneity and reduced particle size (23).

E. Determination of Dietary Starch

The analyses for free glucose and enzymatically released glucose + free glucose may be performed in separate analytical runs. For flow of assay, *see* Figure **2014.10**.

(*1*) Accurately weigh two test portions (W_E, W_F) of 100 to 500 mg each of dried test samples or 500 mg semi-moist, moist, or liquid samples (for all samples, use ≤500 mg, containing ≤100 mg dietary starch; use 500 mg for samples containing <2% dietary starch) into screw-cap glass tubes. Test portion W_E is for the analysis of enzymatically released glucose and W_F is for the determination of free glucose. In addition to unknowns, weigh test portions (W_E, W_F) of D-glucose and purified corn starch, which serve as quality control samples **C**(**g**). Also include two tubes with no test portion to serve as reagent blanks per each analytical run for free glucose or enzymatically released glucose + free glucose.

(*2*) Dispense 30 mL of 0.1 M sodium acetate buffer, **C**(**a**), into each tube.

(*3*) To tubes with test portions designated W_E and to each of the reagent blanks to be used with analysis of enzymatically released glucose + free glucose, add a volume of heat-stable, α-amylase, **C**(**b**), to deliver ca 1800 to 2100 liquefon units or 8200 to 8300 BAU of enzyme activity (typically 0.1 mL enzyme as purchased); do not add the amylase to W_F and to the reagent blanks to be used with free glucose determinations. Cap tubes and vortex to mix.

Note: Vortex tube so that the solution column extends to the cap, washing the entire interior of the tube and dispersing the test portion.

(*4*) Incubate all tubes for 1 h at 100°C in a forced-air oven, vortexing tubes at 10, 30, and 50 min of incubation.

(*5*) Cool tubes at ambient temperature on bench for 0.5 h. At this point, separate tubes designated for free glucose analysis (tubes containing W_F test portions and reagent blanks with no enzyme) from the rest of the run. Those designated for free glucose should skip steps (*6*) and (*7*) and continue with steps (*8*)–(*13*).

(*6*) Add 1 mL of diluted amyloglucosidase solution, **C**(**c**), to W_E test and quality control samples and reagent blanks. Vortex tubes.

(*7*) Incubate tubes for 2 h in a water bath at 50°C, vortexing at 1 h of incubation.

(*8*) Add 20 mL water to each tube. Cap and invert at least 4 times to mix completely. Proceed immediately through steps (*9*)–(*13*).

(*9*) (*a*) *Volume by sum of volume additions*.—Transfer ca 1.5 mL test sample solutions to microcentrifuge tubes, and centrifuge at 1000 x *g* for 10 min. If the sample remains cloudy after centrifugation, centrifuge an additional 10 min at

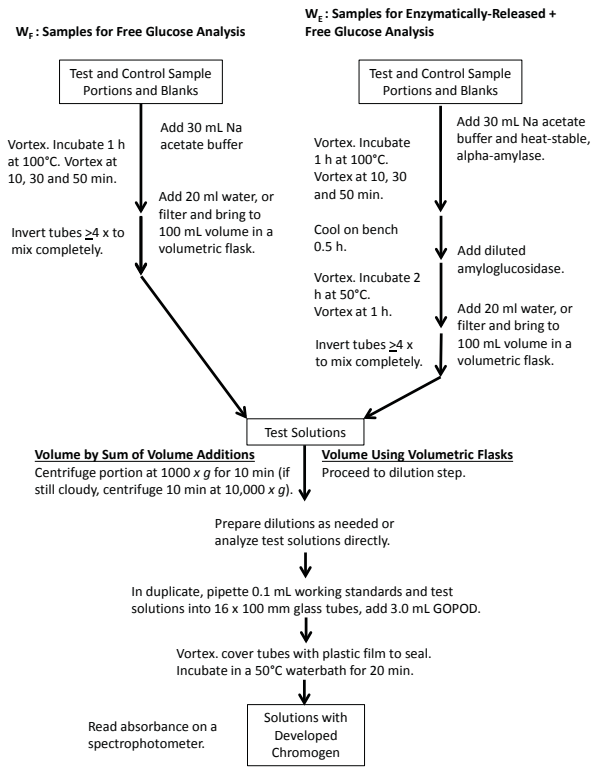


Figure 2014.10. Flow chart of the dietary starch assay.

10 000 × *g* to clarify the solution before proceeding. Solutions may increase in temperature during centrifugation; allow centrifuged solutions to come to room temperature before preparing dilution.

(*b*) *Volume using volumetric flasks*.—Quantitatively transfer test sample solutions with filtration through a hardened paper filter with 22 µm retention and rinses with water to 100 mL volumetric flasks.

(*10*) Prepare dilutions as needed with distilled or deionized water. Solutions from control samples and test samples estimated to give greater than 1000 µg glucose/mL concentrations of free and released glucose should be diluted 1 in 10 if processed as in (*9*)(*a*) or 1 in 5 if processed as in (*9*)(*b*). Reagent blanks should be diluted to provide solutions with the same dilutions as used with the test solutions, so that the diluted reagent blank solutions can be used to make corrections for similarly diluted test solutions. Dilutions may be prepared using volumetric flasks or by accurate pipetting. If done by pipetting, use a minimum of 0.5 mL test sample or control solution to minimize the impact of variation in pipetting small volumes.

(*11*) Pipet 0.1 mL in duplicate of glucose working standard solutions (0, 250, 500, 750, and 1000 µg/mL glucose), **C**(**f**), and reagent blank, quality control sample, and test sample solutions into the bottoms of 16 × 100 mm glass test tubes using two tubes/solution. Add 3.0 mL GOPOD reagent, **C**(**e**)(*1*), to each tube. Vortex tubes. Place tubes in a rack and cover with plastic film to seal.

Note: Alternative to the use of the GOPOD method, proceed with alternate glucose determination method, **C**(**e**)(*2*), for measurement of glucose in working standards, reagent blank, control sample, and test sample solutions.

(*12*) Incubate in a 50°C water bath for 20 min.

(*13*) Set spectrophotometer to measure absorbance at 505 nm. After the incubation is complete, zero the spectrophotometer with the GOPOD-reacted 0 µg/mL working standard solution. Read absorbances of remaining GOPOD-reacted working standard solutions, and reagent blank, control sample, and test sample solutions. All reacted solutions must be read within 30 min of the end of the GOPOD incubation. The duplicate absorbance values are averaged for each reagent blank, test sample, and control sample solution and used in *Calculations*.

F. Calculations

Determine the quadratic equation that fits the absorbances of the working standard solutions. The absorbance values, A_{CF} or A_{CE}, are the independent variables (X), and actual glucose concentrations are the dependent variables (Y). Individual absorbance values of the working standard solutions, not averages, are used. The equation has the form:

$$\mu g\ Glucose/mL = (A_{CF\ or\ CE}{}^2 \times Q + A_{CF\ or\ CE} \times S + I)$$

Calculate dietary starch content in test sample as received as follows:

$$\text{Free glucose, \%} = (A_{CF}{}^2 \times Q + A_{CF} \times S + I) \times V_F \times DF_F \times 1/1\,000\,000 \times 1/W_F \times 162/180 \times 100$$

$$\text{Dietary starch, \%} = [(A_{CE}{}^2 \times Q + A_{CE} \times S + I) \times V_E \times DF_E \times 1/1\,000\,000 \times 1/W_E \times 162/180 \times 100] - \text{free glucose \%}$$

where subscript *F* represents values for samples analyzed for free glucose and subscript *E* represents values for samples treated with amylase and amyloglucosidase; A_{CF}, A_{CE} = absorbance of reaction solutions minus the absorbance of the appropriately diluted reagent blank, values are averages of the two replicates for each test solution; Q = quadratic slope term, S = linear slope term, and I = intercept of the standard curve to convert absorbance values to µg glucose/mL; V_F, V_E = final sample solution volume, ca 50.0 mL for V_F and 51.1 mL for V_E if done by summation of volumetric additions, otherwise, by size of volumetric flask used; DF = dilution factor, e.g., 0.5 mL sample solution diluted into 5.0 mL = 5.0/0.5 = 10; 1 g/1 000 000 µg = conversion from µg to g; W_E, W_F = test portion weight, as received; 162/180 = factor to convert from measured glucose as determined, to anhydroglucose, as occurs in starch.

If test samples are run in duplicate portions, the free glucose % in the dietary starch equation is the average free glucose % value determined for the test sample.

Results and Discussion

Evaluation of the Dietary Starch Method

Initial evaluation of data from all laboratories showed that most outliers occurred in two laboratories (Table 2). Laboratory 14 had significant Cochran's tests for five of the test materials, indicating suspect replicate results within this laboratory. Unlike the other laboratories, Laboratory 14 ran duplicate portions of test materials on separate days, rather than together within the same run. Based on laboratory ranking scores (18), this laboratory was designated as an outlier and its data were

Table 2. Results of collaborating laboratories for dietary starch individual replicate values on an as-received basis

		Collaborating laboratory														
Material	Duplicate	0	1	2	3	4	5	6	7	8	9	10	11[a]	12	13	14[b]
Moist canned dog food	1	1.58	1.42	1.34	1.56	1.58	1.58	1.57	1.64	1.59	1.44	**2.47**[c]	**1.94**[d]	1.55	**1.84**[c]	**0.22**[c]
	2	1.57	1.46	1.36	1.67	1.62	1.59	1.47	1.62	1.60	1.44	**1.59**[c]	**1.94**[d]	1.53	**1.61**[c]	**0.32**[c]
Low starch horse feed	1	7.03	6.29	7.01	7.30	6.78	7.21	6.88	7.33	7.27	6.47	6.68	8.32	7.15	7.02	**5.76**[c]
	2	7.21	6.50	7.44	7.60	6.43	7.61	7.02	7.33	6.98	6.74	7.37	7.87	7.08	6.68	**6.50**[c]
Dry ground corn	1	70.80	63.08	71.80	**58.85**[c]	71.27	68.13	70.18	71.22	71.52	71.25	67.97	**5.84**[d]	70.39	68.98	**60.19**[c]
	2	69.24	63.14	72.89	**26.64**[c]	70.23	67.33	71.47	73.29	71.08	70.04	65.82	**5.93**[d]	70.53	68.74	**65.42**[c]
Complete dairy feed	1	29.19	26.86	28.53	28.90	26.88	28.27	28.39	29.33	29.07	27.59	**26.89**[c]	**37.21**[d]	28.41	25.42	27.85
	2	29.79	26.69	28.49	30.02	26.11	28.70	28.19	29.10	28.89	27.49	**30.90**[c]	**35.45**[d]	28.01	26.10	27.28
Soybean meal	1	1.01	1.04	**1.09**[c]	1.10	0.97	1.13	0.94	1.04	1.06	0.87	1.02	**2.35**[d]	0.82	1.00	**0.02**[c]
	2	1.03	1.11	**1.42**[c]	1.19	0.93	1.11	0.90	0.93	1.09	0.78	1.16	**2.38**[d]	0.84	1.02	**0.82**[c]
Distillers grains	1	4.02	3.90	4.23	4.27	4.05	4.55	4.05	4.16	3.99	4.10	3.81	**4.82**[e]	4.19	3.98	**3.16**[c]
	2	4.07	3.90	4.09	4.30	4.08	4.49	3.94	4.14	4.06	4.06	4.09	**4.85**[e]	4.58	3.79	**3.00**[c]
Poultry feed	1	28.67	28.12	28.57	28.71	26.47	27.99	27.44	29.59	28.78	27.67	27.9	26.50	29.07	25.06	27.51
	2	29.25	27.35	27.95	30.26	28.00	28.27	28.52	29.43	28.83	27.65	30.39	25.18	29.45	24.80	26.56
Corn silage	1	41.10	37.44	39.20	40.92	37.54	39.18	38.08	39.17	40.91	37.00	37.26	36.03	43.50	36.59	37.99
	2	40.34	36.84	39.02	41.59	37.71	38.58	37.65	39.83	40.22	37.34	40.23	35.72	41.31	36.40	36.55
Dog kibble, dry	1	29.87	25.50	24.58	27.73	29.23	27.53	27.37	24.10	27.32	**17.99**[f]	25.73	27.55	28.68	26.30	24.31
	2	27.92	26.45	27.52	24.21	26.57	27.33	25.64	28.00	25.19	**18.35**[f]	27.25	26.93	29.34	25.70	26.25
Alfalfa pellets	1	1.29	1.17	1.56	1.32	1.56	1.59	1.61	1.35	1.33	1.58	1.42	1.31	1.13	1.25	0.60[c]
	2	1.36	1.43	1.43	1.41	1.32	1.61	1.31	1.24	1.34	1.38	1.35	1.13	1.38	1.27	**1.01**[c]

[a] Data for this laboratory was omitted from analysis based on a 7% change in glucose standard absorbances between runs for detection of free glucose and free + enzymatically released glucose. When data were included, four of 10 samples were identified as outliers by the single Grubbs' test, and one by the double Grubbs' test.

[b] Outlier laboratory detected by laboratory ranking.

[c] Outlier detected by the Cochran's test.

[d] Outlier detected by the single Grubbs' test.

[e] Outlier detected by the double Grubbs' test.

[f] Data omitted from analysis because the large test portion used (0.5 g) exceeded the 100 mg α-glucan limit for this assay.

not used in calculation of the study statistics. Laboratory 11 had four outlier values detected by the single Grubbs' test, which would indicate that this laboratory's values for these test samples were substantially higher or lower than those generated by the other laboratories. The very low value for dry ground corn appeared to be a possible error in recording the dilution of the sample, but laboratory records indicated that that was not the case. The basis for the high values for dairy feed, soybean meal, and moist canned dog food was not immediately obvious. The distillers grains results for Laboratory 11 was designated as an outlier based on results of the double Grubbs' test.

Laboratory 11 was not designated as an outlier by the ranking procedure, but test material results were generally higher for this laboratory. A likely basis for the higher dietary starch values was that the absorbances of the glucose standards were lower in the analytical run with the test samples treated with enzyme than were those reported for two other standard curves run for the dietary starch assay in that laboratory. The decrease in absorbance was on the order of 0.029 to 0.089 for 500 and 1000 mg glucose/mL standard solutions. To put this in perspective, the difference in absorbance values between runs represents an almost 8% lower absorbance value for the 1000 mg glucose/mL standard in the assay with enzyme-treated test samples. Standard curves produced from lower absorbance values will give higher calculated glucose and dietary starch values if the absorbances of the test samples are not similarly depressed. Absorbance values for glucose standards are not expected to be identical among analytical runs. However, the glucose oxidase-peroxidase assay used tends to be very consistent. For example, in the Study Director's laboratory, eight glucose standard curves run with dietary starch assays on 4 separate days showed RSD values (SD/mean) of less than 0.8% for absorbance values determined across runs within glucose standard (Table 3). Data from 12 collaborating laboratories that provided absorbance data for more than one standard curve showed the RSD of the absorbances calculated for individual glucose standards and then averaged across all standards were less than 1% for five laboratories, less than 2% for eight,and more than 2% for four (Table 4). Replicate absorbance readings for glucose standards within analytical run showed overall good repeatability for all laboratories. Laboratory 14, which was excluded from the study based on a ranking test, had the

Table 3. Absorbance values for glucose standards analyzed in repeated runs and in the collaborative study

Repeated analyses of glucose standard solutions: values by standard[a]						
Glucose standard, µg/mL	Runs[b]	Mean[c]	SD[d]	CV%[d]	Minimum value	Maximum value
249.4	8	0.285	0.0020	0.69	0.282	0.289
499.4	8	0.568	0.0028	0.49	0.563	0.574
748.7	8	0.848	0.0031	0.36	0.841	0.852
998.7	8	1.125	0.0045	0.40	1.116	1.133
Collaborative study: means across standards of values calculated for individual standards						
Laboratory	Runs	Overall mean[e]	Mean SD[f]	Mean CV, %[g]	Replicate SD[h]	
Study Director	3	0.704	0.0023	0.35	0.001	
7	2	0.688	0.0031	0.46	0.002	
8	4	0.712	0.0040	0.62	0.003	
13	2	0.658	0.0034	0.68	0.003	
2	2	0.855	0.0068	0.79	0.007	
1	6	0.827	0.0083	1.41	0.004	
12	3	0.684	0.0092	1.47	0.004	
3	2	0.736	0.0073	1.49	0.005	
6	2	0.723	0.0121	1.56	0.009	
4	3	0.682	0.0143	2.22	0.008	
5	4	0.727	0.0160	2.28	0.007	
11	3	0.709	0.0287	3.55	0.009	
14	2	0.667	0.0531	8.78	0.004	

[a] Glucose standards were analyzed in the Study Director's laboratory in eight separate analytical runs for the dietary starch assay. Glucose standards were analyzed in duplicate in two separate runs/day on 4 days. Two separate batches of GOPOD reagent were each used for four runs. The same preparations of glucose standards were used for all eight runs.

[b] Number of separate analytical runs in which the glucose standards were analyzed in duplicate.

[c] The mean value of the 16 replicates for each glucose standard.

[d] SD = standard deviation; RSD = 100 × (SD/mean).

[e] Mean of all absorbance values generated by the laboratory.

[f] The mean of all SD of absorbance values calculated for individual glucose standards.

[g] The mean of all RSD of absorbance values calculated for individual glucose standards.

[h] The mean of all SD of absorbance values for replicate pairs of glucose standards.

largest average RSD for absorbances of the glucose standards. Given the good replication for duplicates in this laboratory, the large RSD reflects differences in glucose standard absorbances between analytical runs. The difference this variation would generate in the standard curves could explain the variation detected in test sample replicates for this laboratory, because test sample duplicates were analyzed singly in separate runs, each of which used a different standard curve. Laboratory 11 had the second highest average RSD for absorbances of the standards. Discussions with Laboratory 11 did not uncover the basis for the variation between analytical runs. The dietary starch assay relies on the soundness of the standard curves to give reliable results. For Laboratory 11, because the glucose standard results used with the enzyme-treated samples deviated from two other standard curves they performed, and because the lower absorbances gave a standard curve that appears to have inflated the dietary starch values, the data are suspect and has been omitted from the statistical analysis of this study.

It is important to control the run to run and between replicate variation in analysis of the glucose standards because of the impact these have on accuracy of results. This GOPOD glucose detection assay is highly sensitive to pipetting accuracy. Samples should be read within 30 min of the end of incubation with GOPOD. It is also recommended that the incubated GOPOD-reacted samples be kept out of sunlight as this can degrade the chromagen. In addition to evaluating standard curve data for obvious changes in response, it is recommended that for each batch of GOPOD a log be kept of absorbance data for glucose standards from all runs. Within a glucose standard, calculate the SD of all absorbances. The mean of these SDs across all standards should not be greater than 0.016. Even lower levels of variability in absorbances can be readily achieved with this assay.

Another factor that likely affected accuracy was exceeding the 100 mg of starch limit/test portion in the assay, which was the case for Laboratory 9 when dry dog kibble was analyzed using 0.5 g test portions. The resulting low dietary starch values were likely the result of the enzyme no longer being in the excess required for complete hydrolysis of the dietary starch.

Variability of results may also have been affected by the approach to sample dilution. Laboratory 3 used 0.1 mL of test sample solution and 0.9 mL of water to make a 1 in 10 dilution

Table 4. Statistical data for dietary starch results

Material	Outlier	*n*	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	$2.8 \times s_r$	$2.8 \times s_R$	HorRat	Largest within-lab variance	Largest average lab result	Smallest average lab result
Moist canned dog food	10, 13	11	1.53	0.03	0.09	2.21	5.99	0.10	0.26	1.60	0.01	1.63	1.35
Low starch horse feed		13	7.02	0.23	0.36	3.32	5.19	0.65	1.02	1.74	0.24	7.45	6.40
Dry ground corn	3	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54	1.83	2.31	72.34	63.11
Complete dairy feed	10	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48	1.83	0.64	29.49	25.76
Soybean meal	2	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31	2.79	0.01	1.15	0.83
Distillers grains		13	4.11	0.11	0.20	2.67	4.94	0.31	0.57	1.53	0.08	4.52	3.88
Pelleted poultry feed		13	28.24	0.73	1.34	2.58	4.76	2.04	3.76	1.97	3.10	29.51	24.93
Corn silage		13	39.04	0.80	1.88	2.05	4.82	2.24	5.27	2.09	4.41	42.40	36.49
Dog kibble, dry	9	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46	2.43	7.61	29.01	25.97
Alfalfa pellets		13	1.38	0.12	0.13	8.61	9.69	0.33	0.38	2.54	0.05	1.60	1.25

for the ground corn sample. Even with small differences in pipetted amounts, such an approach could result in the between duplicate difference noted for that sample. Test solutions from the enzymatic hydrolysis procedure can be "sticky", i.e., they do not pipet exactly like water, and require care to pipet accurately. If dilutions are made by pipetting, prewetting of pipet tips and use of larger volumes, such as 0.5 mL of test solution and 4.5 mL of water, are recommended.

The quantity of test material used also may have affected assay variability. Test samples with starch contents of less than 2% generally showed greater variability than test samples that contained more starch (Figure 1 and Table 4) in a pattern nearly identical to that described by Wehling and DeVries (24) for dietary fiber assays. However, among the low starch materials, the moist dog food had RSD values for repeatability and reproducibility that were approximately half those of soybean meal and alfalfa pellets (Table 4); these latter two samples also had the highest HorRat values in the study. In addition to being the only moist, homogenized sample, laboratories were directed to use 0.5 g of the moist dog food as compared to 0.1 g of other samples. The one case in which dietary starch values for the moist dog food were identified by the Cochran test as suspect replicates within laboratory was where Laboratory 10 reported values determined on 0.10 g test samples for this material (Table 2). In the collaborative study, the 0.1 g sample size was used for most samples to minimize the likelihood that the 100 mg limit of dietary starch/test portion would be exceeded, based on the laboratories' prestudy results with the assay; however, it also greatly reduced the concentration of glucose to be detected in low starch test samples. Final glucose concentrations of test sample solutions for 0.1 g enzyme-treated test portions of soybean meal and alfalfa pellets were 22 and 30 μg/mL, respectively as compared to 167 μg/mL for the moist dog food using 0.5 g test portions. These glucose concentrations of the low starch feeds equate to absorbance values of 0.035, 0.054, and 0.221, respectively, as determined in the Study Director's laboratory. Although the glucose detection assay is sensitive and precise, small variations in absorbances of test solutions with very low glucose concentrations will give more variability in calculated glucose values than the same amount of variation will with test solutions with higher glucose concentrations. This can result in greater within and between laboratory variability for low starch test samples for which smaller test portions are used. In the case of the dietary starch assay, as with gravimetric dietary fiber analyses, the increase in RSD as concentrations of the analyte approaches zero may be related to limits of precision of the detection methods themselves. The absorbances and glucose concentrations noted for soybean meal, alfalfa pellets, and moist dog food represent 1.0, 1.4, and 7.7 mg of dietary starch in the respective test portions. It is notable that the distillers grains, for which the 0.1 g test portion would provide approximately 4 mg of dietary starch, had a HorRat value below 2, possibly suggesting a level of dietary starch at and above which precision is improved.

A viable approach to decreasing RSD values for low starch test samples analyzed with the dietary starch method is to increase the size of the test portion in order to increase the amount of analyte to be detected. The idea of increasing the amount of test sample analyzed in order to improve precision by having a greater amount of analyte to measure has been raised (25). Unlike the dietary fiber analyses that may need to restrict test portion size to assure that the extractant remains in excess, starch assays will primarily be restricted by the need to maintain an excess of enzyme to assure complete hydrolysis of the α-glucan. The approach of allowing a range of test portions but a limit on the amount of starch added to the reaction vessel is used by two current AOAC starch methods: AOAC Method **948.02** for starch in plants (26) specifies a use of 0.1–1.0 g of test portion containing approximately 20 mg of starch, and AOAC Method **979.10** for starch in cereals (27) indicates use of a 0.5 g test portion and then specifies "≤1.0 g containing ≤0.5 g starch". In the present method, a limit of 100 mg of dietary starch in each reaction vessel leaves latitude to increase the size of the test portion to that upper limit. Although 0.1 g test portions may be generally adequate, increasing the amount of substrate within the bounds of the assay for feedstuffs with low starch contents may reduce variability of results. The remaining caveat is that as sample quantity is increased, attention must be paid to increasing amounts of interfering substances also brought into the reaction (e.g., antioxidants if the GOPOD assay is used).

With the exceptions of dry ground corn, dairy feed, poultry feed, and corn silage, s_r and s_R were similar within materials (Table 3). The HorRat values obtained in the present study compared favorably to those obtained with AOAC Method **996.11** (10; Table 3). In the collaborative study for that method, starch analyses performed without dimethyl sulfoxide (DMSO)

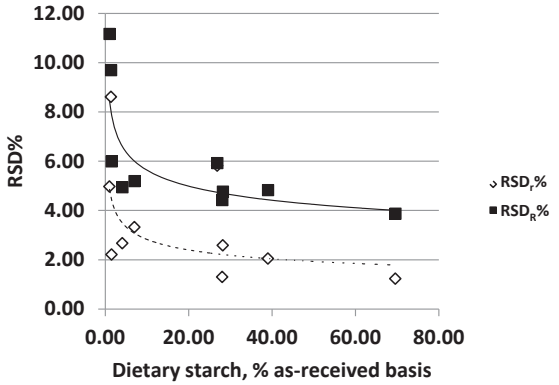


Figure 1. Relationship of dietary starch concentration and RSD values for repeatability within laboratory (RSD_r) and reproducibility between laboratories (RSD_R) obtained in the collaborative study. Equations for the regression lines are RSD_r, % = $4.8616x^{-0.236}$ (R^2 = 0.35; dashed line), and RSD_R,% = $8.4397x^{-0.176}$ (R^2 = 0.66; solid line), where x = dietary starch concentration.

had a starch content of 59.8% as received, and an average HorRat of 2.1 with one value below 2. For the dietary starch collaborative study, the HorRat was less than 2 for six of 10 materials, with an overall average of 2.0 on test materials that averaged 20.7% dietary starch on an as-received basis. Alfalfa pellets and soybean meal had HorRat values of greater than 2.5. As previously discussed, the high RSD_R for these test materials may relate to the combination of their low starch content and the small test portion amount used. Test samples with very low concentrations of the analyte have been reported to give elevated HorRat values (17). The high HorRat value for the dry dog kibble may reflect an issue with homogeneity of the sample, as described previously.

Collaborators' Comments

The collaborators all reported that the assay was not very complicated and was easy to do. They particularly liked additions of all reagents to a single vessel, performing reactions in screw cap tubes, determining total liquid volume as the sum of quantitative volume additions, and making sample solution dilutions by accurate pipetting of volumes. They indicated that they had to work within their laboratories to find tools of acceptable accuracy to make the volume additions, as some of the tools they worked with for other purposes were not adequate. They did report issues with screw cap tube adequacy to hold the needed volume; this was apparently related to differing amounts of glass used by the manufacturers while maintaining the same exterior dimensions of the tubes. That was addressed by describing the screw cap tubes by the volume they needed to contain while allowing adequate room for mixing. With the number of sodium phosphate chemicals available, it was noted that it was crucial to verify and use the exact chemicals specified for the GOPOD reagent. It was also raised that the only extended period to take a break from the assay was during the amyloglucosidase incubation; taking a break after adding water to the fully digested samples resulted in reduced recovery. Development of an approved assay for glucose detection that could be used on a plate reader or automated system was recommended as a way to increase throughput of the assay, which is currently limited by the 30 min period within which samples must be read after incubation in the GOPOD glucose detection assay. Some laboratories had issues with calculating quadratic glucose standard curves; this was resolved by graphing all individual glucose standard solution absorbances data with absorbance on the X-axis and glucose concentration on the Y-axis. Then, a quadratic or second order polynomial regression or "trend" line was graphed through the data. The regression line equation was used for calculation of glucose in test solutions. Collaborators gave extensive input on the method protocol writeup and recommended development of a flow chart for the assay

Recommendations

Based on the results of the collaborative study, the Study Director recommends that the enzymatic-colorimetric method for measurement of dietary starch in animal feeds and pet foods be adopted as Official First Action.

Acknowledgments

I thank Jan Pitas (U.S. Dairy Forage Research Center) for assistance in developing the dietary starch method and assistance with preparing and distributing materials for the study. I thank the Laboratory Methods & Services Committee of the Association of American Feed Control Officials for their orchestration of the effort for defining dietary starch and their support and input in this project. I thank Nancy Thiex, Larry Novotny, and the staff of the Olsen Biochemistry Laboratory at South Dakota State University for assistance in preparing the test samples. Special thanks go to Nancy Thiex for her invaluable guidance and assistance throughout the study. The U.S. Department of Agriculture, Agricultural Research Service provided funding for the materials used in the study. I also thank the following collaborators for their participation in this study:

Robin Johnson, Montana Department of Agriculture and Analytical Laboratory, Bozeman, MT

Brian Steinlicht and David Taysom, Dairyland Laboratories, Arcadia, WI

Courtney Heuer, Don Meyer, Zach Meyer, Lauren Meyer, and John Goeser, Rock River Laboratories, Watertown, WI

Kristi McCallum, Dominika Kondratko, and Tyler Potts, Colorado Dept. of Agriculture/I&CS Biochemistry Laboratory, Denver, CO

Lisa Ruiz, John Jordan, and Tuyen Thi Doan, Eurofins Scientific–Nutritional Analysis Center, Des Moines, IA

Kathryn S. Phillips, C. Andre Odijk, and Kenneth Hodel, NP Analytical Laboratories, St. Louis, MO

Lisa Means, Teresa Grant, and Steven Pleasants, North Carolina Department of Agriculture/Food and Drug Protection Division, Raleigh, NC

Christa Willaredt and Sabrina Trupia, NCERC Analytical Laboratory, Edwardsville, IL

Angela Carlson, SGS Brookings, Brookings, SD

Adela Parganlija-Ramic and Michele Swarbrick, Minnesota Department of Agriculture, St. Paul, MN

Kiley Schwartz, Berthier Jean-Louis, Eduardo Maciel, and Kiley Mulholland, Idaho State Department of Agriculture, Twin Falls, ID

Darren Welch and Audra Gile, Kansas Department of Agriculture Laboratory, Topeka, KS

Sarah Snodgrass and Joshua Arbaugh, West Virginia Department of Agriculture, Charleston, WV

H. Dorota Inerowicz and Keith Binkerd, Office of Indiana State Chemist/Feed Chromatography Laboratory Section, West Lafayette, IN

References

(1) Huntington, G.B. (1997) *J. Anim. Sci.* **75**, 852–867
(2) *Official Methods of Analysis* (2006) 18th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **920.40**
(3) ISO 15914 (E) Animal feeding stuffs – Enzymatic determination of total starch content, 1st Ed. 2004-02-01. International Organization for Standardization, Geneva, Switzerland
(4) Bernal-Santos, G., Perfield II, J.W., Barbano, D.M., Bauman, D.E., & Overton, T.R. (2003) *J. Dairy Sci.* **86**, 3218–3228
(5) Pham, T.-H., Mauvais, G., Vergoignan, C., De Coninck, J., Cachon, R., & Feron, G. (2008) *Biotechnol. Lett.* **30**, 287–294
(6) Approved Methods of the American Association of Cereal Chemists (2000) 10th Ed., AACC International, St. Paul, MN, Method **776-11**
(7) Hodge, J.E., & Osman, E.M. (1976) in *Principles of Food Science, Part 1, Food Chemistry*, O.R. Fennema (Ed.), Marcel Dekker, Inc., New York, NY, p. 58
(8) Bach Knudsen, K.E. (1997) *Anim. Feed Sci. Technol.* **67**, 319–338
(9) Hall, M.B. (2009) *J. AOAC Int.* **92**, 42–49
(10) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **996.11**
(11) Dias, F.F., & Panchal, D.C. (1987) *Starch/Stärke* **39**, 64–66
(12) Karkalas, J. (1985) *J. Sci. Food Agric.* **36**, 1019–1027
(13) *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* (2002) AOAC INTERNATIONAL, Rockville, MD
(14) Hall, M.B., Jennings, J.P., Lewis, B.A., & Robertson, J.B. (2001) *J. Sci. Food Agric.* **81**, 17–21
(15) Hall, M.B., & Keuler, N.S. (2009) *J. AOAC Int.* **92**, 50–60
(16) Wu, X., Beecher, G.R., Holden, J.M., Haytowitz, D.B., Gebhardt, S.E., & Prior, R.L. (2004) *J. Agric. Food Chem.* **52**, 4026–4037
(17) Mertens, D.R. (2002) *J. AOAC Int.* **85**, 1217–1240
(18) Wernimont, G.T. (1990) *Use of Statistics to Develop and Evaluate Analytical Methods*, W. Spendley (Ed.), AOAC INTERNATIONAL, Rockville, MD
(19) Food Chemicals Codex (2014) 9th Ed., The United States Pharmacopeial Convention, Rockville, MD, Appendix V, Enzyme Assays, α-Amylase Activity (Bacterial),1392–1393
(20) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **991.43**
(21) McCleary, B.V. (1999) *Cereal Foods World* **44**, 590–596
(22) McCleary, B.V., Bouhet, G., & Driguez, H. (1991) *Biotechnol. Tech.* **5**, 255–258
(23) Thiex, N., Novotny, L., Ramsey, C., Latimer, G., Torma, L., & Beine, R. (2000) *Guidelines for Preparing Laboratory Samples*, Association of American Feed Control Officials, Inc., Champaign, IL
(24) Wehling, P., & DeVries, J.W. (2012) *J. AOAC Int.* **95**, 1541–1546
(25) Horwitz, W., Albert, R., Deutsch, M., & Thompson, M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 661–680
(26) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **948.02**
(27) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **979.10**

SPECIAL SECTION ON FEED ADDITIVES AND CONTAMINANTS

Factors Affecting Accuracy and Time Requirements of a Glucose Oxidase–Peroxidase Assay for Determination of Glucose

MARY B. HALL
U.S. Department of Agriculture, Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Dr, Madison, WI 53706
NICHOLAS S. KEULER
University of Wisconsin–Madison, Statistics, Computing and Biometry, 1300 University Ave, Madison, WI 53706

Accurate and rapid assays for glucose are desirable for analysis of glucose and starch in food and feedstuffs. An established colorimetric glucose oxidase–peroxidase method for glucose was modified to reduce analysis time and evaluated for factors that affected accuracy. Time required to perform the assay was reduced by approximately 40% by decreasing incubation time and removing steps that do not affect absorbance. Although linear regressions of absorbance and glucose concentrations of standard solutions exceeded R^2 of 0.9997, evaluation of sum of squared residuals, root mean squared error, and significance of the quadratic term indicated that the curves were approximately quadratic in form. Inadequate equilibration of glucose anomers did not appear to be the issue. Historic data suggest that the standard curve is inherently nonlinear. Quadratic curves predicted standard solution glucose concentrations more accurately than did linear forms; overestimations at the midpoint of the curve averaged 0.04, 0.48, and 0.92% for quadratic and linear equations calculated from 5 standard solutions and a linear equation calculated from the 0 and most concentrated standard solution, respectively. A hydrophilic antioxidant at levels no greater than 10 μmol ascorbic acid/0.10 g air-dried sample did not affect absorbance values.

Enzymatic–colorimetric analyses using glucose oxidase and peroxidase (GOPOD) are commonly used for detection of glucose in methods for free glucose (1), starch (2, 3), and resistant starch (4). The assays are both specific and sensitive for the detection of glucose. There are many permutations of GOPOD assays that vary in composition of the GOPOD reagent, incubation times, ratios of sample to reagent, toxicity of reagents, and other elements. The GOPOD method for glucose described by Karkalas (5) avoids the use of potentially carcinogenic reagents such as *o*-dianisidine, and it gives very repeatable within-assay absorbance values. However, increased sample throughput and more economical use of laboratory resources could be achieved through modifications to the assay to reduce the time required for incubation and sample handling. Introduction of modifications warrants the reevaluation of assay performance in terms of its accuracy in predicting glucose concentration and the effect of potentially interfering substances.

The purpose of the present study was to investigate modifications to the Karkalas (5) GOPOD assay for glucose that would reduce the time required for analysis, and to evaluate factors that affect the accuracy of prediction of glucose in the modified assays.

Experimental

Design

The GOPOD method for glucose analysis described by Karkalas (5) was evaluated in a single laboratory with work performed by one technician. Elements evaluated were the effect of temperature and length of incubation, ratio of sample solution to GOPOD reagent, effect of vortexing samples before incubation, effect of cooling samples in the dark post-incubation, effect of time delay between the end of incubation and reading sample absorbance, linearity of absorbance response, time from preparation of standard solutions to analysis and reading, effect of GOPOD reagent type on linearity of response, and application of standard curves based on 5 versus 2 independently prepared standard solutions. Four to 7 standard solutions were analyzed in triplicate for each treatment within each analysis run. Additionally, the interference of a hydrophilic antioxidant (ascorbic acid) on the detection of glucose carried through a starch analysis procedure was evaluated. Each treatment was evaluated in 2 separate runs, thus giving 2 independent results per assay permutation. All possible combinations of factors were not evaluated for each incubation and sample:reagent

Received September 30, 2008. Accepted by EB December 12, 2008.
Corresponding author's e-mail: marybeth.hall@ars.usda.gov
Mention of any trademark or proprietary product in this paper does not constitute a guarantee or warranty of the product by the USDA or the Agricultural Research Service and does not imply its approval to the exclusion of other products that also may be suitable.
This paper is published as part of a themed collection on emerging feed issues organized by the Feed Additives and Contaminants Subgroup of the AOAC Agricultural Materials Community.

Table 1. Effect of delayed reading on sample absorbance values and percentage change in absorbance relative to no time delay[a]

		Time delay to reading, min				
Incubation condition	Glucose, μg/mL	0	15	30	45	60
Standard solution:GOPODk reagent (0.5:2.5)		Absorbance at 505 nm				
35°C for 45 min	0	0.000	0.001	0.001	0.002	0.003
	40	0.239	0.238	0.237	0.234	0.236
	60	0.359	0.357	0.354	0.352	0.351
	100	0.594	0.586	0.582	0.579	0.579
		(Absorbance/0 time delay absorbance) × 100				
	40		99.7%	99.1%	98.1%	98.8%
	60		99.2%	98.6%	97.9%	97.8%
	100		98.7%	98.0%	97.5%	97.5%
		Absorbance at 505 nm				
50°C for 20 min	0	0.000	0.001	0.003	0.003	0.003
	40	0.238	0.240	0.242	0.237	0.235
	60	0.358	0.357	0.356	0.353	0.351
	100	0.589	0.582	0.580	0.577	0.570
		(Absorbance/0 time delay absorbance) × 100				
	40		100.5%	101.4%	99.6%	98.7%
	60		99.9%	99.4%	98.7%	98.0%
	100		98.8%	98.6%	98.0%	96.9%

		Time delay to reading, min				
		0	10	20	30	40
Standard solution:GOPODk reagent (0.1:3.0)		Absorbance at 505 nm				
50°C for 20 min	0	0.000	0.002	0.004	0.000	0.001
	400	0.455	0.453	0.452	0.445	0.444
	600	0.674	0.671	0.671	0.662	0.656
	1000	1.114	1.107	1.098	1.087	1.079
		(Absorbance/0 time delay absorbance) × 100				
	400		99.6%	99.3%	97.8%	97.5%
	600		99.5%	99.5%	98.1%	97.3%
	1000		99.4%	98.6%	97.6%	96.9%

[a] Values presented as least-squares means and calculated percentages.

ratio permutation. The comparisons that were tested were selected based on the outcomes of preceding experiments, with focus on achieving the study goals of reducing the time required for the assay, achieving accurate predictions of glucose concentrations, and evaluating factors that affect the outcome of the modified assays.

Data within each experiment were analyzed as a completely randomized design with method, glucose concentration of the standard solution, and the sample by method interaction included in the statistical model. Numeric factors, such as time to analysis of a solution, were treated as continuous variables to determine statistical significance and classification variables to calculate the least-squares means. If an interaction term was not significant, a reduced model with the interaction term removed was analyzed to determine the significance of the main effects. When appropriate, batches of GOPOD reagent used or assay run were included as random variables. Statistical analysis was performed using the Mixed procedure of the SAS software (SAS Version 8, SAS Institute, Cary, NC). Standard curve equations, residual plots, R^2, root mean squared error, and sum of squared residuals (residual = observed minus predicted value) were determined using the Reg (regression) procedure of SAS.

The effect of the number of glucose standard solutions used on the accuracy of the prediction of quadratic standard curves calculated from the standards was tested. Concentrations of glucose in the standard solutions were predicted using the standard curves and the measured absorbances of the standards. Accuracy of prediction was evaluated using the residuals as the response variable (actual glucose concentration minus predicted glucose concentration). The statistical model included number of glucose standards (3, 4, or 5) used for calculation of the curve within the day in which the analysis was performed, glucose concentration of the standard solutions, and the interaction of these terms. All factors were used as classification variables.

Materials

Purified D-glucose (>99.5% purity; Sigma-Aldrich, Inc., St. Louis, MO; used as purchased) was used to prepare the standard solutions. Average dry matter content of glucose was determined with drying for 15 h at 105°C in a forced-air oven. Glucose values were adjusted for dry matter and purity (determined by manufacturer; 99.8–99.9%).

Apparatus

A spectrophotometer capable of operating at absorbances of 505 and 510 nm was used (Ultrospec 3000 UV-Vis spectrophotometer, Pharmacia Biotech, Model 80-2106-20, Cambridge, UK).

Reagents and Solutions

All reagents and solvents were analytical reagent grade. All references to water are for distilled or equivalent reverse osmosis purified water.

(**a**) *Glucose oxidase–peroxidase–aminoantipyrine buffer mixture.*—(*1*) *GOPODk (for Karkalas method).*—Mixture of glucose oxidase, 7000 U/L; peroxidase, 7000 U/L; and 4-aminoantipyrine (also called 4-aminophenazone, $C_{11}H_{13}N_3O$, CAS 83-07-8; not to be confused with 4-*N*,*N*-dimethyl aminophenazone, also known as aminophenazone or aminopyrine), 0.74 mM in a buffer. Prepared by dissolving 9.1 g Na_2HPO_4 and 5.0 g KH_2PO_4 in ca 300 mL H_2O in a 1 L volumetric flask. Used H_2O to rinse chemicals into bulb of flask. Swirled to dissolve completely. Added 1.0 g phenol and 0.15 g 4-aminoantipyrine. Used H_2O to rinse chemicals into bulb of flask. Swirled to dissolve completely. Added glucose oxidase (7000 U) and peroxidase (7000 U), rinsed enzymes into flask with H_2O, swirled gently to dissolve without causing excessive foaming. Diluted to 1 L with H_2O. Sealed. Inverted repeatedly to mix. Filtered solution through a glass fiber filter with 1.6 μm retention and stored in a sealed amber bottle at ca 4°C. Reagent should be used within 1 month.

(*2*) *GOPODa (for GOPOD assay in AOAC Method **996.11**).*—Mixture of glucose oxidase, 12 000 U/L; peroxidase, 650 U/L; and 4-aminoantipyrine, 0.4 mM in a buffer containing KH_2PO4, NaOH, and 4-hydroxybenzoic acid adjusted to pH 7.4. Buffer was prepared by dissolving 13.6 g KH_2PO_4, 4.2 g NaOH, and 3.0 g 4-hydroxybenzoic acid in 96 mL H_2O. pH was adjusted to 7.4 with either 2 M HCl or 2 M NaOH and solution diluted to 100 mL; sodium azide was not added. The entire buffer mix was transferred to 2 L volumetric flask and the solution made to contain 0.4 mM 4-aminoantipyrine, >12 000 U/L of glucose oxidase, and >650 U/L peroxidase. The solution was gently swirled to dissolve enzymes and chemicals, and was diluted to volume. The reagent was filtered through a glass fiber filter with 1.6 μm retention and stored in a sealed bottle at ca 4°C. Reagent is stable for 2 to 3 months at 4°C.

Note: Glucose oxidase (Sigma-Aldrich product G-6125) contained 21 200 U glucose oxidase/g solid and 0.0461 U catalase/mg solid (manufacturer's analysis).

(**b**) *Glucose standard solutions.*—Standard solutions were made independently, with glucose weighed separately for each solution in order to avoid the issue of improper preparation of a stock solution or pipetting issues affecting the accuracy of the standard solutions. For all standard solutions, glucose was weighed on an analytical balance, and weight was recorded to 0.0001 g; the glucose was quantitatively transferred with rinsing to a volumetric flask, dissolved, and diluted to volume. Dry matter of powdered crystalline glucose (purity ≥99.5%) was determined by drying for 15 h at 105°C in a forced-air oven. The weight of glucose added to a flask was multiplied by dry matter percentage and assayed purity of the glucose (provided by manufacturer) and divided by dilution volume milliliter to calculate actual glucose concentrations of the solutions. For glucose concentrations between 0 and 100 μg/mL, the amount of glucose weighed ranged from 0 to 50 mg and dilution volume was 500 mL; for concentrations between 0 and 1000 μg/mL, the glucose amount ranged from 0 to 250 mg and dilution volume was 250 mL. For samples prepared in benzoic acid solution, 0.2% benzoic acid (w/v) solution was substituted for water.

Table 2. Historic data and results of the present study on effect of standard solution preparation on form of standard regression curve

	Curve form[a]	Intercept	Coefficient for abs[b]	Coefficient for abs squared	R^2	RMSE[c]	Quadratic term *P*-value	Sum of squared residuals
Trinder, 1969 (ref. 8)	L	–5.782	505.536		0.9996	7.03		197.5
	Q	0.559	472.30	16.36	1.0000	1.48	<0.01	6.56
Time, min[d]			Fresh glucose solutions in water, 7 point standard curves					
Standard solution:GOPODk reagent[e] (0.1:3.0)								
45	L	–4.267	899.68		0.9997	4.743		890.0
45	Q	0.342	871.47	25.9859	0.9999	3.402	<0.01	451.3
140	L	–1.794	899.60		0.9997	5.067		1027.0
140	Q	3.338	867.66	29.6173	0.9999	3.342	<0.01	435.6
380	L	–0.318	899.60		0.9998	4.502		810.8
380	Q	3.028	878.92	19.1439	0.9998	3.802	<0.01	563.8
Standard solution:GOPODk reagent (0.5:2.5)								
45	L	–0.277	169.78		0.9999	0.368		5.41
45	Q	–0.007	166.74	5.1439	0.9999	0.321	<0.01	4.03
140	L	–0.205	169.13		0.9997	0.532		11.32
140	Q	–0.001	166.83	3.8836	0.9997	0.520	0.03	10.56
380	L	–0.180	169.99		0.9999	0.369		5.45
380	Q	0.012	167.82	3.6773	0.9999	0.349	0.01	4.76
Time, days			Benzoic acid solutions, 5 point standard curves					
Standard solution:GOPODk reagent (0.1:3.0)								
1	L	–4.054	903.22		0.9998	4.851		305.9
1	Q	0.007	873.80	26.6310	0.9999	3.307	<0.01	131.2
2	L	–5.232	900.70		0.9998	5.183		349.2
2	Q	0.137	861.88	35.0511	1.0000	1.972	<0.01	46.7
3	L	–4.669	900.55		0.9999	3.964		204.2
3	Q	–0.405	869.83	27.6968	1.0000	1.061	<0.01	13.5
Standard solution:GOPODk reagent (0.5:2.5)								
1	L	–0.210	170.87		0.9999	0.442		2.54
1	Q	0.112	166.43	7.6165	0.9999	0.346	0.01	1.44
2	L	–0.064	170.02		0.9999	0.318		1.32
2	Q	0.126	167.40	4.4834	0.9999	0.278	0.04	0.93
3	L	–0.468	170.28		0.9998	0.488		3.10
3	Q	–0.007	163.97	10.7596	0.9999	0.269	<0.01	0.87

[a] Curves represent combined results of 2 replicate assay runs. L = Linear, Q = quadratic.

[b] Abs = Absorbance.

[c] RMSE = Root mean squared error.

[d] Time from standard solution preparation to analysis and reading of absorbance of samples.

[e] GOPODk = Glucose oxidase–peroxidase reagent of Karkalas (ref. 5).

Procedures

In the general procedure used for each assay run, the specified volumes of H_2O and glucose standard solutions were pipetted in triplicate into the bottom of 16 mm diameter glass culture tubes (100 or 150 mm height) to give 3 tubes per standard per treatment. The specified volume of GOPOD was added to each tube using a positive displacement repeating pipet. If tubes were vortexed, it was done at this point. Tubes were covered with plastic film and incubated at the specified temperature and time in a water bath capable of maintaining the temperature ±1°C. Post-incubation cooling was performed after removal from the water bath. The spectrophotometer was zeroed to water, and sample absorbance was read at 505 nm for GOPODk and 510 nm for GOPODa. Absorbance values corrected for the average of the 0 μg glucose/mL solutions for each treatment were calculated and used in calculation of standard curves. Equations for linear and quadratic forms of glucose standard curves were calculated where Y = glucose μg/mL and X = absorbance to reflect the form of the equation used to predict glucose concentrations of unknown samples. Glucose amounts per tube used in this assay (0–100 μg) were within the range in which the original protocol indicated that Beer's law was obeyed (5).

The variations of the GOPODk procedure evaluated were:

(**a**) *Effect of incubation conditions.*—0.5 mL volumes of 0, 40, 60, and 100 μg/mL glucose standard solutions with 2.5 mL GOPODk reagent were mixed on a Vortex mixer, incubated at 35°C for 45 min or 50°C for 20 min, and cooled for 10 min in the dark before having their absorbance read immediately at 505 nm; or, after samples were held on the bench, they were read at 15, 30, 45, and 60 min thereafter. The 35°C for 45 min incubation represents the original method (5). An incubation temperature of 60°C for 20 min was also evaluated, but not pursued because measured absorbances were 13% lower than those obtained at 50°C for 20 min incubations (P <0.01 for effect of temperature; data not shown).

(**b**) *Effect of post-incubation cooling in dark.*—Standard solutions with GOPODk were prepared as in (**a**) for 20 min at 50°C incubation, except that absorbances were read immediately after the incubation or after samples were cooled for 10 min in the dark.

(**c**) *Alteration of the ratio of standard solution:GOPODk reagent volume.*—0.1 mL volumes of 0, 400, 600, and 1000 μg/mL glucose standard solutions with 3.0 mL GOPODk were mixed on a Vortex mixer, incubated at 50°C for 20 min, and cooled for 10 min in the dark before having their absorbance read at 505 nm. Effect of inclusion or omission of the 10 min post-incubation cooling in the dark, and effect of reading absorbances immediately after incubation, or at 10, 20, 30, and 40 min thereafter were evaluated.

(**d**) *Effect of inclusion or omission of vortexing step.*—The effect of inclusion or omission of the step in which standard solution with GOPODk was mixed on a Vortex mixer before incubation was evaluated.

(**e**) *The effect of time delay.*—The effect of time delay from preparation of glucose standard solutions to the time they were analyzed and read on the spectrophotometer was evaluated to indirectly assess the effect of mutarotation of glucose on the form of the standard curves (glucose oxidase is specific for the β-anomer of glucose). Glucose solutions prepared fresh daily in H_2O and glucose in 0.2% w/v benzoic acid solution were used. Freshly prepared solutions contained ca 0, 20, 40, 50, 60, 80, and 100 μg glucose/mL for the standard solution:GOPOD (0.5:2.5) ratio, and 0, 400, 500, 600, 700, 800, and 1000 μg glucose/mL for the standard solution:GOPOD (0.1:3.0) reagent ratio. Benzoic acid solutions contained ca 0, 25, 50, 75, and 100 μg glucose/mL for the standard solution:GOPOD (0.5:2.5) ratio, and ca 0, 250, 500, 750, and 1000 μg glucose/mL for the standard solution:GOPOD (0.1:3.0) ratio. Time from preparation of the solutions to reading absorbance at the end of the glucose assay were approximately 45, 140, and 380 min for freshly prepared solutions, and 1, 2, and 3 days for benzoic acid solutions. All solutions were held at ambient temperature until analysis.

(**f**) *Alternative GOPOD glucose assay.*—An alternative GOPOD glucose assay method (3) using a different GOPOD reagent was evaluated to determine whether the quadratic form of the standard curve was found only in the Karkalas method with GOPODk. The AOAC GOPODa formulation and incubation conditions described in AOAC Method **996.11** (3) were used with glucose solutions prepared with 0.2% benzoic acid solution as described in (**e**) 9 days after the glucose standards were prepared. The GOPODk reagent was also used to analyze the same glucose solutions on the same day for comparison of absorbance per μg glucose/mL.

(**g**) *Effects of form of the standard curve.*—Effects of form of the standard curve and number of standards included in the curve on predictions of starch content of samples were estimated mathematically. Linear and quadratic curves prepared with 5 standard solutions were evaluated, as well as a linear standard curve using only the 0 μg glucose/mL and greatest-concentration standard solutions. Data from the analysis performed using GOPODk and glucose standards prepared in 0.2% benzoic acid 3 days before the assay was performed were used to generate the standard curves. To calculate the effects of deviations in the glucose predictions on sample starch concentrations, the average measured absorbance of each standard solution was entered into the standard curve regression equations to calculate predicted glucose concentrations of the standards. The actual glucose concentrations were subtracted from the predicted values to give μg glucose/mL values for the deviation of the predicted vs actual glucose concentration for each standard. The predicted concentration minus actual μg glucose/mL values were multiplied by 0.9 to convert glucose to a starch basis, then multiplied by a dilution factor (1000 or 100 for 0.5:2.5 and 0.1:3.0 sample solution:GOPODk, respectively), then divided by 1 000 000 to convert from micrograms to grams, and finally divided by 0.09 to represent a 0.1 g sample with a dry matter content of 90%. The calculated value was multiplied by 100 to covert to a percentage basis.

Table 3. Standard curves for AOAC glucose detection method[a]

Run	Curve form[b]	Intercept	Coefficient for abs[c]	Coefficient for abs squared	R^2	RMSE[d]	Quadratic term *P*-value	Sum of squared residuals
Standard solution:GOPODa reagent[e] (0.1:3.0)								
A	L	–4.030	947.27		0.9999	4.084		216.9
A	Q	–0.190	918.14	27.6426	1.0000	2.261	<0.01	61.3
B	L	–7.374	949.99		0.9997	6.465		543.4
B	Q	–0.970	901.44	46.1339	0.9999	3.029	<0.01	110.1
Standard solution:GOPODa reagent (0.5:2.5)								
A	L	–0.438	179.99		0.9999	0.419		2.29
A	Q	–0.041	174.28	10.2969	1.0000	0.232	<0.01	0.64
B	L	–0.576	178.95		0.9995	0.777		7.85
B	Q	0.071	169.61	16.7836	0.9998	0.531	<0.01	3.39

[a] Curves represent individual assay runs.
[b] L = Linear, Q = quadratic.
[c] Abs = Absorbance.
[d] RMSE = Root mean squared error.
[e] GOPODa = Glucose oxidase–peroxidase reagent (ref. 3).

The effect of the number of glucose standards used to calculate the quadratic standard curve on the accuracy of predicted values was tested using data from (**e**) for glucose standards 2, 3, and 9 days after they were prepared (3 analysis runs, 1 per day). Standard curves were calculated for each separate run with data from 3 (highest, lowest, and midpoint), 4 (2 lowest, 2 highest), and 5 (all standards) glucose concentrations. The standard curves were used to calculate predicted values for the glucose concentrations of all 5 standard solutions, and the actual minus predicted residual values for glucose concentrations of the solutions were calculated.

(**h**) *Effect of a hydrophilic antioxidant (ascorbic acid) on glucose detection.*—Ascorbic acid was dosed in μmol quantities reported for hydrophilic antioxidants in foodstuffs (6) into glucose samples carried through a starch assay procedure. A solution of 5000 μmol ascorbic acid/L was prepared with 0.1 M sodium acetate buffer (pH 5.0) used as the diluent. The ascorbic acid solution was pipetted into both reagent blank tubes and tubes containing 100 ± 0.2 mg glucose before addition of 0.1 M sodium acetate buffer (pH 5.0) to achieve a total volume of 30 mL. Ascorbic acid solution was added to provide 0, 1, 2.5, 5, 10, 20, 30, or 50 μmol ascorbic acid per tube. Single treatment tubes for each substrate and ascorbic acid addition were analyzed in each of 2 runs. Sample solutions were analyzed in triplicate using 2 ratios of sample solution:GOPODk (0.1:3.0 and 0.5:2.5) incubated for 20 min at 50°C.

Starch Analysis Method

A modification of the method of Bach Knudsen (7) was performed on D-glucose with ascorbic acid solution additions. Purified D-glucose was accurately weighed into 25 × 150 mm screw-cap glass tubes. The desired volume of 5000 μmol ascorbic acid/L was dispensed into tubes containing glucose or no substrate (reagent blanks). Sodium acetate buffer (0.1 M, pH 5.0) was added to each tube to bring the liquid volume to 30 mL. Heat-stable α-amylase (0.1 mL, ca 2000 Liquefon units; Spezyme Fred, Genencor International, Inc., Rochester, NY; origin: *Bacillus licheniformis*; "Liquefon unit" is a measure of α-amylase activity for which a detailed assay is available from the manufacturer) was pipetted into each tube, which was then capped and mixed on a Vortex mixer. Tubes were incubated for 1 h at 100°C, with vortexing at 10, 30, and 50 min of incubation. After cooling on the bench for 0.5 h, 1 mL amyloglucosidase solution (200 U/mL in 0.1 M sodium acetate buffer, pH 5.0) was added, tubes were mixed on a Vortex mixer, then incubated for 2 h at 60°C, with vortexing at 1 h. After incubation, 20 mL H_2O was dispensed into each tube, and the tubes were recapped and inverted to mix. From each tube 1.5 mL of solution was transferred to a 2 mL microcentrifuge tube, then centrifuged at 1000 × *g* for 10 min. The centrifuged solutions were allowed to come to room temperature before preparing them in 1:1 dilutions with H_2O for glucose analysis.

Results and Discussion

Incubation Conditions

No effect of incubation time and temperature was detected for 0.5 mL of standard solution with 2.5 mL of GOPODk reagent incubated at 35°C for 45 min or 50°C for 20 min, and held 10 min in the dark at ambient temperature before reading (P = 0.79). Nor was there an interaction of incubation conditions and glucose concentration of the standard solutions

Table 4. Absorbance per μg glucose/mL standard solution[a]

	Sample solution:GOPOD reagent				
	0.1:3.0			0.5:2.5	
	Karkalas[b]	AOAC[b]		Karkalas	AOAC
	Glucose μg/mL				
250	0.00116	0.00110	25	0.00617	0.00574
500	0.00113	0.00108	50	0.00604	0.00568
750	0.00113	0.00106	75	0.00598	0.00563
1000	0.00112	0.00106	100	0.00592	0.00557
S_r					
P-value of quadratic term	0.05	0.03		0.02	0.98

[a] Values are least-squares means.
[b] Karkalas: GOPODk used (ref. 5); AOAC: GOPODa used (ref. 3).

($P = 0.47$), indicating that the standard curves did not diverge over the range of 0–100 μg glucose/mL concentrations. The respective standard curves were glucose μg/mL = $169.25x - 0.16$ at 35°C for 45 min (adjusted $R^2 = 0.9997$), and $169.78x - 0.17$ at 50°C for 20 min (adjusted $R^2 = 0.9998$), where x = the measured absorbance.

Delayed reading after incubation altered the absorbance of standard solutions ($P < 0.01$): those containing glucose declined, but the 0 μg/mL standard increased slightly over time (Table 1). The absolute decrease in absorbance with time was greater for greater concentrations of glucose ($P < 0.01$). Incubation conditions ($P = 0.66$) and the interaction of incubation conditions and glucose concentration ($P = 0.16$) did not alter the effect of delayed time to reading. Absorbance declined at approximately 0.5% each 15 min. The recommendation in the original protocol (5) that samples be read before 30 min would allow a decrease to ca 99% of the initial absorbance. With comparable responses in absorbance and delay to reading, the shorter incubation time at warmer temperature could be used to give results equivalent to those of the original method.

Allowing samples time to cool in the dark between incubation and absorbance reading did not affect results for the standard solution:GOPODk (0.5:2.5) samples incubated at 50°C for 20 min. Neither reading samples immediately after removal from the water bath, nor cooling the samples in the dark (least-squares means for absorbances 0.299 and 0.298, respectively; $P = 0.92$), nor the interaction of cooling in the dark by glucose concentration ($P = 0.62$) affected sample absorbance. Mixing the sample solution with GOPODk on a Vortex mixer before incubation also not did not affect sample absorbance for the 20 min at 50°C incubated samples (least-squares means for absorbances: 0.296 vortexed, 0.296 not vortexed; $P = 0.89$; glucose concentration × vortex, $P = 0.54$).

Ratio of Standard Solution:GOPODk Reagent (0.1:3.0)

Changing the ratio of standard solution:GOPODk reagent from the 0.5:2.5 described in the original protocol to 0.1:3.0 incubated at 50°C for 20 min with glucose concentrations of 0–1000 μg/mL increased the amount of glucose added per reaction tube, but was still within the range reported to obey Beer's law in the original protocol (5). By using a smaller sample volume and a broader range of glucose concentrations for the standard curve, the need for or extent of sample dilution is reduced (3). The standard curves produced with this modification had much greater slopes and intercepts (e.g., glucose μg/mL = $896.24x - 3.39$; $R^2 = 0.9999$); x = measured absorbance.

Absorbance values were reduced for samples that were cooled in the dark compared to those read immediately, with the difference increasing with increasing glucose concentration ($P < 0.01$; for μg glucose/mL of 0, 399, 600, and 999, absorbance at 505 nm: 0.019, 0.471, 0.694, and 1.130 for samples read immediately, and 0.020, 0.469, 0.690, and 1.120 for those cooled in the dark for 10 min, respectively; values are least-squares means; interaction of post-incubation cooling in the dark by glucose concentration, $P < 0.01$). Unlike the 0.5:2.5 ratio of standard solution:GOPODk, for which results were not affected, use of a cooling period is not recommended for the 0.1:3.0 ratio.

As with the 0.5:2.5 ratio of standard solution:GOPODk with delayed reading of samples, the absorbance of the 0 μg/mL solutions increased slightly over time, while the glucose solutions declined at a rate of approximately 1% every 20 min (Table 1). Vortexing the sample solution with GOPODk reagents before incubation not did not affect sample absorbance (vortex, $P = 0.78$; glucose concentration × vortex, $P = 0.94$).

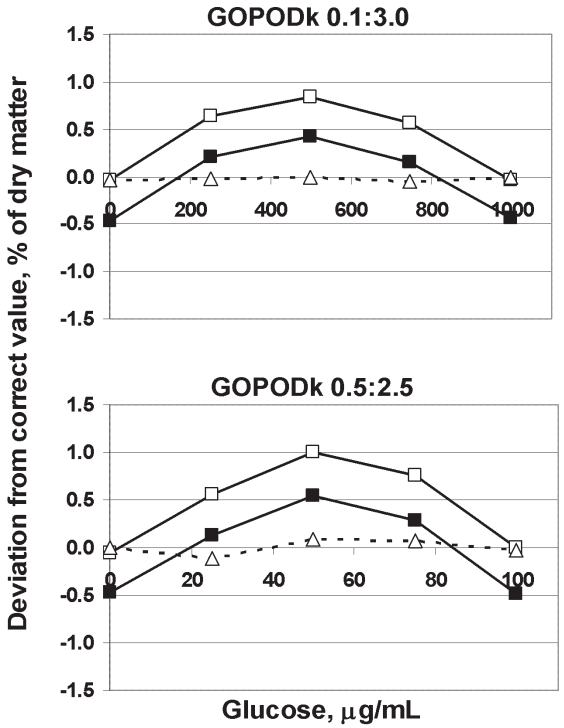


Figure 1. Effect of deviations in predicted glucose concentrations on calculated estimates of sample starch content as related to form of standard curve used. GOPODk = GOPOD reagent of (ref. 5), 0.1:3.0 and 0.5:2.5 are the ratios of standard solution:GOPOD used. ■ = 5-point linear equation, □ = 2-point linear equation, and △ = 5-point quadratic equation.

Linearity of Response

A problematic aspect of the regression equations produced from all approaches used with GOPODk was that all the linear equations had R^2 of nearly 1.0 (0.9998 to 1.0), suggesting a very good fit to the linear form, but the intercept was not 0. Thus, when the standard curves were used to predict glucose concentrations of the standard solutions used to produce them, the predicted values were frequently incorrect. It was determined that a quadratic form fit the standard curves better than a linear form (Table 2), based on significance of the quadratic term in the regression equation, the reduction in the root mean squared error of the standard curve, and the relative decrease in residual sums of squares (residual = observed minus predicted) between the linear and quadratic equations, and evaluation of the residual vs predicted value plots. Other nonlinear forms were not explored. Review of data from one of the original GOPOD assays for glucose (8; Table 2), as well as of glucose assays performed with the original GOPODk method (5) at 3 different institutions with different equipment over the course of 13 years frequently showed the non-zero intercept and quadratic pattern of the standard curve (data not shown). Presence of catalase in the glucose oxidase enzyme did not seem to be implicated as the ratio of peroxidase to catalase in the GOPODk reagent in the present study was 460:1. Catalase has a considerably lower Km for H_2O_2 than does peroxidase (93 and <5 mM, respectively; 9) and the maximal millimolar concentration of glucose in the standard solution + GOPODk reaction mixes was 32.3.

In the original work (5), absorbance of glucose solutions was measured against a 0 μg glucose/mL solution to which GOPODk had been added, though the author did not indicate whether the 0 standard was included in the standard curve. Even with exclusion of the 0 μg glucose/mL absorbances from calculation of the standard curves in the present data set, the quadratic term remained significant, and the pattern of residuals for the linear form of the curve still suggested that the curves were not linear (data not shown). The quadratic/nonlinear form of the curve does not appear to be due to inclusion of a 0 standard.

Investigations into the need for equilibration of α- and β-anomers of glucose in the standard solution, as evaluated by allowing different periods of time to elapse between preparation of the glucose solutions and their analysis, and effects of different GOPOD reagents suggest that the nonlinear/quadratic absorbance response to glucose is inherent in this assay. Both for the standard solutions prepared fresh daily and those made in benzoic acid solution, and for the different ratios of standard solution to GOPODk reagent, the quadratic terms of the curves were significant, and the values for the sum of squared residuals and root mean square error were smaller for the quadratic than for the linear forms of the equations (Table 2).

Specific to the standards prepared fresh daily with H_2O, time from preparation of the standards to reading of samples did not affect absorbances for the 0.1:3.0 ratio of standard solution:GOPODk reagent (time × glucose concentration, $P = 0.96$; reduced model time, $P = 0.15$; least-squares means for absorbance: 0.639, 0.636, and 0.634, for 45, 140, and 380 min, respectively; standard error of the difference = 0.0031; Table 2). For the ratio of standard solution:GOPODk (0.5:2.5), the interaction of glucose concentration and time was not significant ($P = 0.35$), but time did affect absorbance ($P < 0.01$ in the reduced model; least-squares means for absorbance: 0.298, 0.296, and 0.295, for 45, 140, and 380 min, respectively; standard error of the difference = 0.0007). This result is in contrast to results in the original protocol in which α- and β-anomers were reported to have equilibrated by 40 min into the 35°C incubation (5). The quadratic terms of all standard curves were significant.

For glucose standards prepared in 0.2% benzoic acid solution, the time between preparation of the standards and their analysis and reading did not affect the standard solution:GOPODk (0.1:3.0) samples (time by glucose concentration, $P = 0.53$, reduced model time $P = 0.22$; least-squares means for absorbance: 0.557, 0.560, and 0.560 for 1, 2, and 3 days after standard preparation, standard error of the difference: 0.0009; Table 2). For the sample solution:GOPODk (0.5:2.5) samples, the time to analysis did

Table 5. Effect of number of glucose standards used for calculation of quadratic standard curves on accuracy of prediction of glucose concentrations in the standards[a]

		Actual minus predicted glucose, μg/mL	
		Sample solution:GOPODk reagent	
Day of analysis	No. of glucose standards	0.1:3.0	0.5:2.5
2	3	–0.243	0.064
2	4	0.161	–0.044
2	5	<0.001	<0.001
3	3	0.170	0.047
3	4	–0.113	–0.032
3	5	<0.001	<0.001
9	3	–0.615	–0.46
9	4	0.542	0.032
9	5	0.017	<0.001
Standard error of the mean		0.456	0.062

[a] Values are least-squares means for each standard curve.

affect absorbances (time by glucose concentration, $P = 0.44$; reduced model time, $P < 0.01$; least-squares means: 0.293, 0.294, and 0.296 for 1, 2, and 3 days after standard preparation; standard error of the difference: 0.0005). Overall, the standard solution:GOPODk (0.1:3.0) standards appeared to be less affected by time of standard preparation than were the 0.5:2.5 samples.

The use of the GOPODa reagent that used more units of glucose oxidase, used fewer units of peroxidase, and used 4-hydroxybenzoic acid rather than phenol gave similar results to the GOPODk assay (Table 3). All standard curves produced with GOPODa were more quadratic than linear, as determined on the basis of significance of the quadratic term, reduction in root mean squared error, and sum of squared residuals between the linear and quadratic forms of the curves.

Although the original assay reported a linear response in absorbance through 200 μg glucose/mL (5), the nonlinear nature of the relationship of the absorbance per unit of glucose and non-zero intercept of the linear equations indicate that this is perhaps not the best model (Table 4). In agreement with the original study, the relationship between absorbance and glucose concentration in the present study became grossly nonlinear and in violation of Beer's Law (absorbance response plateaued or declined with increasing glucose concentrations) at approximately 300 and 1500 μg glucose/mL for the ratios of sample solution:GOPODk (0.5:2.5 and 0.1:3.0), respectively (data not shown). The original basis for presuming linearity of the responses at glucose concentrations <200 μg glucose/mL probably lies in the very high R^2 for the linear form of the curves, and in that the absorbance per unit of glucose values differ in the fourth or fifth decimal place. While the quadratic form seems to fit better than the linear form, we do not necessarily consider it to be the "true" or "best" form of the relationship. The quadratic form is presented as a clear improvement over linearity, but it is possible that other functional forms could fit as well as or better than the quadratic.

Impact of Standard Curves on Prediction and Implications

Linear or not, the value of an assay is in its ability to predict with the desired accuracy the content of an analyte in a substrate. Both the GOPODk and GOPODa methods showed similar patterns when the impact of predicted minus actual glucose concentrations of standard solutions was calculated to apply to determination of the starch content of a 0.1 g sample of 90% dry matter (Figure 1; GOPODk data only). Quadratic curves produced from 5 glucose standards showed no more than 0.1% deviation from the correct value, whereas linear curves produced from the same data over-predicted glucose concentration and calculated starch content through the middle of the range of standard solutions by up to 0.5% of sample dry matter, and under-predicted by the same amount at the highest and lowest concentrations. The linear curve produced from the highest and zero glucose standards gave accurate predictions at these 2 points, but overestimated in the middle of the standard curve by up to 1% of dry matter. The different standard solution:GOPOD ratios behaved similarly when 100× and 1000× dilution factors were used for the 0.1:3.0 and 0.5:2.5 ratios, respectively. These dilution factors allow samples containing 0.09 g of pure starch (e.g., pure starch with a dry matter of 90%) to fall into the range of the standard curve. Greater dilution of such samples may allow them to be read in the middle of the standard curve; however, increasing the dilution factor also multiplies the size of the error [e.g., compared to 1000×, a 2000× dilution factor would double the overestimation midrange on the 5-point linear curve for the sample solution:GOPOD (0.5:2.5) ratio]. Use of greater sample size while staying within the 0.09 g of starch limit can also reduce error as the greater sample weight is divided into the starch estimate (e.g., a 0.2 g sample would have half the predicted minus actual deviation of a 0.1 g sample). The error will vary somewhat depending upon the standard curve run.

Depending on the desired accuracy, linear or quadratic standard curves can be used, but the quadratic equation gives more accurate predictions. With possible deviations of –1 to +1, or 0 to +2 percentage units from the accurate value depending on how the standard curve is run, dilution factor, and where in the standard curve the sample absorbances fall, interpretation of single measures, such as clinical blood glucose values, would be little affected whether linear or quadratic equations are used. However, such deviations could skew interpretation of results or mask differences when values are used for comparison, such as for starch contents among grain varieties or efficiency of yield of ethanol from starch in

Table 6. Effect of ascorbic acid additions on absorbance of glucose samples carried through a starch analysis[a]

	Sample solution:GOPODk reagent, mL:mL			
	0.1:3.0		0.5:2.5	
Ascorbic acid, μmol	Absorbance, 505 nm[b]	0 μmol ascorbic acid absorbance, %	Absorbance, 505 nm[c]	0 μmol ascorbic acid absorbance, %
0	1.080	100.0	0.566	100.0
1	1.084	100.4	0.567	100.2
2.5	1.079	99.9	0.569	100.5
5	1.080	100.0	0.566	100.0
10	1.072	99.3	0.565	99.8
20	1.047	97.0	0.559	98.7
30	1.036	96.0	0.549	97.0
50	0.982	91.0	0.534	94.3

[a] Values are least-squares means.
[b] Standard error of the difference for least-squares means = 0.0049.
[c] Standard error of the difference for least-squares means = 0.0017.

batches of corn grain. Another way that use of linear GOPOD standard curves may affect accuracy is by compensating for or adding to other errors in assays in which the GOPOD method is incorporated. For example, in starch assays in which samples are gelatinized and hydrolyzed with heat-stable α-amylase at neutral pH, maltulose formation should decrease recovery of starch as released glucose (10). However, overestimation of glucose in the middle of the linear standard curves may provide a compensating error, allowing values for purified starches to measure closer to 100%.

Evaluation of the effect of the number of glucose standard solutions used to generate a quadratic standard curve within a given run showed that use of standard curves produced using 3, 4, or 5 standard solutions did not differ in their accuracy of prediction [$P > 0.80$ for ratios of sample solution:GOPODk (0.1:3.0 and 0.5:2.5); Table 5]. Neither the effect of glucose concentration ($P > 0.58$) nor the interaction of glucose concentration and number of standards used to generate the curves ($P > 0.87$) were significant for either sample solution:reagent ratio. The curves generated from 5 glucose standards had the numerically smallest residuals, but even the largest residual [–0.615 μg glucose/mL for sample solution:reagent (0.1:3.0)] was small. Even though the accuracy was acceptable, we do not recommend using 3 glucose concentrations to describe a quadratic curve, as this is overfitting the data and risks generation of an erroneous curve if one of the glucose standards is not properly prepared. Use of 4 glucose standards to produce standard curves gives acceptable results.

Repeatability

Repeatability of absorbance values on triplicate samples of standard solutions was very good within run and is a key reason that the small deviations from linearity could be detected. For the glucose standards prepared in benzoic acid, the standard solution:GOPODk ratio (0.1:3.0) gave standard errors of 0.0022, 0.0012, 0.0018, 0.0021, and 0.0030 for 0, 250, 500, 750, and 1000 μg glucose/mL standards (overall coefficient of variation % for glucose-containing solutions = 0.31%). Standard error values for the 0.5:2.5 ratio were 0.0011, 0.0016, 0.0018, 0.0013, and 0.0016 for 0, 25, 50, 75, and 100 μg glucose/mL standards (overall coefficient of variation % for glucose-containing solutions = 0.57%). Values for the freshly prepared samples were similar.

Limit of Determination

Limits of determination for glucose measurement were calculated from absorbance values of 0 μg glucose/mL standards analyzed in triplicate from 4 assay runs in which the glucose standards were prepared in 0.2% benzoic acid solution. Values were calculated as mean blank value + 3 × blank standard deviations (11). For standard solution:GOPODk (0.5:2.5 and 0.1:3.0) samples, the mean absorbance ± standard deviation of undiluted blanks were 0.0002 ± 0.0010 for a detection limit of 0.0029 absorbance, and 0.0002 ± 0.0020 for a detection limit of 0.0063 absorbance, respectively. Using average quadratic standard curves calculated for each preparation, glucose detection limits are 0.53 and 5.12 μg/mL for standard solution:GOPODk (0.5:2.5 and 0.1:3.0), respectively. The detection limits represent approximately 0.5% of the range of the glucose standards in each case.

Effect of Antioxidants

Addition of ascorbic acid to tubes containing glucose and subject to a modification of the Bach Knudsen (7) assay for starch showed a linear decrease in absorbance at additions of >10 μmol of ascorbic acid [effect of ascorbic acid on absorbance for 0–10 μmol of ascorbic acid, $P = 0.30$ and 0.37 for sample:GOPODk (0.1:3.0 and 0.5:2.5), respectively;

effect of ascorbic acid for 10–50 μmol: linear P <0.01 and quadratic P <0.01 for 0.1:3.0 and 0.5:2.5 sample:GOPODk, respectively; Table 6]. The effect was relatively small through 20 μmol ascorbic acid. Investigations into the antioxidant content of foodstuffs (6) showed that most of the high starch or leafy vegetable foods had hydrophilic antioxidant values that would be equivalent to <10 μmol of ascorbic acid per 0.1 g dry matter. Exceptions included foods high in phenolic compounds (e.g., beets, red sorghum grain, antioxidant content approximately equivalent to 23 and 14 μmol ascorbic acid, respectively). Because of the interference in the GOPOD assay, another method for measuring glucose should be considered for feeds or foods exceeding 10–20 μmol of hydrophilic antioxidant per 0.1 g dry matter.

Recommendations

Based on its lesser sensitivity to time of sample preparation, the ratio of standard solution:GOPODk reagent (0.1:3.0) incubated at 50°C for 20 min is the preferred approach among those tested. With the reduced incubation time, and no need to mix samples on a Vortex mixer or cool them in the dark after incubation, a reduction in 30–40% of the time needed to perform the assay can be realized. Absorbance of samples should be read within 30 min of incubation. Use of a quadratic form of the standard curve produced using a minimum of 4 standard solutions differing in glucose concentration will give greater accuracy of prediction as compared to linear equations, but the choice in form of the equation depends on the accuracy required for the application. Use of standard solutions prepared in advance in 0.2% benzoic acid solution reduces the time needed to run the assay and avoids potential issues with solubilization or equilibration of glucose. This assay may be used to analyze materials with ≤10 μmol of hydrophilic antioxidant per 0.1 g of air-dried sample without appreciable reduction in glucose values, and reductions are small through 20 μmol, but an alternative glucose assay should be considered for use on samples containing more antioxidant.

References

(1) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **969.39**

(2) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **979.10**

(3) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **996.11**

(4) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2002.02**

(5) Karkalas, J. (1985) *J. Sci. Food Agric.* **36**, 1019–1027

(6) Wu, X., Beecher, G.R., Holden, J.M., Haytowitz, D.B., Gebhardt, S.E., & Prior, R.L. (2004) *J. Agric. Food Chem.* **52**, 4026–4037

(7) Bach Knudsen, K.E. (1997) *Anim. Feed Sci. Technol.* **67**, 319–338

(8) Trinder, P. (1969) *J. Clin. Pathol.* **22**, 158–161

(9) BRENDA, The comprehensive enzyme information system Release 2008.2, Braunschweig Department of Bioinformatics, http://brenda-enzymes.org

(10) Dias, F.F., & Panchal, D.C. (1987) *Starch/Stärke* **39**, 64–66

(11) *Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* (2002) AOAC INTERNATIONAL, Gaithersburg, MD

SPECIAL SECTION ON FEED ADDITIVES AND CONTAMINANTS

Determination of Starch, Including Maltooligosaccharides, in Animal Feeds: Comparison of Methods and a Method Recommended for AOAC Collaborative Study

MARY B. HALL
U.S. Department of Agriculture, Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Dr, Madison, WI 53560

Starch is a nutritionally important carbohydrate in feeds that is increasingly measured and used for formulation of animal diets. Discontinued production of the enzyme Rhozyme-S required for AOAC Method 920.40 invalidated this method for starch in animal feeds. The objective of this study was to compare methods for the determination of starch as potential candidates as a replacement method and for an AOAC collaborative study. Many starch methods are available, but they vary in accuracy, replicability, and ease of use. After assays were evaluated that differed in gelatinization method, number of reagents, and sample handling, and after assays with known methodological defects were excluded, 3 enzymatic–colorimetric assays were selected for comparison. The assays all used 2-stage, heat-stable, α-amylase and amyloglucosidase hydrolyses, but they differed in the gelatinization solution (heating in water, 3-(*N*-morpholino) propanesulfonic acid buffer, or acetate buffer). The measured values included both starch and maltooligosaccharides. The acetate buffer-only method was performed in sealable vessels with dilution by weight; it gave greater starch values (2–6 percentage units of sample dry matter) in the analysis of feed/food substrates than did the other methods. This method is a viable candidate for a collaborative study.

In the last decade, interest has increased in the measurement of dietary carbohydrates that may affect animal performance and health. Starch, in particular, has received much attention because of its influence on nutrient supply and ruminal acidosis (1). AOAC Method **920.40** for starch in animal feeds (2) is no longer valid because of discontinued production of the enzyme Rhozyme-S (Rohm and Haas, Philadelphia, PA) specified in the procedure. Accordingly, another approved method for starch in animal feeds is needed.

A definition of "starch" for the nutritional description of feedstuffs is essential to the selection of a method and for an accurate description of what the analytical values represent. However, this requirement becomes problematic when we consider how starch has been defined, the variety of potentially digestible α-linked glucose carbohydrates that are present in feedstuffs, and what the enzymatic methods measure. Starch is defined as a natural vegetable polymer consisting of long, linear unbranched chains of 1,4-α-D-glucose units (amylose) and/or long α-1,6-branched chains of α-1,4-linked glucose units (amylopectin; 3). However, amyloglucosidase used in enzymatic starch methods releases glucose from α-glucans present in animal (e.g., liver or muscle glycogen; "animal starch"; 4) or microbial (e.g., glycogen in yeast; 5) products because these carbohydrates contain α-(1,4) and α-(1,6) linkages as does starch, although in different proportions. Accordingly, enzymatic starch methods do not measure plant starch alone (6), unless animal and microbial ingredients and the feedstuffs that contain them are excluded from analysis. Maltooligosaccharides are also detected by enzymatic starch assays if the oligosaccharides are not extracted from samples before analysis. If starch is measured to give a nutritional description of a feedstuff, the inclusion of glycogen with starch more completely describes the pool of homoglucan that is potentially available to digestion by small intestinal enzymes (7). It remains open to discussion whether there is a nutritional basis to include or exclude maltooligosaccharides from the nutritional fraction that includes starch. Recognizing the aim of nutritional characterization and the limitations of the specificity of the methods, we have defined "starch" as α-glucan from which glucose can be released after gelatinization through the use of purified amylases and amyloglucosidases that are specifically active only on α-(1,4)

Received March 20, 2008. Accepted by EB July 2, 2008.
Corresponding author's e-mail: marybeth.hall@ars.usda.gov
Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.
This paper is published as part of a themed collection on emerging feed issues organized by the Feed Additives and Contaminants Subgroup of the AOAC Agricultural Materials Community.

Table 1. Results for determinations of free glucose and starch + maltooligosaccharides corrected for free glucose in purified substrates (dry matter basis)

		Hot water		Acetate buffer		Extension of AOAC **996.11**	
Sample	DM, %[a]	Mean	s_r[b]	Mean	s_r	Mean	s_r
			Free glucose				
Glucose	100.0	91.8	1.78	90.8	0.32	86.0	0.16
Sucrose	100.0	0.10	0.01	0.17	0.00	0.17	0.00
Dextrin	91.8	2.27	0.04	2.17	0.01	1.65	0.04
Corn starch	89.3	0.04	0.01	0.04	0.00	0.06	0.04
Potato starch	90.0	0.02	0.01	0.04	0.01	0.08	0.01
			Starch				
Glucose	100.0	90.4	0.3	90.8	0.3	86.0	0.2
			Starch + maltooligosaccharides corrected for free glucose[c]				
Sucrose	100.0	0.3	1.9	0.7	0.0	1.4	0.0
Dextrin	91.8	46.9	0.2	50.0	0.1	49.8	0.3
Corn starch	89.3	93.9	1.9	98.3	0.3	93.4	1.1
Potato starch	90.0	91.2	0.3	97.0	0.3	94.8	1.3
			Average for dextrin, corn starch, and potato starch				
Mean		77.3		81.8		79.3	
s_r		0.82		0.22		0.88	
CV, %[d]		0.94		0.26		1.02	

[a] DM = Dry matter.

[b] s_r = Standard deviation of replicates.

[c] Measured values for sucrose, corn starch, and potato starch represent starch content, not starch + maltooligosaccharide content, of these substrates.

[d] CV = Coefficient of variation (s_r/mean).

and α-(1,6) linkages, and exclusive of maltooligosaccharides that are extractable from feedstuffs with aqueous ethanol.

The candidate method for the determination of starch in animal feeds should be accurate, repeatable, and robust, and should avoid known analytical defects. The efficacy of enzymatic starch assays is affected by their level of complexity, specificity of release of glucose from starch alone, and factors causing incomplete starch hydrolysis. Increasing assay complexity or number of steps increases the potential variability of the results because the accuracy with which each dilution, transfer, or neutralization is accomplished affects the final measurement.

The release of glucose from nonstarch carbohydrates gives erroneously high starch values. It can be caused by enzyme preparations that are not specific for starch hydrolysis (8), run conditions that result in chemical hydrolysis, or the presence of appreciable quantities of maltooligosaccharides. Maltooligosaccharide content may be elevated when starchy foodstuffs have been subjected to enzymic or acidic hydrolysis (7), or when the oligosaccharides have been specifically added. Acid additions commonly used to quench enzymatic activity can hydrolyze sucrose to release glucose. Pre-extraction of interfering carbohydrates with aqueous ethanol (3, 9, 10), or avoiding the use of problematic run conditions and enzyme preparations can reduce or eliminate the release of glucose from nonstarch sources.

Reduced starch recovery due to incomplete hydrolysis can have physical or chemical causes. Examples of procedures that could lead to the formation of physical barriers to the interaction of enzyme and substrate include the formation of microgel or lumps with the addition of dimethyl sulfoxide to feeds (3), gelatinization without agitation, or insufficient grinding of samples, resulting in too coarse a particle size for efficient extraction of starch. A chemical reaction that results in incomplete starch hydrolysis is the isomerization of the reducing end glucose to fructose when starch is heated in water or buffer at neutral pH (11). Amyloglucosidase hydrolyzes the starch molecule up to the glucose–fructose disaccharide, but it leaves this remaining disaccharide, maltulose, unhydrolyzed. The first step in many starch assays is hydrolysis of starch with heat-stable α-amylase at neutral pH, which produces large numbers of reducing ends and can

lead to increased production of maltulose and decreased starch values. Performing the hydrolysis at slightly acidic pH reduces maltulose formation (11). Use of moderately acid tolerant α-amylases (12) allows the starch hydrolyses to be performed enzymatically under mildly acidic conditions.

Methods of enzymatic starch analysis differ primarily in method of gelatinization, with relatively similar enzymatic digestions by amyloglucosidase with or without a predigestion with amylase. In a preliminary study, starch analysis methods using heating with heat-stable α-amylase in water (modified from ref. 13), or acetate buffer (modified from ref. 14), or gelatinization in hot alkali followed by neutralization (15) were used to analyze corn starch, dextrin, glucose, and sucrose to evaluate the assays for accuracy and ease of use. The assay using acetate buffer gave a corn starch value (95.2% of dry matter) of 2–4 percentage units of dry matter greater than those of the other assays, 100% recovery of glucose, sucrose as 0.1% of dry matter, and a value for dextrin (49.4% of dry matter) 2–10 percentage units greater than those of the other analyses. Hot alkali destroyed 97% of the purified glucose substrate. With its greater recovery with starch and ease of use, a modification of the acetate buffer assay (AB; 14) was compared with starch assays using traditional hot water gelatinization (HW; 13) and an extension of the AOAC method for starch in cereal grains (ExtAOAC; 10) across a variety of substrates. Although the samples analyzed likely had a low content of maltooligosaccharides, without the use of pre-extraction to remove oligosaccharides, this evaluation is only able to compare starch + maltooligosaccharide measurements among the methods, except where the use of glucose, purified starch, or sucrose ensures the absence of these oligosaccharides.

Experimental

Design

Three methods of starch analysis were tested in a single laboratory over the same range of samples. All samples were run in duplicate within each analysis run. Additionally, glucose and corn starch were analyzed as control samples in duplicate within each run. Purified samples and feed/food samples were analyzed in separate runs, thus giving 2 independent results per assay. Data were analyzed in a completely randomized design, with method, sample, and the sample by method interaction included in the statistical model. Statistical analysis was performed by using the general linear model of SAS (SAS Version 8, SAS Institute, Cary, NC) with mean separation by the Bonferroni method. Starch assay data for glucose and sucrose were evaluated separately from other purified substrates to assess the efficacy of the methods with the other substrates that contained carbohydrate that is measured as starch.

Materials

Purified substrates—including corn starch, glucose, dextrin, potato starch, and sucrose—were analyzed to evaluate the recoveries of glucose and starch + maltooligosaccharides, and the hydrolysis of sucrose when the feed matrix provided no barrier to analysis. The feed/food substrates of alfalfa silage, soybean meal, corn silage, split green peas, high-moisture ensiled corn grain, wheat flour, and medium grain rice were selected as representative feeds likely subject to starch analysis, with the first 2 selected as representative of low starch, the next 2 representative of intermediate starch, and the remainder representative of high starch feeds. Silages and high-moisture corn were dried to a constant weight at 55°C in a forced-air oven. The purified substrates and flour were used as purchased, and the remaining samples were ground to pass the 1 mm screen of an abrasion (cyclone) mill (Udy Corp., Fort Collins, CO). The average dry matter content of samples was determined after drying for 15 h at 105°C in a forced-air oven.

Apparatus

(**a**) *Grinding mill*.—Cyclone mill equipped with a 1 mm screen (gives particle size equivalent to a cutting or Wiley mill with a 0.5 mm screen).

(**b**) *Bench centrifuge.*—Capable of holding 2 mL microfuge tubes, with a rating of ca 1000–12 000 × *g*.

(**c**) *Water bath.*—Capable of maintaining 35 and 50 ± 1°C.

(**d**) *Boiling water bath.*—Capable of boiling at 95–100°C.

(**e**) *Vortex mixer.*

(**f**) *pH meter.*

(**g**) *Stop-clock timer (digital).*

(**h**) *Top-loading balance*.—Capable of weighing accurately to ±0.01 g.

(**i**) *Analytical balance.*—Capable of weighing accurately to ±0.0001 g.

(**j**) *Laboratory ovens*.—With forced convection; capable of maintaining 105 ± 1°C for determining the dry weight of the test sample; capable of maintaining 92, 100, and 60 ± 1°C for incubations.

(**k**) *Spectrophotometer*.—Capable of measuring absorbances at 505 and 510 nm.

(**l**) *Pipets.*—Capable of delivering 0.1, 0.5, and 1.0 mL; with disposable tips.

(**m**) *Positive-displacement repeating pipet.*—Capable of accurately delivering 0.1, 0.2, 1.0, 2.5, 3.0, 4.0, 5.0, and 8.0 mL.

(**n**) *Dispenser.*—1000 mL capacity, capable of delivering 20 and 30 mL.

(**o**) *Glass test tubes*.—16 × 100, 18 × 150, and 16 × 150 mm.

(**p**) *Glass tubes.*—25 × 150 mm (approximate volume, 55 mL), with polytetrafluoroethylene (PTFE)-lined screw caps.

(**q**) *Glass beakers.*—50 mL.

(**r**) *Aluminum foil.*

(**s**) *Plastic film, or similarly nonreactive material.*

(**t**) *PTFE-coated magnetic stir bars.*—2.5 cm.

(**u**) *Magnetic stir plate.*

Reagents and Solutions (Specific to HW and AB Methods)

(**a**) *Acetate buffer.*—(*1*) *100 mM, pH 4.5*.—Weigh 6.0 g glacial acetic acid, and transfer immediately with distilled water rinses to a flask. Bring volume to ca 850 mL. Adjust pH

Table 2. Results for determinations of free glucose and starch + maltooligosaccharides corrected for free glucose in food and feed samples (dry matter basis)

Sample	DM, %[a]	Hot water		Acetate buffer		Extension of AOAC **996.11**	
		Mean	s_r[b]	Mean	s_r	Mean	s_r
		Free glucose					
Alfalfa silage	90.0	0.12	0.00	0.14	0.00	0.12	0.01
Corn grain, ensiled	93.5	0.44	0.00	0.38	0.00	0.18	0.00
Corn silage	91.1	0.05	0.00	0.07	0.00	0.07	0.00
Rice	90.4	0.18	0.01	0.20	0.01	0.02	0.02
Soybean meal	92.2	0.08	0.00	0.04	0.00	0.05	0.00
Split peas	91.4	0.07	0.00	0.04	0.00	0.02	0.02
Wheat flour	90.9	0.17	0.02	0.13	0.00	0.05	0.02
		Starch + maltooligosaccharides corrected for free glucose					
Alfalfa silage	90.0	1.5	0.1	1.6	0.0	1.4	0.0
Corn grain, ensiled	93.5	69.0	0.1	75.6	0.8	70.4	0.2
Corn silage	91.1	7.3	0.8	37.1	0.4	34.2	0.9
Rice	90.4	82.0	0.4	83.3	1.3	82.4	0.1
Soybean meal	92.2	1.4	0.0	0.8	0.0	0.9	0.0
Split peas	91.4	48.4	0.3	50.1	0.7	48.1	0.1
Wheat flour	90.9	74.3	0.5	79.6	0.2	74.8	0.1
		Average of feed/food samples					
Mean		40.6		46.9		44.6	
s_r		0.31		0.48		0.20	
CV, %[c]		2.57		1.55		0.78	

[a] DM = Dry matter.
[b] s_r = Standard deviation of replicates.
[c] CV = Coefficient of variation (s_r/mean).

to 4.5 with 1 M NaOH solution. Dilute to 1 L with water. (*2*) *100 mM, pH 5.0*.—Same procedure as for (**a**)(*1*), except pH is adjusted to 5.0.

(**b**) *Heat-stable α-amylase solution.*—Heat-stable α-amylase, ca 20 000 liquefon units/g, specific gravity 1.25 (Product Multifect AA 21L, Genencor International, Rochester, NY; origin: *Bacillus licheniformis*; pH optima, 5.5–5.8).

(**c**) *Amyloglucosidase solution.*—(*1*) *7 U/8 mL*.—Pipet 0.067 mL concentrated amyloglucosidase and dilute to 250 mL with 100 mM sodium acetate buffer, (**a**)(*1*). (*2*) *100 U/mL*.—Dilute 0.77 mL concentrated amyloglucosidase with 100 mM sodium acetate buffer, (**a**)(*2*), to 25 mL (concentrated amyloglucosidase, 3260 U/mL, Product E-AMGDF, Megazyme International Ireland, Ltd, Bray Co., Wicklow, Ireland; origin: *Aspergillus niger*; optimum pH, 4.0; stable pH, 4.0–5.5).

(**d**) *Glucose oxidase–peroxidase–aminoantipyrine buffer mixture.*—Mixture of glucose oxidase, 7000 U/L; peroxidase, 7000 U/L; and 4-aminoantipyrine, 0.74 mM. Prepare by dissolving 9.1 g Na_2HPO_4 and 5.0 g KH_2PO_4 in ca 300 mL distilled water in a volumetric flask. Use distilled water to rinse chemicals into bulb of flask. Swirl flask to dissolve completely. Add 1.0 g phenol (ACS grade) and 0.15 g 4-aminoantipyrine. Use distilled water to rinse chemicals into bulb of flask. Swirl flask to dissolve completely. Add glucose oxidase (7000 U) and peroxidase (7000 U), rinse enzymes into flask with distilled water, and gently swirl flask to dissolve contents without causing excessive foaming. Dilute to 1 L with distilled water. Seal flask and invert repeatedly to mix. Filter solution through a glass fiber filter with 1.6 μm retention. Store in a sealed amber bottle at ca 4°C. Determine standard curve for the reagent, using a 4-point standard curve with distilled water and (**e**), according to *Preparation of Reagent Blanks and Standard Curves*, (**b**)(*1*).

(**e**) *Glucose standard solutions.*—40, 60, and 80 μg/mL. Determine the dry matter of powdered crystalline glucose (purity ≥99.5%). Weigh approximately 20, 30, and 40 mg glucose, and record weight to 0.0001 g. Dissolve each portion separately in distilled water. Dilute each solution to 500 mL to obtain 3 independent glucose standard solutions. Multiply the weight of glucose by the percentage of dry matter, and divide

by 500 mL to calculate the glucose concentrations of the solutions.

Reagents and Solutions (Specific to ExtAOAC Method)

(**f**) *3-(N-Morpholino)propanesulfonic acid (MOPS) buffer.*—pH 7.0. Contains 50 mM MOPS and 5 mM calcium chloride. In 1 L volumetric flask, dissolve 11.55 g MOPS in 900 mL water, and adjust pH to 7.0 with 1 M HCl (ca 17 mL). Add 0.74 g $CaCl_2 \cdot 2H_2O$. Dilute to volume with water.

(**g**) *Heat-stable α-amylase solution.*—3000 U/mL. Dilute 1 mL α-amylase solution (in 50% glycerol) to 30 mL with MOPS buffer, (**f**) (origin: *Bacillus licheniformis*; optimum pH, 6.0–6.5; stable pH, 4.5–8.0). This reagent was supplied in the Total Starch Assay Kit (AOAC Method **996.11**; Megazyme International Ireland, Ltd).

(**h**) *Amyloglucosidase solution.*—AOAC method, 200 U/mL. Use directly without dilution. Solution is viscous; for dispensing, use positive displacement dispenser. This reagent was supplied in the Total Starch Assay Kit (AOAC Method **996.11**, Megazyme International Ireland, Ltd; origin: *Aspergillus niger*; optimum pH, 4.0; stable pH, 4.0–5.5).

(**i**) *Glucose oxidase–peroxidase–aminoantipyrine buffer mixture.*—Mixture of glucose oxidase, 12 000 U/L; peroxidase, 650 U/L; and 4-aminoantipyrine, 0.4 mM in a buffer containing KH_2PO_4, NaOH, 4-hydroxybenzoic acid, and sodium azide. This reagent was supplied in the Megazyme Total Starch Assay Kit (AOAC Method **996.11**, Megazyme International Ireland, Ltd).

(**j**) *Aqueous ethanol*.—About 80% (v/v). Dilute 80 mL 95% ethanol (laboratory grade) to 95 mL with distilled water.

(**k**) *Sodium acetate buffer, 200 mM, pH 4.5.*—Pipet 11.8 mL glacial acetic acid (ACS grade, 1.05 g/mL) into 900 mL water. Adjust pH to 4.5 with 1 M NaOH solution. Dilute to 1 L with water.

(**l**) *Glucose standard solution.*—1 mg/mL. This reagent was used as supplied in the Total Starch Assay Kit (AOAC Method **996.11**; Megazyme International Ireland, Ltd).

Preparation of Reagent Blanks and Standard Curves

(**a**) *Reagent blank.*—For each assay, tubes or beakers containing no sample and only the reagents added for each method were carried through the entire procedure. Absorbance values for the reagent blanks were subtracted from sample absorbance values.

(**b**) *Standard curves.*—(*1*) *HW and AB methods.*—Pipet 0.5 mL water and 40, 60, and 80 μg/mL glucose standard solutions, (**e**), in duplicate into the bottom of 16 × 150 mm glass culture tubes. Add 2.5 mL glucose oxidase–peroxidase reagent, (**d**), to each tube, using a positive displacement repeating pipet. Mix tubes on a Vortex mixer. Cover tubes with plastic film. Incubate in a 35°C water bath for 45 min. Cool in the dark for 10 min. Read absorbance at 505 nm. Calculate slope and intercept of Y = glucose (μg/mL) and X = absorbance at 505 nm. Use this standard curve to calculate glucose μg/mL in sample solutions. A new standard curve should be prepared with each new batch of reagent. (*2*) *ExtAOAC method*.—With each set of analyses, pipet 0.1 mL glucose standard, (**l**), into the bottom of each of four 16 × 100 mm glass tubes. Carry through analysis with glucose oxidase–peroxidase reagent, (**i**), with samples.

Procedures

(**a**) *HW method.*—Run D-glucose, corn starch, and a reagent blank with each set of test samples.

(*1*) Accurately weigh 90–100 mg purified and high starch samples or 190–200 mg of other test samples into the bottom of 50 mL glass beaker.

(*2*) Add a magnetic stir bar to beaker.

(*3*) Add 5 mL water to beaker with positive displacement repeating pipet and stir on magnetic stir plate to wet sample. Once sample is uniformly blended with water, add 15 mL water, and stir on magnetic stir plate to mix.

(*4*) Add 0.1 mL heat-stable α-amylase, (**b**). Stir on magnetic stir plate to mix.

(*5*) Seal beakers with aluminum foil, and incubate at 92°C in a forced-air oven for 1 h.

(*6*) After incubation, cool on bench for 0.5 h.

(*7*) Filter sample through glass wool in a funnel into a 100 mL volumetric flask, using water to rinse beaker and funnel, and quantitatively transfer the sample to the flask. Dilute to volume with water. Seal flask, and invert repeatedly to mix.

(*8*) Pipet 1 mL sample into a 50 mL volumetric flask. Add 8 mL 0.1 M sodium acetate buffer (pH 4.5), (**c**)(*1*), containing 7 U amyloglucosidase, and swirl gently to mix.

Note: Selection of flasks with volumes other than 50 mL can be used to achieve solution glucose concentrations that are readable within the standard curve.

(*9*) Cap flask tightly with foil, and incubate at 60°C in a forced-air oven for 30 min, swirling flasks every 10 min to mix.

(*10*) Cool flask on bench for 30 min; then dilute to volume with water. Seal flask, and invert repeatedly to mix. This solution is used directly in step *11*.

(*11*) Pipet 0.5 mL water (0 μg/mL glucose standard) and sample solutions into the bottoms of 16 × 150 mm glass test tubes in duplicate; use 2 tubes/sample solution. Add 2.5 mL glucose oxidase–peroxidase reagent, (**d**), to each tube, using a positive displacement repeating pipet. Mix tubes on a Vortex mixer. Place tubes in a rack, and cover with plastic film.

(*12*) Incubate in a 35°C water bath for 45 min. Cool in the dark for 10 min. Read absorbance at 505 nm. Use 0 μg/mL standard to zero the spectrophotometer. Average absorbance values for each sample, and use in *Calculations*.

Note: Free glucose is determined in samples carried through steps *1*–*7*, except that no α-amylase is added. Sample solutions are then subjected to steps *11* and *12*.

(**b**) *AB method.*—Run D-glucose, corn starch, and a reagent blank with each set of test samples.

(*1*) Accurately weigh 90–100 mg purified and high-starch samples or 190–200 mg of other test samples into 25 × 150 mm screw-cap glass tubes.

Table 3. Mean percentage recovery from starch analysis of purified glucose and starch samples[a]

	Hot water		Acetate buffer		Extension of AOAC **996.11**	
Sample	Mean	SD[b]	Mean	SD	Mean	SD
Glucose	100.5	0.4	100.9	0.4	95.6	0.2
	Starch corrected for free glucose					
Corn starch	93.9	1.9	98.3	0.3	93.4	1.1
Potato starch	91.2	0.3	97.0	0.3	94.8	1.3

[a] Recovery, %, was calculated as (measured/actual) × 100 by using values from duplicate analyses.
[b] SD = Standard deviation.

(*2*) Weigh tube, cap, and sample on a top-loading balance; record weight to 0.01 g.

(*3*) Dispense 30 mL 0.1 M sodium acetate buffer (pH 5.0), (**a**)(*2*), into the tube.

(*4*) Add 0.1 mL heat-stable α-amylase, (**b**). Cap tube, and mix on a Vortex mixer.

Note: Mix on a Vortex mixer so that the solution column extends to the cap, washing the entire interior of the tube.

(*5*) Incubate tube for 1 h at 100°C, mixing tube on a Vortex mixer at 10, 30, and 50 min of incubation.

(*6*) Cool tube on bench for 0.5 h.

(*7*) Add 1 mL amyloglucosidase solution, (**c**)*(2)*. Mix tube on a Vortex mixer.

(*8*) Incubate tube for 2 h at 60°C, mixing on a Vortex mixer at 1 h.

(*9*) Add 20 mL distilled water to tube, recap, and invert to mix.

(*10*) Weigh tube, cap, and contents on top-loading balance; record weight to 0.01 g.

(*11*) Transfer ca 1.5 mL sample solution to 2 mL microcentrifuge tube, and centrifuge at 12 000 × *g* for 10 min. Allow centrifuged solution to come to room temperature before preparing dilution.

(*12*) Prepare dilutions by weight of sample solutions so that they fall within the standard curve.

Note: Preparing dilutions by weight is useful with solutions that present pipetting difficulties, such as those that adhere to the interior of pipet tips, or rise several millimeters into rinsed pipet tips when the tip is placed vertically into the sample solution. Densities of sample solutions can be determined for the remainder of a sample by centrifuging it at ca 2060 × *g* to sediment particles, allowing the centrifuged solution to come to room temperature, and determining the weight of solution held by a 10 mL volumetric flask; these density values did not differ from those of the 12 000 × *g* centrifuged solutions. Sample solution densities have ranged from 0.997 to 1.00 g/mL. Water density has averaged 0.995 g/mL at 22–24°C. Dilutions may be prepared by volumetric methods if accuracy of sample solution pipetting is not an issue.

(*13*) Analyze diluted samples for glucose according to steps *11* and *12* in the HW method.

Note: Free glucose is determined for samples carried through steps *1–6* except that no α-amylase is added. Sample solutions are then subjected to steps *9–13*.

(**c**) *ExtAOAC method.*—The ExtAOAC method was performed according to instructions provided with the Total Starch Assay Kit (AOAC Method **996.11**; Megazyme International Ireland, Ltd). The kit method deviates from the AOAC protocol in that marbles were not used to cover tubes for the 30 min incubation described in (*5*), and samples containing >10% starch were diluted by pipetting (1 mL sample solution and 9 mL water) as described in *(6)* rather than by using volumetric flasks.

(*1*) Run D-glucose, corn starch, and reagent blanks with each set of test samples.

(*2*) Accurately weigh 90–100 mg ground test portion directly into glass test tube. Tap tube gently on laboratory bench to ensure that all particles drop to bottom of tube.

(*3*) Add 0.2 mL 80% aqueous ethanol, (**j**), to tube, and stir on Vortex mixer to ensure that test portion is wet. Add 3.0 mL heat-stable α-amylase, (**g**), and mix contents of tube on a Vortex mixer to ensure complete dispersion.

(*4*) Immediately place tube in boiling water bath for a 6 min incubation, mixing the tube vigorously on a Vortex mixer after 2 and 4 min.

(*5*) Place tubes in water bath set at 50°C, and let equilibrate 5 min. Add 4.0 mL 200 mM sodium acetate buffer, (**k**), and 0.1 mL amyloglucosidase solution, (**h**), and vigorously mix contents on Vortex mixer. Incubate 30 min at 50°C.

(*6*) Adjust the volume of each tube to 10 mL by adding 2.8 mL water by pipet. For samples containing 10–100% starch, an aliquot (1.0 mL) of the 10 mL volume is diluted to 10 mL with distilled water, and the resulting dilution is mixed thoroughly before proceeding. An aliquot of each sample solution was centrifuged at 3000 rpm (1000 × *g*) for 10 min.

(*7*) Carefully and accurately transfer a 0.1 mL aliquot of each supernatant to the bottom of a separate test tube; use 2 tubes/supernatant.

(*8*) Add 3.0 mL glucose oxidase–peroxidase–aminoantipyrine buffer mixture, (**i**), to each tube, and incubate 20 min at 50°C.

(*9*) Measure and record absorbance, *A*, of each test solution at 510 nm versus reagent blank. Average *A* values for each test and use in *Calculations*.

(*Note:* To correct the starch + maltooligosaccharide values for free glucose present in the sample, another set of duplicate tubes for all samples was processed through the method, but without addition of amylase or amyloglucosidase.)

Calculations

(**a**) *HW and AB methods*.—Calculate total starch + maltooligosaccharide content (percent, on a dry matter basis) in test sample as follows:

$$\text{Total starch + maltooligosaccharides, \%} = (A \times S + I) \times V \times DF \times 1/1000000 \times W \times DM \times 162/180$$

where A = absorbance of reaction solutions minus the absorbance of the reagent blank; S = slope and I = intercept of the standard curve to convert absorbance values to μg glucose; V = final sample solution volume; DF = dilution factor, e.g., 0.1 mL sample solution diluted to 5 mL = 5/0.1 = 50; 1/1 000 000 = conversion from μg to g; W = sample weight, as is; DM = dry matter content of the sample as a decimal; 162/180 = factor to convert free glucose, as determined, to anhydroglucose, which is present in starch.

Correction of enzymatic starch assay values for free glucose (on a starch basis as glucose × 0.9) = result as % of sample dry matter from enzymatic starch assay-free glucose on a starch basis as % of sample dry matter.

Final sample solution volume after all liquid additions for method AB were calculated as follows:

[(Final weight of tube, cap, sample, and reagents, g) – (initial weight of tube, cap, and sample, g)] /(average density of sample solutions, g/mL)

(**b**) *ExtAOAC method*.—Calculate total starch + maltooligosaccharide content (percent, on a dry matter basis) in test sample as follows:

$$\text{Total starch + maltooligosaccharide, \%} = A \times F \times V \times 1/1000 \times 100/W \times 162/180$$

where A = absorbance of reaction solutions read versus reagent blank; F = factor to convert absorbance values to μg glucose = 100 mg glucose/absorbance value for 100 mg glucose; V = volume correction, e.g., 0.1 mL taken from 100 mL = 1000, or 0.1 taken from 10 mL = 100; 1/1000 = conversion from μg to mg; 100/W = conversion to 100 mg test portion; 162/180 = factor to convert from free glucose, as determined, to anhydroglucose, which is present in starch.

Correction of enzymatic starch assay values for free glucose (on a starch basis as glucose × 0.9) = result as % of sample dry matter from enzymatic starch assay-free glucose on a starch basis as % of sample dry matter.

Results and Discussion

Standard Curves

Analysis of standard curves used with the HW and AB methods did not show linear ($P = 0.90$) or quadratic ($P = 0.13$) patterns for the residuals (actual minus predicted values, $n = 6$ standard curves). The mean ± standard deviation of the residuals for the curves was 0.0048 μg glucose/mL ± 0.127 over a range of 0–80 μg glucose/mL. The standard curves had an average slope of 172.23 ± 1.35, an intercept of –0.131 ± 0.146, and an R^2 of 0.9999 ± 0.0001 for the linear form of the curve. The single point 100 μg glucose/mL standard for ExtAOAC had a mean residual value of –0.0003 ± 0.196 (n = 3 standard determinations), and an average value of 93.02 ± 0.89. Two data points, one each from 2 runs were omitted for ExtAOAC. Their removal decreased the standard deviation of the standard determination in the runs from 1.72 to 0.27 and from 0.87 to 0.21. In these cases, a minimum of 3 data points remained to determine the value of the glucose standard. No data were omitted from the standard curves for HW or AB.

Recovery

Mean total free glucose values expressed on a starch basis (glucose × 0.9) as a percentage of dry weight are shown in Tables 1 and 2. Method ExtAOAC gave lower free glucose values for the food and feed samples tested than did the other 2 assays ($P = 0.05$). Recovery of purified glucose determined with the starch methods was greater for HW and AB than for ExtAOAC ($P = 0.05$), with HW and AB giving approximately 100% recovery (for glucose expressed on a starch basis, 90% of dry matter = 100% recovery; Table 3). Free glucose is a contaminant that must be corrected for in the starch assay, but failure to recover or destroy it completely in this assay is not desirable. A high recovery offers some assurance that glucose released from starch is not destroyed or undetected in the assay.

Method HW gave the lowest starch + maltooligosaccharide values for purified (Table 1) and food and feed substrates (Table 2), followed by ExtAOAC, with AB giving the greatest values ($P = 0.05$). Recovery values for corn starch and potato starch were greatest for AB and did not differ between HW and ExtAOAC ($P = 0.05$; Table 3). The lower values for procedures HW and ExtAOAC may be related to maltulose formation from starch during hydrolysis with heat-stable α-amylase at high temperatures at close to neutral pH. It is not certain why the corn silage starch + maltooligosaccharide value for HW was low, but it may have been an effect of the acids in the silage reducing pH and depressing enzyme function; this is an effect that buffers in the other assays would have reduced.

Limits of Determination

Limits of determination for starch based on absorbance values of undiluted reagent blanks used for methods AB and ExtAOAC were calculated as mean blank value + 3 × blank standard deviations (16). For AB, based on 1:1 to 1:3 dilution of reagent blanks converted back to an undiluted basis, and including results from assays performed outside of this study (5 blanks with 2 readings each), the mean absorbance ± standard deviation of undiluted blanks was 0.016 ± 0.004 for a detection limit of 0.028 absorbance. Undiluted reagent blank absorbance values for ExtAOAC (2 blanks with 2 readings each) read with the spectrophotometer zeroed versus distilled water were 0.024 ± 0.003, giving a detection limit of 0.033 absorbance. By using the calculations specific to each method

and average values for standard curves, starch detection limits were 0.2% for AB and 0.3% for ExtAOAC analysis of sample dry matter based on a 100 mg sample of 90% dry matter that required no dilution of the final volume for each assay.

Selectivity

All methods gave very low starch values for sucrose, indicating that run conditions and enzyme preparations used did not appreciably hydrolyze this common feed component, which has been shown to interfere with starch analysis (Table 1; 8). Use of separate free glucose determinations allowed correction for free glucose and background absorbance associated with each sample. The final detection method is specific for glucose, which limits interference from other carbohydrates. Without use of an aqueous ethanol pre-extraction, maltooligosaccharides present in the samples would be determined as starch, but the error should be consistent across methods and should be small with the sample types used in this study. Unless samples are known to contain no maltooligosaccharides, or the explicit intent is to measure starch + maltooligosaccharides, a pre-extraction with an aqueous ethanol solution should be performed to remove these oligosaccharides to exclude them from starch analysis (3, 10).

Repeatability

The standard deviations of replicates for starch + maltooligosaccharide analysis of the food and feed substrates and for purified substrates were low and did not differ among assays (Tables 1 and 2; $P > 0.36$). These values are comparable to or less than the repeatability standard deviation values (1.6–2.2) previously reported for method ExtAOAC when food or feed samples were analyzed (17).

Ease of Use

Both methods AB and ExtAOAC had the advantage that they allowed all additions to samples to be made in tubes, and they did not require a transfer of sample until the final dilution and measurement of glucose. With method AB, care did not need to be taken to ensure that samples were tapped to the bottom of the tube, as with method ExtAOAC, because the entire interior of the tube was rinsed with solution during mixing on a Vortex mixer. Measurement of the density of sample solutions in method AB may not be necessary, because of the consistency of the value over time (0.999 g/mL, standard deviation = 0.002, $n = 120$, from 16 analysis runs over 16 months). Dilution by weight used in method AB offered an accurate way to handle the sample solutions that present pipetting difficulties, as well as a check on the accuracy of dilution that is not possible with volumetric methods. Use of the same temperature for the amyloglucosidase and glucose analysis incubations in method ExtAOAC provided an economical use of laboratory resources that would be worthwhile to consider if revising method AB.

Recommendations

On the basis of achievement of greater values and recovery for starch + maltooligosaccharide analysis, very good repeatability among replicates, ease of handling of samples, and avoidance of known defects that can reduce the accuracy of other starch assays, the modification of the acetate buffer assay developed by Bach Knudsen (14) appears to be a viable candidate for full single-laboratory validation and collaborative study to establish an official method for determination of starch in animal feeds.

References

(1) Huntington, G.B. (1997) *J. Anim. Sci.* **75**, 852–867

(2) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **920.40**

(3) ISO 15914E (2004) *Animal Feeding Stuffs–Enzymatic Determination of Total Starch Content*, 1st Ed., International Organization for Standardization, Geneva, Switzerland

(4) Bernal-Santos, G., Perfield II, J.W., Barbano, D.M., Bauman, D.E., & Overton, T.R. (2003) *J. Dairy Sci.* **86**, 3218–3228

(5) Pham, T.H., Mauvais, G., Vergoignan, C., De Coninck, J., Cachon, R., & Feron, G. (2008) *Biotechnol. Lett.* **30**, 287–294

(6) *Approved Methods of the American Association of Cereal Chemists* (2000) 10th Ed., AACC International, St. Paul, MN, Method **776-11**

(7) Hodge, J.E., & Osman, E.M. (1976) in *Principles of Food Science, Part 1, Food Chemistry*, O.R. Fennema (Ed.), Marcel Dekker, Inc., New York, NY, p. 58

(8) Hall, M.B., Jennings, J.P., Lewis, B.A., & Robertson, J.B. (2000) *J. Sci. Food Agric.* **81**, 17–21

(9) Gaillard, B.D.E. (1958) *J. Sci. Food Agric.* **9**, 170–177

(10) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **996.11**

(11) Dias, F.F., & Panchal, D.C. (1987) *Starch/Stärke* **39**, 64–66

(12) Antrim, R.L., Solheim, B.A., Solheim, L., Auterinen, A.-L., Cunefare, J., & Karppelin, S. (1990) *Starch/Stärke* **43**, 355–360

(13) Holm, J., Björck, I., Drews, A., & Asp, N.-G. (1986) *Starch/Stärke* **38**, 224–226

(14) Bach Knudsen, K.E. (1997) *Anim. Feed Sci. Technol.* **67**, 319–338

(15) Application Note 322 (2000) YSI Inc., Yellow Springs, OH

(16) *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* (2002) AOAC INTERNATIONAL, Gaithersburg, MD

(17) McCleary, B.V., Gibson, T.S., & Mugford, D.C. (1997) *J. AOAC Int.* **80**, 571–579

J. Dairy Sci. 98:6340–6360
http://dx.doi.org/10.3168/jds.2015-9379

Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs

R. J. Higgs, L. E. Chase, D. A. Ross, and M. E. Van Amburgh[1]
Department of Animal Science, Cornell University, Ithaca, NY 14853

ABSTRACT

The Cornell Net Carbohydrate and Protein System (CNCPS) is a nutritional model that evaluates the environmental and nutritional resources available in an animal production system and enables the formulation of diets that closely match the predicted animal requirements. The model includes a library of approximately 800 different ingredients that provide the platform for describing the chemical composition of the diet to be formulated. Each feed in the feed library was evaluated against data from 2 commercial laboratories and updated when required to enable more precise predictions of dietary energy and protein supply. A multistep approach was developed to predict uncertain values using linear regression, matrix regression, and optimization. The approach provided an efficient and repeatable way of evaluating and refining the composition of a large number of different feeds against commercially generated data similar to that used by CNCPS users on a daily basis. The protein A fraction in the CNCPS, formerly classified as nonprotein nitrogen, was reclassified to ammonia for ease and availability of analysis and to provide a better prediction of the contribution of metabolizable protein from free AA and small peptides. Amino acid profiles were updated using contemporary data sets and now represent the profile of AA in the whole feed rather than the insoluble residue. Model sensitivity to variation in feed library inputs was investigated using Monte Carlo simulation. Results showed the prediction of metabolizable energy was most sensitive to variation in feed chemistry and fractionation, whereas predictions of metabolizable protein were most sensitive to variation in digestion rates. Regular laboratory analysis of samples taken on-farm remains the recommended approach to characterizing the chemical components of feeds in a ration. However, updates to the CNCPS feed library provide a database of ingredients that are consistent with current feed chemistry information and laboratory methods and can be used as a platform to formulate rations and improve the description of biology within the model.

Key words: feed composition, Cornell Net Carbohydrate and Protein System, modeling, methods, sensitivity

INTRODUCTION

Obtaining useful outputs from any biological model is very dependent on the quality of the information being used to perform a simulation (Haefner, 2005). The feed library in the Cornell Net Carbohydrate and Protein System (**CNCPS**) contains information not routinely available from commercial laboratories such as AA profiles, FA profiles, digestion rates (**kd**), and intestinal digestibilities (Tylutki et al., 2008). The feed library also provides commonly analyzed fractions that can be used as they are or updated by the user. Correct estimation of these chemical components is critical in enabling the CNCPS to best predict the ME, MP, and other specific nutrients available from a given ration (Offner and Sauvant, 2004; Lanzas et al., 2007a,b). Regular laboratory analysis of feeds will reduce the variation in model inputs to that derived from the sampling process, sample handling, preparation, and the variation of the assay itself (Hall and Mertens, 2012). However, in some situations, this is not possible and feed library values have to be relied on. In other situations, feed compositions are very consistent, meaning library values provide a reasonable estimation without laboratory analysis. The CNCPS feed library consists of approximately 800 ingredients, including forages, concentrates, vitamins, minerals, and commercial products, and serves as the reference database for describing the chemical composition of a diet. The origin of the feed library is from the work of Van Soest (1994, 2015), Sniffen et al. (1992), and related publications. The addition of AA to the feed library began

Received January 24, 2015.
Accepted May 25, 2015.
[1]Corresponding author: mev1@cornell.edu

with the publication of O'Connor et al. (1993). Many of the feed ingredients have been updated since that time, using data from more contemporary sources such as the National Research Council publications and other commercial feed additions through the CPM Dairy (University of Pennsylvania, Kennett Square, PA) effort, but not in a systematic or comprehensive manner. The objective of the current study was to evaluate and revise the CNCPS feed library to ensure that it is consistent with values being generated and used as inputs from commercial laboratories. A multistep approach was designed and used to combine current feed library information with new information and predict uncertain values. The intended methods for analyzing each major chemical component for use in the CNCPS are reported, as well as a sensitivity analysis of model outputs to variation in feed library inputs. An evaluation of model outputs and sensitivity relative to animal data is provided in a companion paper (Van Amburgh et al., 2015).

MATERIALS AND METHODS

Feed Chemistry

The chemical components considered in our study were those routinely analyzed by commercial laboratories and required by the CNCPS for evaluation and formulation of nutrient adequacy and supply. These include DM, CP, soluble protein (**SP**), ammonia, acid detergent-insoluble CP (**ADICP**), neutral detergent-insoluble CP (**NDICP**), acetic acid, propionic acid, butyric acid, lactic acid, other organic acids, water-soluble carbohydrates (**WSC**), starch, ADF, NDF, lignin, ash, ether extract (**EE**), and soluble fiber. Amino acids were also reviewed and updated. A list of the expected analytical procedures for measuring each chemical component and the units required by the CNCPS v6.5 are described in Table 1. Fractionation of chemical components from Table 1 into the pool structure of the CNCPS are described by Tylutki et al. (2008) and summarized in Table 2.

Calculation Procedure

To complete the analysis, data sets were provided by 2 commercial laboratories (Cumberland Valley Analytical Services Inc., Maugansville, MD, and Dairy One Cooperative Inc., Ithaca, NY). The compiled data set included 90 different ingredients and >100,000 individual samples. Additional means and standard deviations (**SD**) of individual feeds were sourced from the laboratory websites. The online resource for both laboratories includes >10 yr of data and an extensive collection of different ingredients. Each feed was evaluated for internal consistency and consistency against laboratory data. Internal consistency required each feed to adhere to the fractionation scheme summarized in Table 2. Briefly, equation [1] (Table 2) provides the relationship between carbohydrates (**CHO**), CP, EE, and ash. Carbohydrates are characterized as NDF, acetic, propionic, butyric, isobutyric, lactic, and other organic acids, WSC, starch, and soluble fiber. From equations [1], [4], and [5] in Table 2, equation [16] can be derived for the *j*th feed in the library:

$$100 = \mathrm{CP}_j + \mathrm{EE}_j + \mathrm{ash}_j + \mathrm{NDF}_j + \mathrm{acetic}_j + \mathrm{propionic}_j + \mathrm{isobutyric}_j + \mathrm{lactic}_j + + \text{other organic acids}_j + \mathrm{WSC}_j + \mathrm{starch}_j + \text{soluble fiber}_j. \quad [16]$$

Soluble fiber (CB2) is calculated in the CNCPS by difference (equation [5]). This means any error in the estimation of the CA1 (volatile fatty acids), CA2 (lactic acid), CA3 (other organic acids), CA4 (WSC)], or CB1 (starch) fractions will result in an over- or underestimation of soluble fiber. Also, error in the estimation of CP, EE, ash, or NDF will cause error in soluble fiber through the calculation of CHO (equation [1]) and the subsequent calculation of NFC (equation [4]). Other components, such as alcohols, are also included in soluble fiber within the current structure of the model. Overestimation of components in equation [16] can cause a situation where soluble fiber is forced to 0 and the sum of the equation is greater than 100% DM, which, theoretically, is chemically impossible. Feeds that did not adhere to the assumptions of equation [16] were updated. This rule can be problematic when the N content of protein deviates from 16%, in which a factor of 6.25 was used to convert the amount of N to an equivalent weight of protein (Van Soest, 1994). The mass of all proteins in the CNCPS are calculated as N × 6.25 despite the proper factor varying according to feed type (Van Soest, 1994). Therefore, for feeds high in NPN (urea, ammonium salts), equation 16 was allowed to exceed 100% DM. This is a legacy issue with the CNCPS and other formulation systems and would require considerable recoding to an N basis to overcome. However, future versions of the model will address this problem. Likewise, NDF in the data sets provided were not ash-corrected as recommended in Table 1, as these data were not available at time the analysis was conducted. The distributions of corn silage ash and NDF are in Figure 1. Both distributions are skewed to the left, which in the case of NDF, indicates ash contamination (Mertens, 2002). Over-estimation of NDF through

Table 1. Expected wet chemistry methods for analyzing feeds used in Cornell Net Carbohydrate and Protein System (CNCPS) v6.5

			Expected wet chemistry method for use in the CNCPS v6.5	
Chemical component	Abbreviation	Unit	Base reference[1]	Brief description
Dry matter	DM	%	AOAC 934.01	Gravimetric difference between dry and wet sample weights.
Crude protein	CP	% DM	AOAC 968.06	Nitrogen measured using a combustion N analyzer and multiplied by a factor of 6.25.
Soluble protein	SP	% CP	Procedure 3 of Licitra et al. (1996)	Crude protein soluble in borate-phosphate buffer including sodium azide. Nonprotein nitrogen is not subtracted. This is corrected within the framework of the model.
Ammonia	Ammonia	CPE[2] (% SP)	AOAC 941.04	Nitrogen measured by Kjeldahl on fresh feed samples and multiplied by a factor of 6.25 to convert to CPE.
Acid detergent-insoluble crude protein	ADICP	% CP	Procedure 4 of Licitra et al. (1996)	Residual nitrogen measured by combustion or Kjeldahl after completing the ADF procedure described below.
Neutral detergent-insoluble crude protein	NDICP	% CP	Procedure 4 of Licitra et al. (1996)	Residual nitrogen measured by combustion or Kjeldahl after completing the NDF procedure described below.
Volatile fatty acids, lactic acid and other organic acids	Acetic, propionic, butyric, isobutyric, lactic and other OA	% DM	(Siegfried et al., 1984)	A fresh sample (25 g) is weighed into an Erlenmeyer flask with 200 mL of distilled water, mixed, and refrigerated overnight. The sample is then blended and filtered through a 25-μm filter. The extract is then analyzed by HPLC according to Siegfried et al. (1984).
Water-soluble carbohydrate	WSC	% DM	(Hall, 2014)	Water-soluble carbohydrates analyzed using a phenol-sulfuric acid assay after a water extraction for 1 h at 40°C.
Starch	Starch	% DM	(Hall, 2015)	Enzymatic analysis after gelatinization with acetate buffer.
Acid detergent fiber	ADFom	% DM	AOAC 973.18	Acid detergent fiber, excluding ash, measured gravimetrically after an extraction with acid detergent and filtration on a 1.5-μm glass filter.
Neutral detergent fiber	aNDFom	% DM	(Mertens, 2002)	Neutral detergent fiber, excluding ash, measured gravimetrically after an extraction with neutral detergent, heat stable amylase, sodium sulfite, and filtration on a 1.5-μm glass filter.
Lignin	Lignin	% NDF	AOAC 973.18[3]	Acid detergent lignin applied to the fiber residue after completing an ADF extraction. Measured gravimetrically on an ash free basis.
Undigested neutral detergent fiber	uNDFom	% NDF	(Raffrenato, 2011)	Undigested aNDFom after completing a 240 h in vitro NDF digestibility and filtration on a 1.5-μm glass filter.
Ether extract	EE	% DM	AOAC 920.39	Measured gravimetrically after extraction with diethyl ether.
Soluble fiber	Soluble fiber	% DM	Not available	Calculated by difference within the model.
Ash	Ash	% DM	AOAC 942.05	Gravimetric difference between dry sample weight and dry sample weight after ashing.
Essential AA excluding Met and Trp	Arg, His, Ile, Leu, Lys, Phe, Thr, Val	% CP	AOAC 994.12	Sample is hydrolyzed with 6 *N* HCl for 21 h. An internal standard is added and HCl is evaporated. Hydrolysates are diluted with lithium citrate buffer and individual AA are measured by ion exchange chromatography.
Met	Met	% CP	AOAC 988.15	Sample is oxidized with performic acid for 16 h to form methionine sulfone, then hydrolyzed with 6 *N* HCl for 21 h and analyzed by ion exchange chromatography.
Trp	Trp	% CP	(Landry and Delhaye, 1992)	Sample is hydrolyzed with barium hydroxide for 16 h using 5-Methyltryptophan as an internal standard and analyzed by chromatography with fluorescence detection.

[1]AOAC methods were taken from AOAC International (2005).

[2]CPE = crude protein equivalents.

[3]Raffrenato and Van Amburgh (2011) provide details on improving recovery during filtration.

Table 2. Equations used by the Cornell Net Carbohydrate and Protein System (CNCPS) to calculate carbohydrate and protein fractions

Fraction[1]	Description	Equation[2,3]	Equation no.
CHOj	Carbohydrates	100 − CPj − EEj − Ash$j$	[1]
CCj	Indigestible fiber	(aNDFom$j$ × (Lignin$j$ × aNDFom$j$) × 2.4)/100 or, aNDFom$j$ × uNDFom$j$	[2]
CB3j	Digestible fiber	aNDFom$j$ − CCj	[3]
NFCj	Nonfiber CHO	CHOj − aNDFom$j$	[4]
CB2j	Soluble fiber	NFCj − CA1j − CA2j − CA3j − CA4j − CB1j	[5]
CA1j	Volatile fatty acids	Acetic$j$ + Propionic$j$ + (Butyric + Isobutyric)j	[6]
CA2j	Lactic acid	Lactic$j$	[7]
CA3j	Other organic acids	Organic acids$j$	[8]
CA4j	WSC	WSCj	[9]
CB1j	Starch	Starch$j$	[10]
PA1j[4]	Ammonia	Ammonia$j$ × (SPj/100) × (CPj/100)	[11]
PA2j	Soluble true protein	SPj × CPj/100 − PA1j	[12]
PB1j	Insoluble true protein	CPj − (PA1j − PA2j − PB2j − PCj)	[13]
PB2j	Fiber-bound protein	(NDICPj − ADICPj) × CPj / 100	[14]
PCj	Indigestible protein	ADICPj × CPj / 100	[15]
SUMj	Sum of composition	100 = CPj + EEj + ash$j$ + NDFj + acetic$j$ + propionic$j$ + isobutyric$j$ + lactic$j$ + + other organic acids$j$ + WSCj + starch$j$ + soluble fiber$j$	[16]

[1]Subscript j means the jth feed in the library.

[2]EE = ether extract; WSC = water-soluble carbohydrates; SP = soluble protein; ADICP = acid detergent-insoluble CP; NDICP = neutral detergent-insoluble CP; aNDFom = NDF assay with amylase, sodium sulfite and ash correction; uNDFom = undigested NDFom after a 240-h in vitro fermentation and ash correction.

[3]Chemical components are expressed as percent DM except: SP = % CP; ADICP = % CP; NDICP = % CP; ammonia = % SP; lignin = % NDF; uNDFom = unavailable aNDFom, % NDF.

[4]Previous versions of the CNCPS feed library use NPN for the PA1 fraction. This has been replaced with ammonia.

ash contamination could also influence estimates of kd. Commercial laboratories have addressed this issue through determination of NDF digestibility on an OM basis. Using NDF assay with amylase, sodium sulfite and ash correction (**aNDFom**) in future updates of the library is recommended to remove variance associated with ash contamination. Evaluation against laboratory data compared each individual feed in the feed library to the mean and SD of the corresponding feed in the databases available from the commercial laboratories. Each component within each feed was required to fall within 1 SD of the mean value from the laboratory data

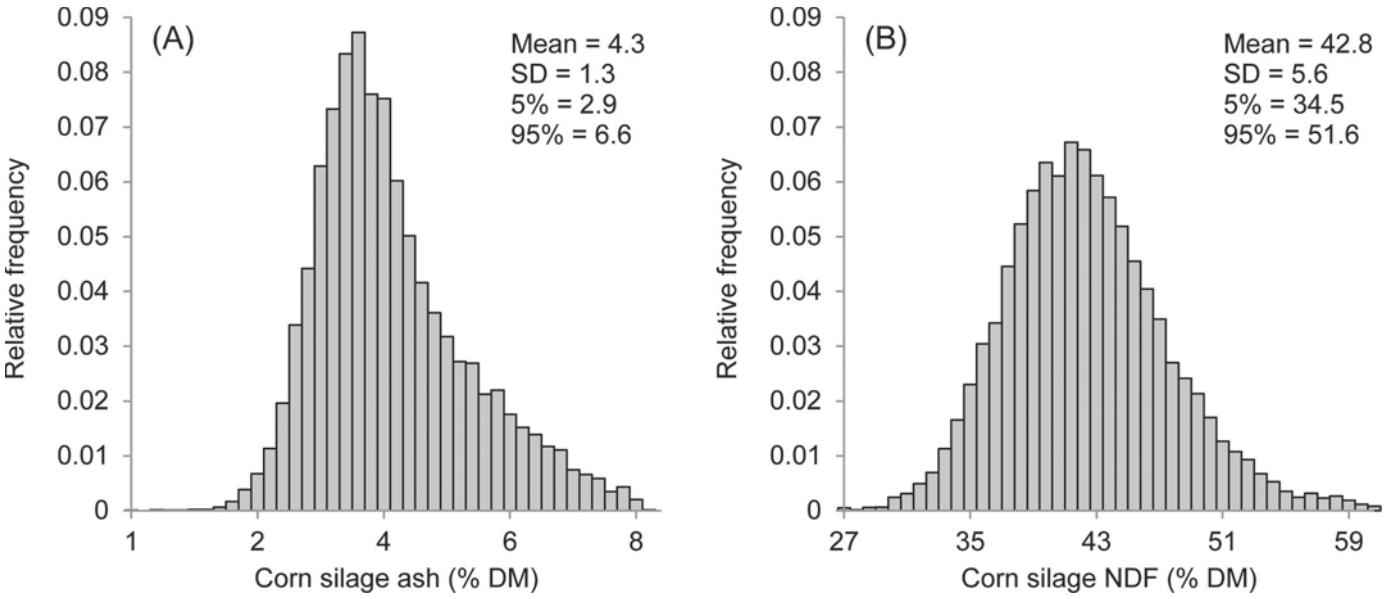


Figure 1. Frequency distributions for corn silage ash (A) and NDF (B) generated using commercial laboratory data sets (n = 21,000; Cumberland Valley Analytical Services Inc., Maugansville, MD, and Dairy One Cooperative Inc., Ithaca, NY).

Table 3. Predicting chemical components[1] of feeds using simple and multiple linear regression ($Y = A + BX_1 + CX_2 + DX_3$)

Feed name	Y	X_1	X_2	X_3	A	B	C	D	RMSE[2]	R^2
Barley silage	ADF	NDF	Lignin		−7.15	0.69	0.5		1.53	0.90
Corn silage	ADF	NDF			−3.67	0.68			1.28	0.89
Corn silage	Starch	NDF	CP		96.18	−1.18	−1.62		2.6	0.87
Fresh grass (high NDF)	ADF	NDF	Lignin	CP	0.47	0.54	0.75	−0.27	2.54	0.67
Fresh grass (low NDF)	ADF	NDF	Lignin	CP	5.84	0.45	0.51	−0.17	2.11	0.83
Fresh legume	ADF	NDF	Lignin		−6.31	0.69	0.52		1.53	0.88
Grass hay	ADF	NDF			3.57	0.57			3.21	0.69
Grass silage	ADF	NDF	Lignin		−0.25	0.57	0.47		1.79	0.85

[1]Expressed as percent DM, except lignin, which is expressed as percent NDF.
[2]RMSE = root mean square error.

set, or the entire feed would be updated. The calculation procedure consisted of 4 steps.

Step 1: Setting Descriptive Values

Chemical components used to differentiate different forms of the same feed were held constant during the recalculation process. The CNCPS has multiple options for many of the feeds in the feed library to give users the flexibility to pick the feed that best matches what they are feeding on the farm. For example, the feed library has 24 different options for processed corn silage that are differentiated on the basis of DM and NDF. Therefore, in this example, DM and NDF were maintained as they were in the original library whereas other components were recalculated.

Step 2: Linear Regression

In the second step, the data set provided was used to establish relationships among feed components using linear regression ($Y = A + BX_1 + CX_2 + DX_3$). Regression was used if components could be robustly predicted by other components within a feed ($R^2 > 0.65$). Regression equations were derived using the general linear modeling function in SAS (2010). Examples of some of the regression equations used are in Table 3.

Step 3: Matrix Regression

In the third step, factors that could not be predicted using standard linear regression were calculated using a matrix of regression coefficients derived from data generated using a Monte Carlo simulation (Law and Kelton, 2000). The Monte Carlo simulation was completed using @Risk version 5.7 (Palisade Corporation, Ithaca, NY). To complete the analysis, probability density functions were fit to each chemical component of each feed using the data provided by the commercial laboratories and the distribution fitting function in @Risk (Palisade, 2010a). A detailed description of the distributions used can be found in Palisade (2010a). Distributions were ranked on how well they fit the input data using the Chi-squared goodness of fit statistic. Equiprobable bins were used to adjust bin size in the Chi-square calculation to contain an equal amount of probability (Law and Kelton, 2000). The distribution with the lowest Chi-square was assigned to each component. Examples of the distribution derived for each chemical component for a range of feeds are in Table 4.

Components within each feed were then correlated with each other using laboratory data and the "define correlation" function in @Risk (Palisade, 2010a). If components were not correlated, they would change randomly relative to each other during the Monte Carlo simulation. Correlating the components meant that for each iteration, components changed in tandem relative to each other with the magnitude of the change depending on the assigned correlation coefficient (Law and Kelton, 2000). Spearman rank order correlations were used which determine the rank of a component relative to another by its position within the min-max range of possible values. Rank correlations can range between −1 and 1, with a value of 1 meaning components are 100% positively correlated, −1 meaning components are 100% negatively correlated, and 0 meaning no relationship exists between components (Law and Kelton, 2000). The correlation coefficients derived for a range of feeds used in the Monte Carlo simulation are in Table 5.

Once the probability density functions had been fit to each component, and components within each feed correlated, a Monte Carlo simulation was performed with 30,000 iterations. Various sampling techniques are available in @Risk to draw the sample from the probability density function (Palisade, 2010a). The Latin Hypercube technique was used to divide the distribution into intervals of equal probability and then randomly take a sample from each interval, forcing the simulation to represent the whole distribution (Shapiro, 2003). The raw data from the simulation was then used

Table 4. Mean, SD, distribution, and distribution parameters for each chemical component of each feed used to perform Monte Carlo simulations

Feed name and chemical components[1,2]	Mean	SD	Distribution	Distribution parameters[3] A	B	C	D
Corn silage							
CP	8.0	0.90	Loglogistic	0.6	7.4	14.1	
SP	56.4	9.61	BetaGeneral	75.0	7.2	−238.8	84.3
ADICP	7.7	1.86	Loglogistic	−0.1	7.6	7.1	
NDICP	14.0	3.24	Pearson5	16.9	214.0	0.6	
NDF	42.5	5.08	Loglogistic	14.5	27.8	9.4	
Lignin	7.1	1.00	Loglogistic	−5.4	12.5	21.6	
Starch	33.0	7.11	Weibull	10.1	65.7	−29.7	
WSC	1.6	0.97	Pearson5	3.4	3.9	0.0	
EE	3.3	0.48	Logistic	3.3	0.3		
Ash	4.3	1.14	Extvalue	3.8	1.0		
Alfalfa silage							
CP	21.7	2.83	Normal	21.7	2.9		
SP	60.0	9.07	Logistic	60.1	5.3		
ADICP	7.2	2.10	Loglogistic	1.9	5.0	4.3	
NDICP	14.6	4.95	Pearson5	13.2	224.6	−3.6	
NDF	42.5	5.24	Loglogistic	−17.0	59.3	19.5	
Lignin	17.2	2.34	Logistic	17.3	1.3		
Starch	1.9	0.88	Loglogistic	−0.6	2.4	4.8	
WSC	3.4	1.95	Loglogistic	0.1	2.9	2.8	
EE	3.7	0.81	Lognorm	77.3	0.8	−73.6	
Ash	11.0	1.80	Loglogistic	4.8	6.0	5.9	
Grass hay							
CP	10.9	3.46	Lognorm	15.0	3.7	−3.9	
SP	31.3	6.21	Loglogistic	−43.6	74.7	20.8	
ADICP	9.1	4.12	Pearson5	6.9	64.7	−1.5	
NDICP	32.6	7.68	Loglogistic	−22.3	54.5	12.2	
NDF	62.6	7.95	Logistic	62.6	4.6		
Lignin	8.7	2.37	Loglogistic	1.3	7.1	5.5	
Starch	2.2	1.27	Invgauss	3.3	17.7	−1.1	
WSC	6.8	2.69	Loglogistic	−22.8	29.4	18.2	
EE	2.5	0.72	Pearson5	46.3	226.4	−2.5	
Ash	7.7	2.27	Logistic	7.7	1.3		
Corn grain							
CP	8.6	0.70	Loglogistic	3.6	5.0	12.5	
SP	17.6	5.59	Logistic	17.6	3.3		
ADICP	7.1	2.28	Loglogistic	−0.5	7.4	5.4	
NDICP	11.9	3.81	Lognorm	13.0	4.1	−1.0	
NDF	11.4	1.30	Loglogistic	−0.8	12.2	16.4	
Lignin	15.7	4.73	Loglogistic	1.9	13.4	4.6	
Starch	72.1	1.49	Logistic	72.1	0.8		
WSC	2.5	0.62	Loglogistic	−1.6	4.0	11.3	
EE	3.7	0.52	Logistic	3.7	0.3		
Ash	1.5	0.29	Loglogistic	0.7	0.8	5.2	
Soybean meal							
CP	53.1	1.72	Logistic	53.1	1.0		
SP	24.3	6.75	Lognorm	61.6	7.0	−37.2	
ADICP	2.8	1.45	Loglogistic	−1.0	3.6	4.2	
NDICP	13.4	4.17	Logistic	13.0	2.8		
NDF	11.1	1.91	Pearson5	11.0	65.7	4.7	
Lignin	9.1	3.69	Logistic	9.1	2.5		
Starch	1.1	0.49	Loglogistic	−1.2	2.3	7.5	
EE	1.7	0.68	Loglogistic	−0.2	1.8	4.6	
Ash	7.6	0.77	Logistic	7.6	0.4		
Blood meal[4]							
CP	104.5	3.57	Weibull	14.1	45.2	60.8	

[1]WSC = water-soluble carbohydrates; SP = soluble protein; ADICP = acid detergent-insoluble CP; NDICP = neutral detergent-insoluble CP; EE = ether extract.

[2]Chemical components are expressed as % of DM except: SP = % of CP; ADICP = % of CP; NDICP = % of CP; Lignin = % of NDF.

[3]A, B, C and D are the parameters that define the characteristics of each distribution: BetaGeneral, A = Shape, B = Shape, C = Min value, D = Max value; ExtValue, A = Location, B = Scale; Invgauss, A = Mean, B = Variance, C = Shift; Logistic, A = Location, B = Scale, Loglogistic, A = Location, B = Scale, C = Shape; Lognorm, A = Mean, B = Variance, C = Shift; Normal, A = Mean, B = SD; Pearson5, A = Shape, B = Scale, C = Shift; Weibull, A = Shape, B = Scale, C = Shift.

[4]Blood meal CP can be >100% DM if nitrogenous components are >16% N.

Table 5. Spearman rank correlation coefficients for the chemical components of feeds used to perform Monte Carlo simulations[1,2]

Item	CP	SP	ADICP	NDICP	NDF	Lignin	Starch	WSC	EE	Ash
Corn silage										
CP	1.00									
SP	0.11	1.00								
ADICP	−0.19	−0.27	1.00							
NDICP	−0.12	−0.55	0.39	1.00						
NDF	0.18	−0.10	0.41	0.46	1.00					
Lignin	0.08	−0.09	0.25	0.15	0.05	1.00				
Starch	−0.37	0.09	−0.39	−0.38	−0.91	−0.10	1.00			
WSC	0.07	−0.30	0.09	0.11	0.09	−0.06	−0.25	1.00		
EE	0.18	0.37	−0.27	−0.27	−0.29	−0.01	0.30	−0.28	1.00	
Ash	0.35	−0.08	0.26	0.12	0.35	0.30	−0.50	0.07	−0.16	1.00
Alfalfa silage										
CP	1.00									
SP	0.18	1.00								
ADICP	−0.52	−0.23	1.00							
NDICP	−0.31	−0.57	0.67	1.00						
NDF	−0.62	−0.18	0.54	0.56	1.00					
Lignin	0.27	0.13	0.23	−0.02	−0.21	1.00				
Starch	−0.25	−0.15	0.01	0.01	−0.08	−0.13	1.00			
WSC	0.17	−0.62	−0.27	−0.14	−0.42	−0.10	0.18	1.00		
EE	0.27	0.45	−0.16	−0.14	−0.12	−0.16	−0.07	−0.56	1.00	
Ash	0.18	0.20	0.02	−0.16	−0.12	0.22	−0.18	−0.17	0.05	1.00
Grass hay										
CP	1.00									
SP	0.07	1.00								
ADICP	−0.43	−0.21	1.00							
NDICP	−0.11	−0.42	0.48	1.00						
NDF	−0.51	−0.11	0.27	0.36	1.00					
Lignin	0.04	−0.03	0.55	0.25	−0.04	1.00				
Starch	−0.10	−0.07	0.10	−0.04	−0.24	0.10	1.00			
WSC	0.09	0.24	−0.48	−0.46	−0.65	−0.31	0.13	1.00		
EE	0.51	−0.13	−0.27	−0.11	−0.60	0.05	0.09	0.34	1.00	
Ash	0.50	0.10	−0.16	−0.06	−0.55	−0.18	−0.01	0.01	0.23	1.00
Corn grain										
CP	1.00									
SP	0.17	1.00								
ADICP	−0.10	−0.19	1.00							
NDICP	−0.18	−0.11	0.43	1.00						
NDF	0.05	0.02	0.10	0.34	1.00					
Lignin	0.19	−0.07	0.17	−0.07	−0.24	1.00				
Starch	−0.40	−0.16	0.13	0.00	−0.56	0.01	1.00			
WSC	0.03	0.34	−0.11	−0.05	0.04	0.16	−0.20	1.00		
EE	0.21	0.22	−0.25	−0.16	0.24	0.14	−0.48	0.23	1.00	
Ash	0.15	0.23	0.00	0.03	−0.01	−0.02	−0.14	0.00	0.22	1.00
Soybean meal										
CP	1.00									
SP	−0.03	1.00								
ADICP	0.10	−0.62	1.00							
NDICP	−0.36	−0.39	0.14	1.00						
NDF	−0.15	−0.31	0.06	0.20	1.00					
Lignin	−0.03	−0.09	0.32	−0.35	−0.18	1.00				
Starch	−0.02	0.00	−0.16	−0.54	−0.18	0.27	1.00			
WSC[3]										
EE	0.08	−0.24	−0.03	0.44	0.21	−0.14	−0.19		1.00	
Ash	−0.26	−0.06	0.04	−0.01	−0.34	0.10	0.04		0.03	1.00

[1]WSC = water-soluble carbohydrates; SP = soluble protein; ADICP = acid detergent-insoluble CP; NDICP = neutral detergent-insoluble CP; EE = ether extract.

[2]Chemical components are expressed as % of DM except: SP = % of CP; ADICP = % of CP; NDICP = % of CP; Lignin = % of NDF.

[3]Row left blank because insufficient data was available to perform the analysis.

to construct a matrix of regression estimates in the arrangement shown below and according to the general form $Y_{ij} = A + BX_i$, where Y is the response variable and column vector for the ith component in the jth feed with n entries, A is the intercept arranged in an $n \times p$ matrix, B is the predictor variable arranged in an $n \times p$ matrix, and X is the regression coefficient and row vector for the ith component with n entries:

$$Y = \begin{pmatrix} Y_1 \\ Y_2 \\ \vdots \\ Y_n \end{pmatrix}, A = \begin{pmatrix} A_{11} \cdots A_{1p} \\ A_{21} \cdots A_{2p} \\ \vdots \ddots \vdots \\ A_{n1} \cdots A_{np} \end{pmatrix}, B = \begin{pmatrix} B_{11} \cdots B_{1p} \\ B_{21} \cdots B_{2p} \\ \vdots \ddots \vdots \\ B_{n1} \cdots B_{np} \end{pmatrix}, X = \begin{pmatrix} X_1 \\ X_2 \\ \vdots \\ X_n \end{pmatrix},$$

In this arrangement, if $Y_n = X_n$, $A_{np} = 0$ and $B_{np} = 1$. For example, if Y_1 was the response variable CP, then the predictor variable X_1 would also be CP and the relationship would have an intercept of 0 and slope of 1. Therefore, equations where $Y_n = X_n$ were excluded from the matrix. The weighted mean of response variables were calculated across each row of the matrix. The coefficients used to correlate each probability density function for the Monte Carlo simulation (Table 5) were normalized to sum to 1 and then used as weights (W) in the weighted mean $\left(\text{i.e., } \sum_{i=1}^{n} W_i = 1, \text{ and therefore } \bar{Y} = \sum_{i-1}^{n} W_i X_i\right)$. Using correlation coefficients as weights meant components within a specific feed that were more highly correlated had more influence on the mean and vice versa.

Components calculated using this method varied depending on the data available for a specific feed. To avoid confounding, components within a feed that were calculated by the matrix were not used as predictor variables for other components in the matrix. Therefore, the number of components calculated using the matrix was limited to avoid running out of predictor variables. Typically, nitrogenous components (SP, ammonia, NDICP, ADICP) not calculated in the preceding steps and not factors in equation [16] were calculated in this step.

Step 4: Optimize to a Final Solution

Lastly, components that were not assigned values in any of the preceding steps were calculated using an optimization. RISKOptimizer version 5.7 (Palisade Corporation) was used to perform the optimization, which uses a genetic algorithm simulation to find solutions when uncertainty exists around the values (Palisade, 2010b). Minimum and maximum boundaries for each component within a feed were set to constrain the optimizer to a likely range of values. The data used to calculate the range in each component was taken from the databases available online from the commercial laboratories. Each range was calculated as the mean plus or minus the SD of each component multiplied by global coefficient that was adjusted to allow the optimizer to converge. Typically, the coefficient used was between 0.5 and 1.5, meaning the range for each component was the mean plus or minus 0.5 to 1.5 times the SD of each component. An example of the constraints used to optimize corn silage is in Table 6.

The second constraint applied to the optimization was the relationship described by equation [16]. Components included in the optimization were, therefore, adjusted within the calculated range to the most likely

Table 6. Minimum and maximum boundaries used to constrain the chemical components of corn silage during optimization in step 4 of the procedure used to update the CNCPS feed library

			Optimizer boundaries (1.5 × SD)	
Chemical component[1]	Mean	SD	Minimum	Maximum
DM	33.8	10.3	18.3	49.2
CP	8.2	1.0	6.7	9.8
SP (% of CP)	53.4	10.1	38.3	68.5
Ammonia (% of SP)	13.4	6.2	4.1	22.7
ADICP (% of CP)	7.5	1.8	4.8	10.2
NDICP (% of CP)	15.2	3.8	9.6	20.9
Acetic	2.4	1.5	0.1	4.6
Propionic	0.3	0.3	0.0	0.9
Butyric	0.0	0.0	0.0	0.2
Lactic	4.7	2.2	1.4	8.1
Other OA	0.0	0.0	0.0	0.0
WSC	2.1	1.3	0.2	4.0
Starch	31.3	7.5	20.0	42.6
ADF	26.1	4.1	20.0	32.2
NDF	44.1	6.0	35.1	53.1
Lignin (% of NDF)	7.6	1.5	5.3	9.9
Ash	4.2	1.2	2.5	6.0
EE	3.3	0.5	2.6	4.0

[1]Expressed as % of DM unless otherwise stated. WSC = water-soluble carbohydrates; SP = soluble protein; ADICP = acid detergent-insoluble CP; NDICP = neutral detergent-insoluble CP; Other OA = other organic acids; EE = ether extract.

values in which equation [16] summed to 100% DM. The optimization step was completed last in the calculation process to fit the components within each feed together within the described constraints. The process was dynamic in that the values calculated in the optimization fed back into the matrix and regression calculations described above. Typically, the optimizer had to be run numerous times before it would converge and stabilize. If insufficient data were available to perform any of the calculation steps described above, current CNCPS library values were retained. The approach was not acceptable for proprietary feeds due to a lack of robust data of chemical components or the functional nature of some ingredients beyond the nutrient content. For example, products such as Met analogs are partially absorbed through the rumen wall and do not completely flow to the small intestine, yet the supply of Met to the animal or the conservation of the AA due to the supplementation of the analog is documented (Chen et al., 2011). Conventional chemical analysis does not adequately estimate the true nutrient supply for these types of feeds. Current library values were retained in these circumstances. Approximately 75% of the feeds in the feed library were updated and 25% remained unchanged. Those remaining unchanged were primarily commercial products, minerals, and vitamins, along with unusual feeds with little information within the databases.

AA

In addition to the chemical components described above, each feed in the CNCPS feed library includes a profile of the 10 essential AA. Amino acid profiles were updated using data sets provided by Evonik Industries AG (Hanau, Germany), Adisseo (Commentry, France), and taken from the NRC (2001). Data provided were mean values from analyses completed in the respective companies' laboratories or published in the NRC (2001). In all cases, AA analyses were completed on the whole feed and are expressed in the CNCPS on a percent CP basis. This differs from previous versions of the CNCPS, where AA were expressed as a percent of the buffer-insoluble residue (O'Connor et al., 1993). The most appropriate profile was assigned based on data availability and was used as received by the source without alteration. If profiles for specific feeds were not available in the data sets provided, current CNCPS values were retained. Proprietary feeds were not changed.

Model Sensitivity

The sensitivity of model outputs to variation in feed library inputs was also evaluated. The analysis was split into 2 parts. Part 1 looked at the likely range in 6 major chemical components in the diet: (1) CP, (2) starch, (3) NDF, (4) lignin, (5) ash, and (6) EE; and 4 model outputs: (1) ME-allowable milk, (2) MP-allowable milk, (3) MP from RUP, and (4) MP from bacteria. To complete this part of the analysis, a reference diet was constructed in a spreadsheet version of the CNCPS (Van Amburgh et al., 2013). The diet was formulated using ingredients typically found in North American dairy cattle rations and was balanced to provide enough ME and MP for a mature, nonpregnant, 600-kg cow in steady state (0 energy balance) to produce 35 kg of milk containing 3.1% true protein and 3.5% fat (Table 7). Probability density functions were fit to chemical components within each feed in the reference diet (Table 4) and correlated to each other with Spearman rank order correlations (Table 5) using @Risk version 5.7 (as previously described). Frequency distributions for model outputs were then generated using a Monte Carlo simulation with 10,000 iterations to describe the range of possible outcomes for each output and the relative likelihood of occurrence.

Part 2 of the analysis investigated which feed library inputs for the feeds in the reference diet had the most influence on selected model outputs: (1) ME-allowable milk, (2) MP-allowable milk, (3) MP from RUP, and (4) MP from bacteria. The feed library inputs investigated were those described in part 1 of the analysis, as

Table 7. Diet ingredients, chemical composition, and model predicted ME and MP for the reference diet used to analyze model sensitivity

Item[1]	Unit
Diet ingredient (kg of DM)	
Corn silage	4.76
Alfalfa silage	3.14
Grass hay	4.03
Corn grain ground fine	6.48
Soybean meal solvent extracted	2.58
Blood meal	0.20
Minerals and vitamins	0.50
Total DMI	21.69
Diet composition (% of DM unless stated)	
CP	16.7
SP (% of CP)	35.3
ADICP (% of CP)	6.4
NDICP (% of CP)	15.6
WSC	3.5
Starch	29.0
NDF	31.8
Lignin (% of NDF)	11.5
EE	3.0
Ash	7.7
Model outputs	
ME (Mcal/d)	53.7
MP (g/d)	2,385

[1]WSC = water-soluble carbohydrates; SP = soluble protein; ADICP = acid detergent-insoluble CP; NDICP = neutral detergent-insoluble CP; EE = ether extract.

well as kd for the carbohydrate and protein fractions summarized in Table 2. Probability density functions were fit to each chemical component within each feed as previously described. Program Evaluation and Review Technique (**PERT**) distributions (Cottrell, 1999) were used to describe the variation in kd. The PERT distribution is similar to a β or triangular distribution and is useful to describe variation in a situation where limited data exists (Johnson, 1997). The PERT distribution requires 3 estimates: (1) the most likely result; (2) the minimum expected result; and (3) the maximum expected result. Most likely results were set as CNCPS feed library values. Minimum and maximum values were set as the most likely value ±2 SD to encompass approximately 95% of the expected data without including extreme results (Table 8). Data on kd are scarce and, other than the CB3 fraction, are not routinely estimated for model input. Variation in kd changes proportionally to changes in mean values (Weiss, 1994). Therefore, in situations where data were not available, the proportional variation relative to the mean of other known feeds was used as a proxy to calculate the minimum and maximum values of unknown feeds. The CB3 kd was calculated for the forage feeds in the reference diet using lignin × 2.4 and 30-h in vitro NDF digestibility as described by Van Amburgh et al. (2003). Variation in kd for fractions other than CB3 were estimated from literature values. Fractions CA1–4 and CB1–2 kd were estimated from the soluble and potentially degradable fractions presented in Offner et al. (2003). The PB2 fractions (fiber-bound protein) were set to equal the CB3 fractions as described by Van Amburgh et al. (2007), The PB1 values were taken from the NRC (2001) and PA2 values were estimated from Broderick (1987). Correlation coefficients among components were not assigned for this part of the analysis as the interest was in understanding model sensitivity to individual components independent of correlated changes in composition. To complete the analysis, a Monte Carlo simulation with 10,000 iterations was performed. Changes in model outputs resulting from a 1 SD increase in model inputs were captured and are presented in Figures 2, 3, and 4.

RESULTS AND DISCUSSION

Analytical Techniques and Fractionation

The required procedures to most appropriately characterize the chemical components of feeds for version 6.5 of the CNCPS are described in Table 1. Chemical components and fractionation of feeds in the updated library were maintained in the format described by Tylutki et al. (2008) with the exception of the protein A1 fraction. Previously this has been classified as NPN, which is measured as the nitrogen passing into the filtrate after extraction of the soluble component with borate-phosphate buffer and precipitation of the true protein fraction from the supernatant with 10% trichloroacetic acid (Krishnamoorthy et al., 1982). The protein A1 fraction is typically assumed completely degraded in the rumen (Lanzas et al., 2007b). However, small peptides and free AA not precipitated by this method are still nutritionally relevant to the animal if they escape rumen degradation and flow through to the small intestine (Givens and Rulquin, 2004). Choi et al. (2002) suggested 10% of the AA flowing through to the small intestine originated from dietary NPN sources that, under the previous approach within the CNCPS, were unaccounted for. Reynal et al. (2007) conducted a similar study and measured soluble AA flows at the omasum ranging from 9.2 to 15.9% of total AA flow. Likewise, Velle et al. (1997) infused free AA into the rumen at various rates and showed that up to 20% could escape degradation and flow through to the small intestine, which is in agreement with data from Volden et al. (1998). Van Amburgh et al. (2010) suggested it might be more appropriate to redefine the protein A1 fraction from NPN as described by Krishnamoorthy et al. (1982) to ammonia. This would shift small peptides and free AA currently associated with the A1 fraction into the A2 fraction, where they could contribute to MP supply, and also refines the prediction of rumen N balance as less N is degraded in the rumen. Ammonia has the advantage of being easily measured and available from most commercial laboratories. Therefore, the NPN fraction in previous feed libraries has been updated to ammonia in version 6.5 (Van Amburgh et al., 2013).

Amino acid profiles from the original feed database (O'Connor et al., 1993) were determined on the insoluble protein residue and analyzed using a single acid hydrolysis with 6 *N* HCl for 24 h (Macgregor et al., 1978; Muscato et al., 1983). During acid hydrolysis, Met is partially converted to methionine sulfoxide, which cannot be quantitatively recovered, and Trp is completely destroyed (Allred and MacDonald, 1988). Methionine is typically considered one of the most limiting AA in dairy cattle diets (Schwab et al., 1992; Armentano et al., 1997; Rulquin and Delaby, 1997) and is frequently the target of supplementation (Schwab, 1996). Therefore, updating AA profiles in the feed library, particularly Met, was an important part of improving overall model predictions. The AA profiles used to update the feed library were analyzed on a whole-feed basis, rather than on the insoluble protein residue. The insoluble protein residue was originally assumed to have a greater probability of escaping the rumen and was more likely to

Table 8. Parameters used to specify program evaluation and review technique (PERT) distributions (mean, minimum, and maximum) and SD for the digestion rates of carbohydrate and protein fractions of feeds in the reference diet used to analyze model sensitivity

	Carbohydrate and protein fractions[1]												
Item	CA1	CA2	CA3	CA4	CB1	CB2	CB3	CC	PA1	PA2	PB1	PB2	PC
Corn silage													
Mean	0.0	7.8	5.6	22.3	35.7	33.5	3.8	0.0	200.0	50.0	20.0	3.8	0.0
SD	0.0	3.5	2.5	10.0	16.1	15.1	0.7	0.0	15.1	6.6	5.2	0.7	0.0
Minimum	0.0	0.2	0.1	0.2	0.6	0.7	1.9	0.0	161.1	32.8	6.8	1.9	0.0
Maximum	0.0	18.2	13.0	52.4	82.8	78.6	5.6	0.0	238.4	66.8	33.4	5.7	0.0
Alfalfa silage													
Mean	0.0	7.0	5.0	20.0	30.0	35.0	7.0	0.0	200.0	45.0	16.0	7.0	0.0
SD	0.0	1.4	1.0	4.0	6.0	7.0	1.4	0.0	15.1	6.0	5.0	1.4	0.0
Minimum	0.0	3.4	2.5	9.9	14.6	17.1	3.5	0.0	161.3	29.7	3.3	3.4	0.0
Maximum	0.0	10.5	7.6	30.1	45.2	52.8	10.5	0.0	238.9	60.2	28.6	10.5	0.0
Grass hay													
Mean	0.0	7.0	5.0	40.0	30.0	30.0	4.5	0.0	200.0	20.0	14.0	4.5	0.0
SD	0.0	1.4	1.0	8.0	6.0	6.0	1.0	0.0	15.1	2.7	5.1	1.0	0.0
Minimum	0.0	3.5	2.4	19.8	14.6	14.8	1.9	0.0	161.4	13.2	0.7	1.9	0.0
Maximum	0.0	10.6	7.6	60.7	45.3	45.3	7.1	0.0	238.9	26.8	27.1	7.1	0.0
Corn grain													
Mean	0.0	7.0	5.0	40.0	15.0	20.0	6.0	0.0	200.0	16.0	9.0	6.0	0.0
SD	0.0	2.4	1.7	14.0	5.2	7.0	1.2	0.0	15.1	2.1	2.8	1.2	0.0
Minimum	0.0	0.8	0.4	4.1	1.6	2.3	2.8	0.0	161.0	10.6	1.9	2.8	0.0
Maximum	0.0	13.2	9.5	76.7	28.6	38.0	9.2	0.0	238.8	21.4	16.1	9.1	0.0
Soybean meal													
Mean	0.0	7.0	5.0	40.0	25.0	30.0	6.0	0.0	200.0	24.0	11.0	6.0	0.0
SD	0.0	2.2	1.6	12.5	7.8	9.4	1.2	0.0	15.1	3.2	2.7	1.2	0.0
Minimum	0.0	1.4	1.0	7.9	5.2	5.8	2.9	0.0	161.3	15.9	4.2	2.8	0.0
Maximum	0.0	12.5	9.0	71.9	45.3	53.9	9.1	0.0	238.8	32.1	17.8	9.2	0.0
Blood meal													
Mean	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	200.0	13.5	3.7	0.0	0.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.1	1.8	1.9	0.0	0.0
Minimum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	161.4	8.9	0.0	0.0	0.0
Maximum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	238.4	18.1	9.7	0.0	0.0

[1]CA1 = acetic + propionic + butyric + isobutyric; CA2 = lactic; CA3 = other organic acids; CA4 = WSC; CB1 = starch; CB2 = soluble fiber; CB3 = digestible fiber; CC = indigestible fiber; PA1 = ammonia; PA2 = soluble true protein; PB1 = insoluble true protein; PB2 = fiber-bound protein; PC = indigestible protein.

match the AA profile of the RUP fraction (Macgregor et al., 1978). However, Tedeschi et al. (2001) investigated this hypothesis and found no differences in AA profiles of feeds analyzed with or without extraction of the soluble fraction. Furthermore, the soluble fraction of feeds has been shown to contribute 10 to 20% to the flow of AA to the small intestine (Velle et al., 1997; Volden et al., 1998; Choi et al., 2002). Extracting the insoluble protein residue requires soaking samples in borate-phosphate buffer to remove the soluble fraction (Krishnamoorthy et al., 1982) and adds another step to AA analysis. Therefore, it was decided using AA profiles determined on a whole-feed basis was simpler, more feasible for commercial laboratories, biologically more relevant, and provided access to much larger data sets than using profiles from the insoluble residue.

Revision of the Feed Library

The process of evaluating and updating the feed library was designed specifically to pool data from various sources and combine it to estimate likely values. Although the data set used in our analysis encompassed a large number of samples from a wide range of situations, information on environmental and management factors implicit in the composition of individual samples were not available. Many external factors affect the nutrient composition of feeds both pre- and postharvest. When considering forages, preharvest environmental factors such as temperature, light intensity, nitrogen availability, water, and predation affect quality and composition (Van Soest et al., 1978). Postharvest, management factors such as packing density, particle size, silo type, silo filling rate, and the way in which the face of the silo is managed can affect ADF, NFC, ADICP, SP, ammonia, pH, surface temperature, and aerobic instability (Ruppel et al., 1995). Furthermore, biological processes during ensiling such as plant respiration, plant enzymatic activity, clostridial activity, and aerobic microbial activity will affect levels of rapidly fermentable CHO, AA, and NPN and can lead to heating and Maillard reactions (Muck, 1988). Analytically, elevated levels

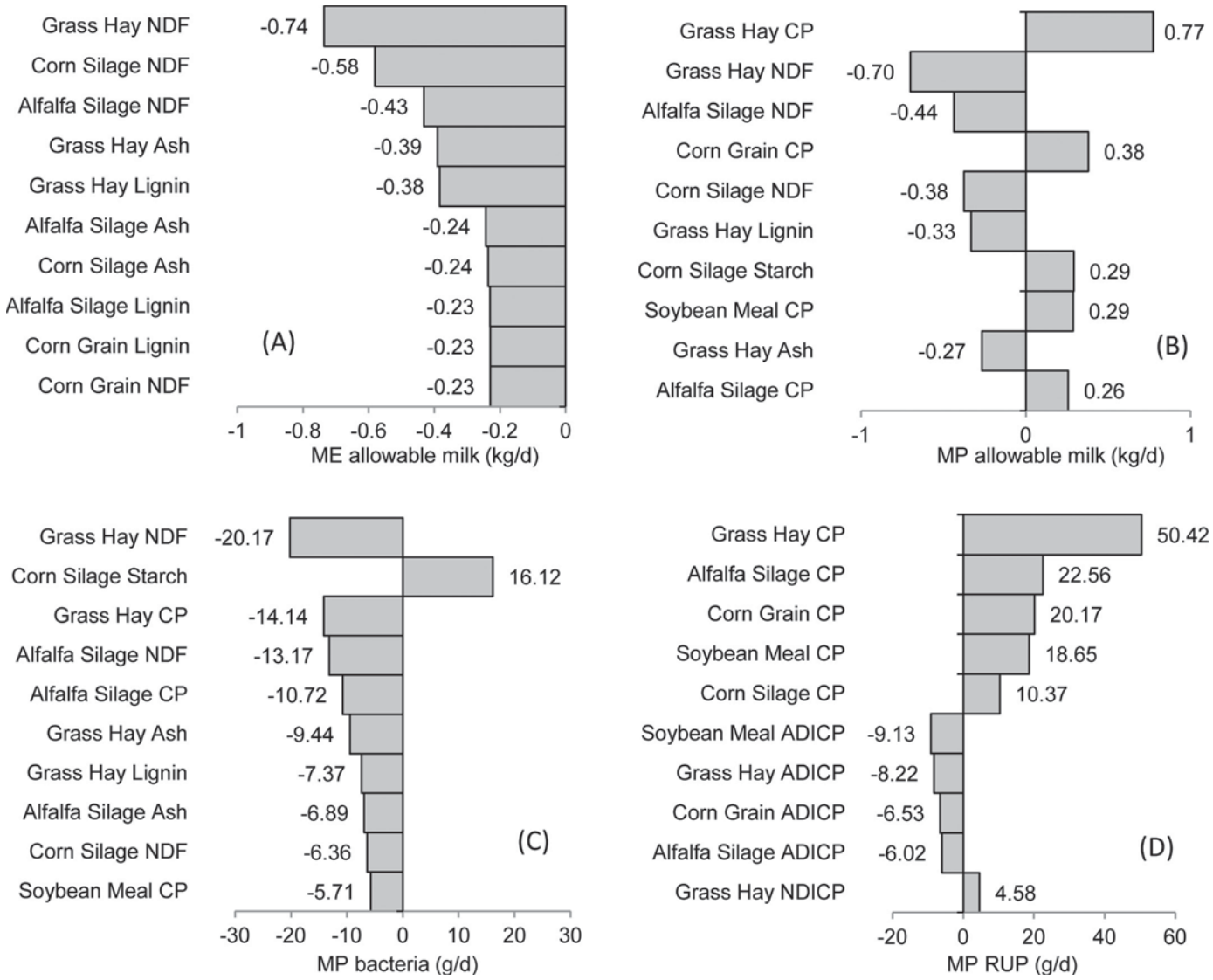


Figure 2. Change in model output from a 1 SD increase in the chemical components of feeds used in the reference diet ranked in order of importance. ADICP = acid detergent-insoluble CP; NDICP = neutral detergent-insoluble CP.

of ADICP are indicative that Maillard reactions have occurred and are common in many heat-dried feeds and fermented feeds where excessive heating occurred (Van Soest and Mason, 1991). Given the importance of external factors on the composition of different feeds, the process used in our project was not sensitive enough to accurately predict the composition of feeds on a sample-by-sample basis. However, it was capable of producing estimated compositions under average conditions in an efficient and repeatable manner that was useful for reviewing and updating a large database such as the CNCPS feed library.

Examples of the changes made to selected forages and concentrates are in Figures 5 and 6. The figures were constructed so that the 0 point on the y-axis represents the mean of the data set used to update the composition (given in brackets on the x-axis) and the error bars represent ±1 SD from the mean. The new and old values for each chemical component within the example feeds are presented relative to the mean and SD. For forage feeds, typically multiple options were available for each feed in the feed library. Therefore, some deviation from the mean could be expected, as the variation is what makes the individual option unique (e.g., high NDF, low NDF). In contrast, the concentrate feeds typically had only one option. In this case, the composition could be expected to be similar to the mean (Figure 6). Noteworthy changes that

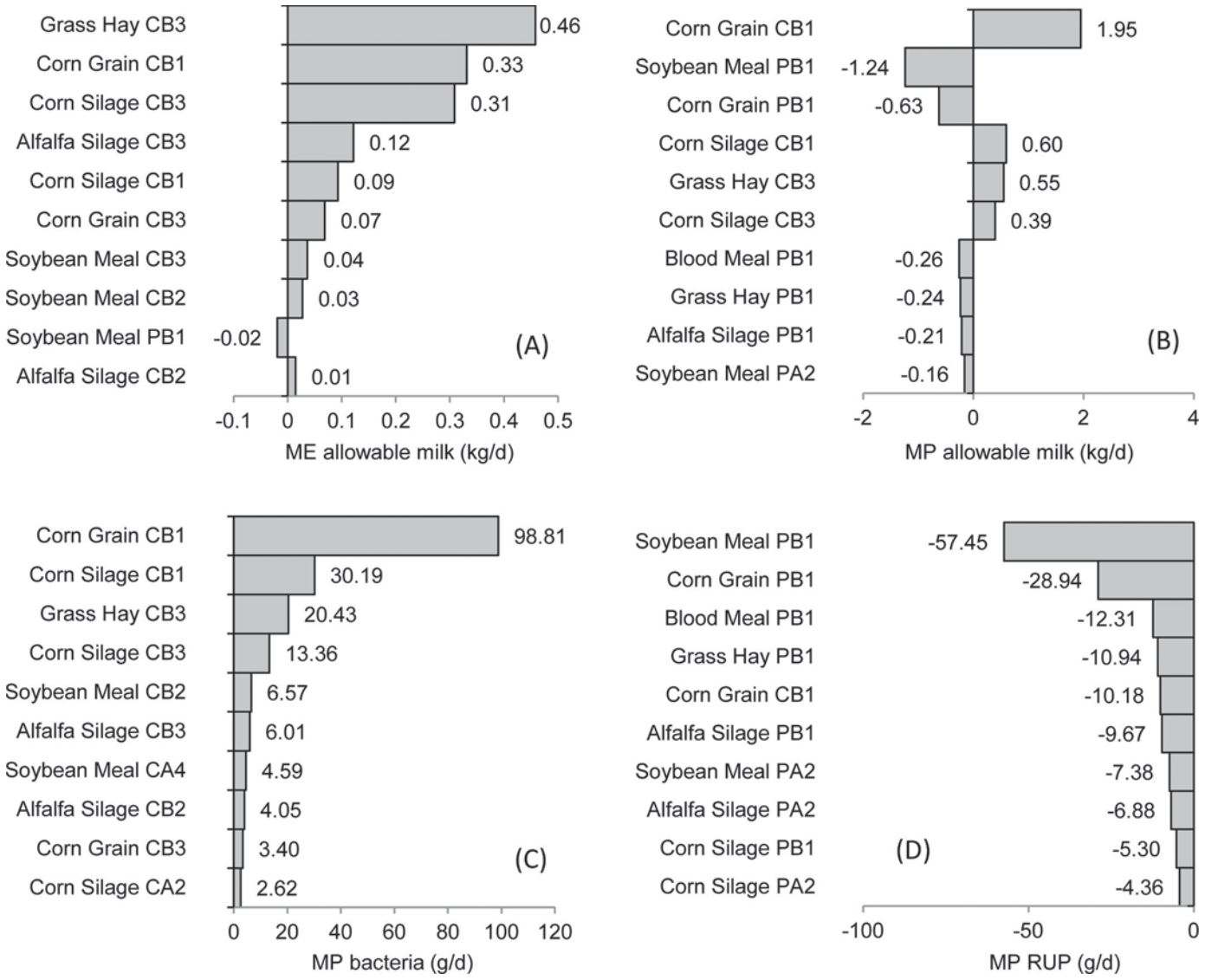


Figure 3. Change in model output from a 1-SD increase in the digestion rates of carbohydrate and protein fractions of feeds used in the reference diet ranked in order of importance. CA2 = lactic acid; CA4 = water-soluble carbohydrates (WSC); CB1 = starch; CB2 = soluble fiber; CB3 = digestible fiber; PA2 = soluble true protein; PB1 = insoluble true protein.

reflect some of the relationships observed in the data set include a reduction in starch for the corn silage in Figure 5A. Starch and NDF in corn silage have a strong reciprocal relationship (r = −0.91; Table 5) and NDF in the example is approximately 6 units greater than the mean. Based on the correlation, starch in this example should be a similar magnitude below the mean, which is reflected by the updated composition. In another example, the composition of canola meal in the old feed library (Figure 6B) was similar to mean values for all components other than starch, which was considerably higher, and outside the expected range. In this case the recalculation procedure reduced starch to within 1 SD of the mean. Similar adjustments were made on a feed-by-feed basis for the entire feed library.

Model Sensitivity to Variation in Feed Chemistry and Digestion Kinetics

Analyzing model sensitivity to variation in inputs can help users understand where emphasis should be placed when requesting feed analyses and also help identify target areas for investigation if model outputs deviate from expected or observed outcomes. The variation in our study represents an entire population of samples for each feed analyzed over numerous growing seasons.

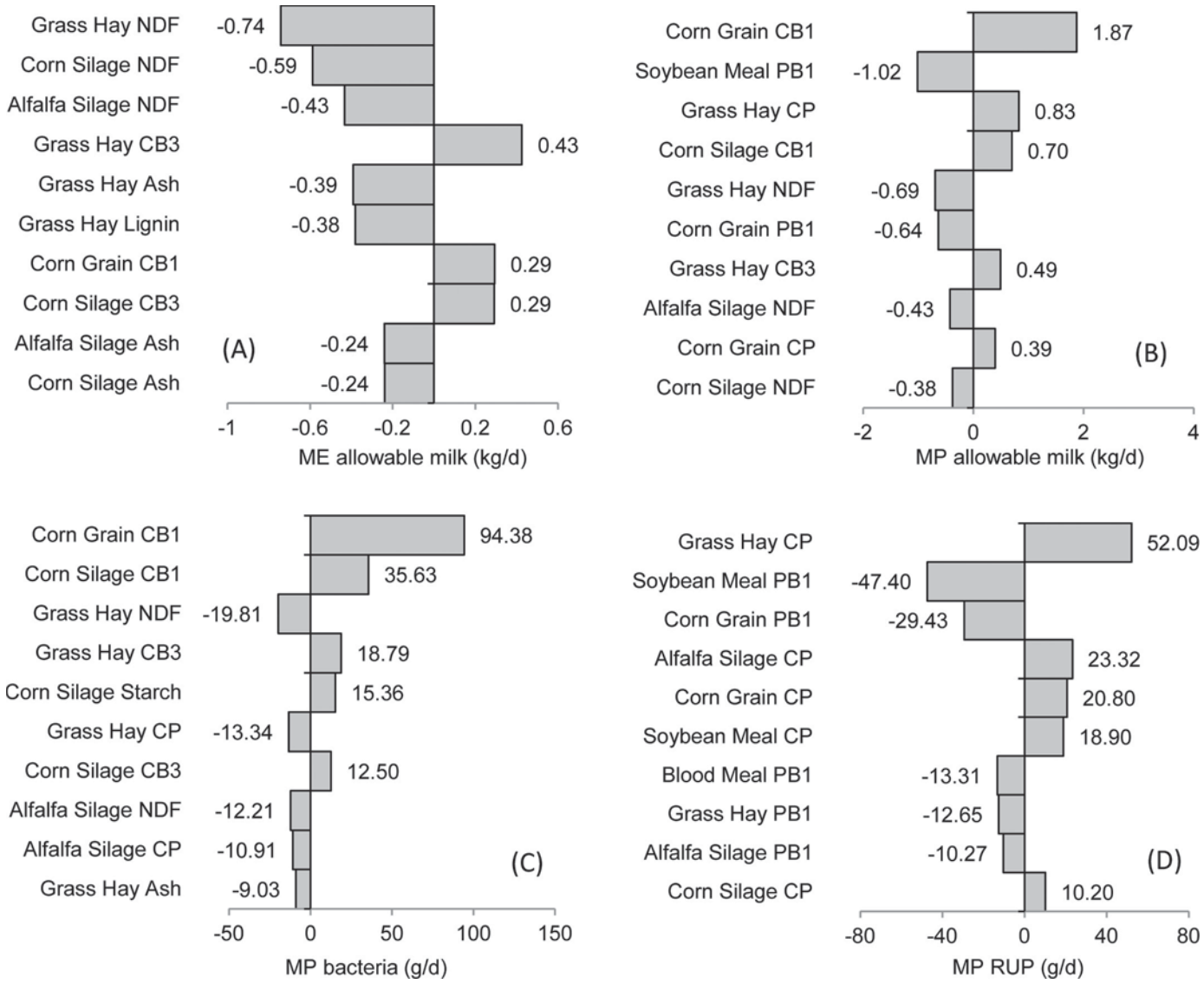


Figure 4. Change in model output from a 1-SD increase in both the chemical components and digestion rates of carbohydrate and protein fractions of feeds used in the reference diet. Items are ranked in order of importance. CB1 = starch; CB2 = soluble fiber; CB3 = digestible fiber; PB1 = insoluble true protein.

Therefore, the variation encompassed is what might be expected if a user ran a simulation in the CNCPS using feeds from the feed library with no information on actual feed chemistry. The mean, SD, and distribution for the components considered in our analysis are in Table 4 and are similar to other reports where the same components and feeds are presented (Kertz, 1998; Lanzas et al., 2007a,b). Data rarely fit a normal distribution and were more commonly represented by a loglogistic distribution, similar to the findings of Lanzas et al. (2007a,b). The data of some components were skewed and were better represented by distributions, such as the Beta, Pearson, or Weibull (Table 4). When data are skewed, the mean and SD are less appropriate in describing centrality and dispersion of a population (Law and Kelton, 2000). Outputs of deterministic models, such as the CNCPS, represent an average (Lanzas et al., 2007b); however, when input variation is accounted for, the mean value may no longer represent the most likely value. For example, in Figure 7A, the mean value for ME allowable milk is 34.1 kg/d; however, the most likely value based on frequency of occurrence is 36.3 kg/d. These types of considerations are particularly important when conducting model evaluations, as studies rarely report adequate information to complete a robust model simulation (Higgs et al., 2012, Pacheco

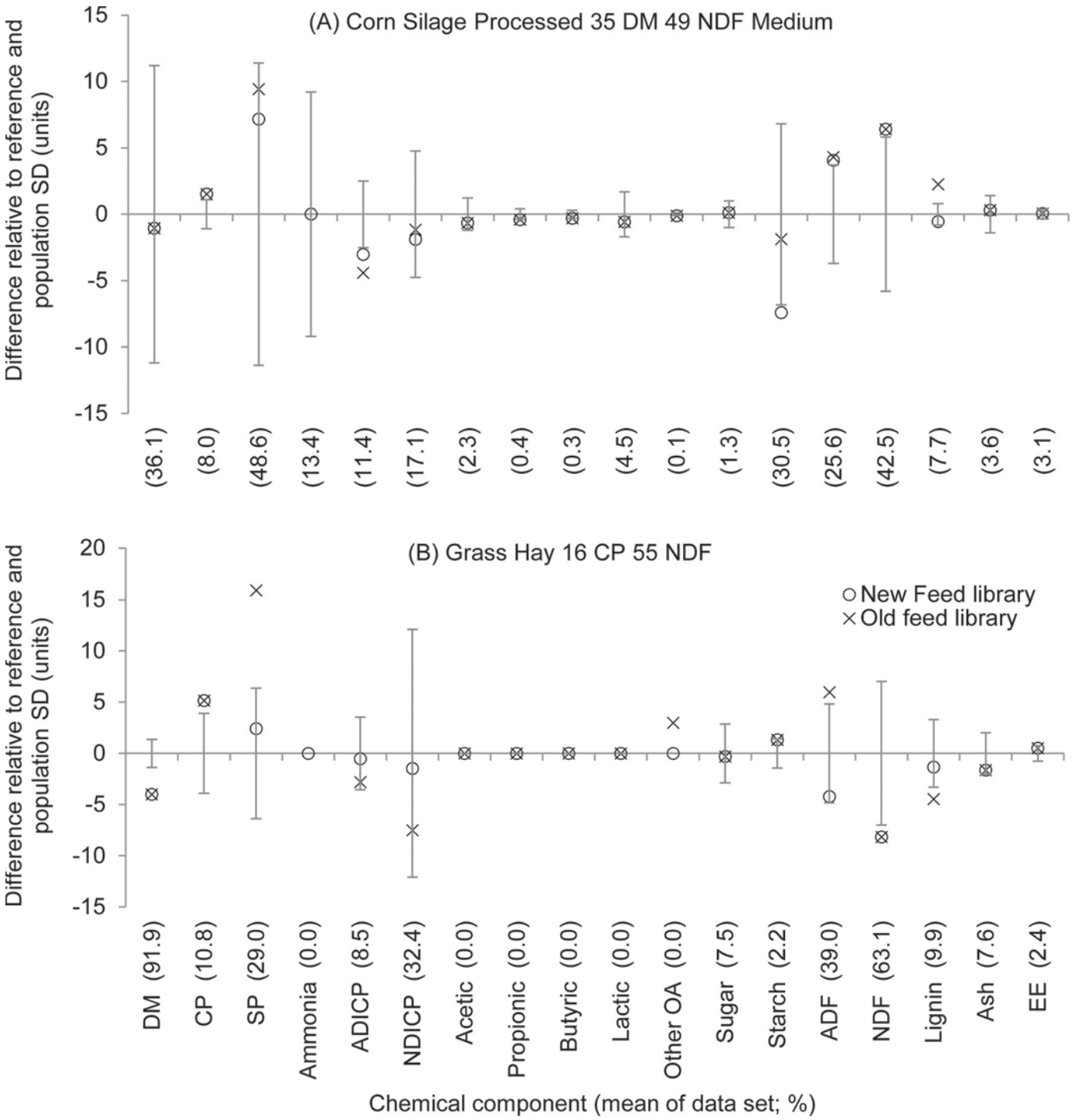


Figure 5. Comparison of the relative difference in chemical composition between the old (×) and new (○) Cornell Net Carbohydrate and Protein System feed library for 2 forages (A = corn silage processed 35% DM, 49% NDF medium processed; B = grass hay 16% CP, 55% NDF) using the mean and SD of commercial laboratory data sets as a reference (Cumberland Valley Analytical Services Inc., Maugansville, MD, and Dairy One Cooperative Inc., Ithaca, NY). All components are expressed as percent DM with the exception of soluble protein (SP; percent CP), ammonia (percent SP), acid detergent-insoluble CP (ADICP; percent CP), neutral detergent-insoluble CP (NDICP; percent CP), and lignin (percent NDF). OA = organic acids; EE = ether extract.

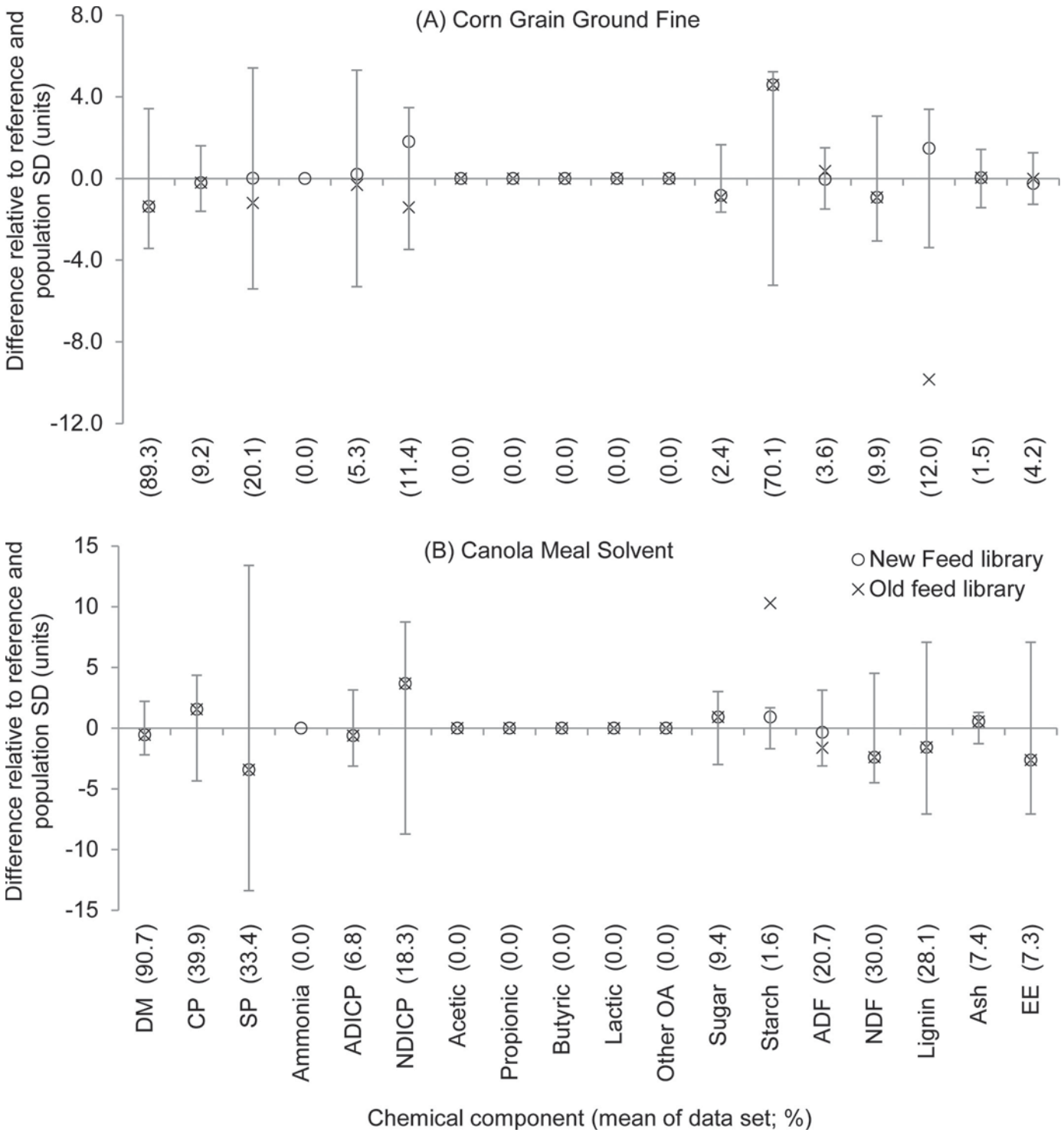


Figure 6. Comparison of the relative difference chemical composition between the old (×) and new (○) feed library of 2 concentrate feeds (A = corn grain ground fine; B = canola meal solvent) using the mean and SD of the online laboratory data sets as a reference (Cumberland Valley Analytical Services Inc., Maugansville, MD, and Dairy One Cooperative Inc., Ithaca, NY). All components are expressed as percent DM with the exception of soluble protein (SP; percent CP), ammonia (percent SP), acid detergent-insoluble CP (ADICP; percent CP), neutral detergent-insoluble CP (NDICP; percent CP), and lignin (percent NDF). OA = organic acids; EE = ether extract.

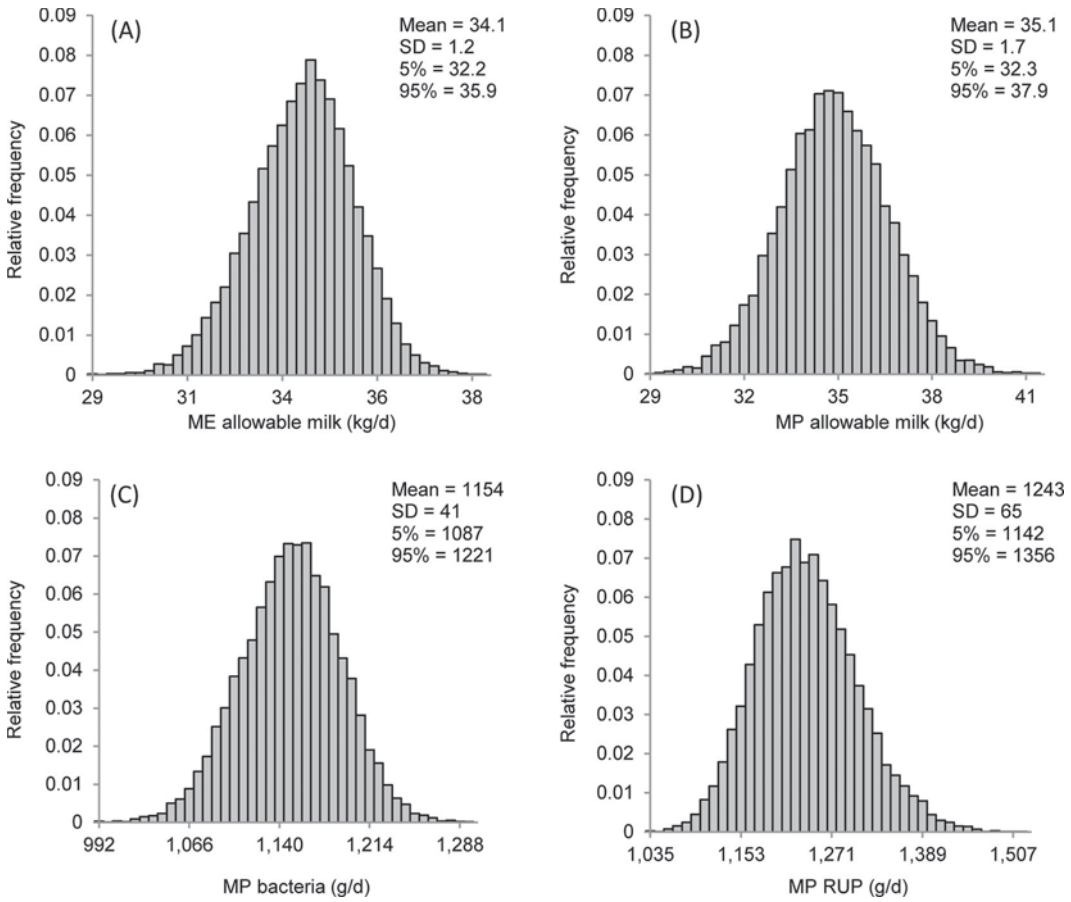


Figure 7. Frequency distributions generated from a Monte Carlo simulation for selected model outputs from the reference diet. Each graph displays the range of possible outcomes for each component and the relative likelihood of occurrence.

et al., 2012). Feed library defaults are typically used in place of reported data leading to the type of variation and bias reported in Figures 7 and 8. Presenting model outputs in the CNCPS as frequency distributions, similar to Figures 7 and 8, could be useful for aid users in managing risk, particularly when balancing rations close to animal requirements. Estimating the variation associated with the sampling process, sample handling, preparation, and the variation of the assay itself within and among labs could be challenging (Hall and Mertens, 2012).

The relative importance of specific model inputs was also investigated. This part of the analysis included variation from both feed composition and the kd values for the CHO and protein fractions. For this analysis, correlations were not fit to chemical components meaning, during the simulation, values were drawn from probability density functions independently of each other. The rationale for treating components as independent was to understand model behavior irrespective of biological relationships in feed composition. In doing this, insight can be gained into the laboratory analyses that are most critical to predict target model outputs.

The chemical components the model was most sensitive to differed among the outputs considered (Figure 2). Prediction of ME allowable milk was most sensitive to forage NDF, lignin and ash whereas MP allowable milk was most sensitive to CP along with CHO components and ash. Interestingly, ME-allowable milk was negatively correlated with all the items it was most sensitive to, with a 1-SD increase in grass hay NDF resulting in a 0.74 kg/d reduction in ME-allowable milk (Figure 2A). This behavior can be attributed to aspects of the models internal structure; ME in the CNCPS is calculated using the apparent TDN system described by Fox et al. (2004) where the net energy derived from the diet is empirically calculated from an estimate of total-tract nutrient digestion. In this system, carbohydrate intake is calculated by difference according to equation 1 in Table 2, and total-tract nutrient digestion is calculated as the difference between nutrient intake and fecal output. Error in laboratory analysis that

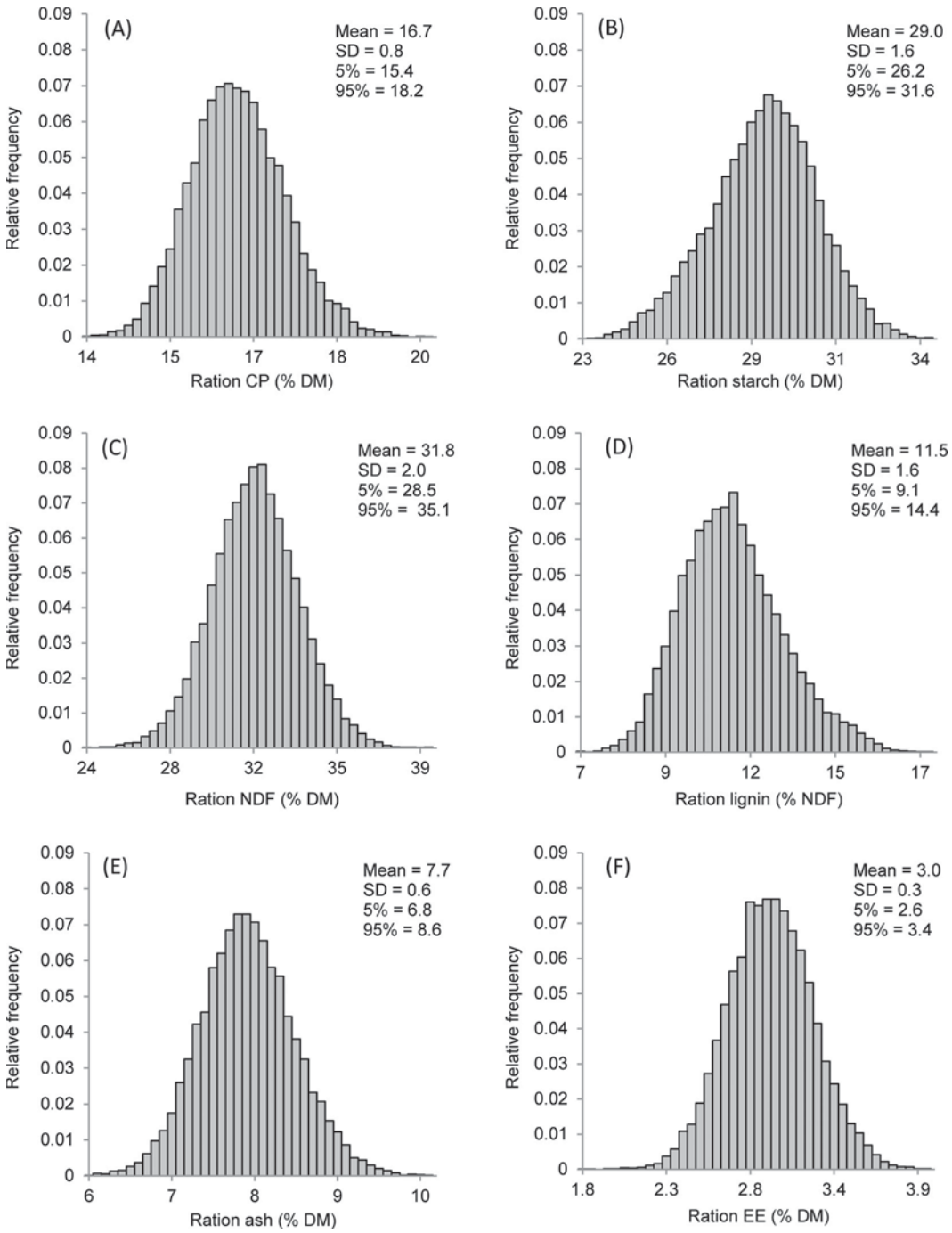


Figure 8. Frequency distributions generated from a Monte Carlo simulation for selected chemical components in the reference diet. Each graph displays the range of possible outcomes for each component and the relative likelihood of occurrence. EE = ether extract.

forces equation [16] to a sum >100% DM leads to an overestimation of fecal appearance and an underestimation of apparent TDN. Further, because soluble fiber is also calculated by difference (equation [5]; Table 2), an increase in the concentration of any component less digestible than soluble fiber (i.e., NDF) results in an increase in fecal nutrient appearance and decrease in apparent TDN. For these reasons, ensuring laboratory results are internally consistent and adhering to the framework of equation [16] is critical for the accurate prediction of ME. Furthermore, due to the potential to obscure the true NDF content of forages, the adoption

of the aNDFom procedure is strongly suggested for current formulation and diet evaluations, as suggested by Sniffen et al. (1992). Although not a part of the library edits, evaluations of ME and MP predictions were improved when aNDFom was used, especially in cases where ash contamination of the NDF was significant. Metabolizable protein is derived from a combination of microbial protein and RUP (Sniffen et al., 1992). Predictions of microbial yield are directly related to ruminal CHO digestion (Russell et al., 1992). The prediction of microbial growth was most sensitive to components that affect the quantity and digestibility of CHO in the rumen (Figure 2C). In contrast, sensitivity in RUP prediction was most affected by CP concentration and the concentration of ADICP, which defines the indigestible protein fraction (Figure 2D).

Ruminal digestion of CHO and protein fractions in the CNCPS are calculated mechanistically according to the relationship originally proposed by Waldo et al. (1972), where digestion = kd/(kd + kp), where kp is the rate of passage. Estimations of kd are, therefore, fundamental in predicting nutrient digestion and subsequent model outputs. With the exception of the CB3 kd (Table 2), which can be calculated according to Van Amburgh et al. (2003), kd values are not routinely estimated during laboratory analysis. Various techniques exist to estimate kd (Broderick et al., 1988, Nocek, 1988); however, technical challenges restrict their application in commercial laboratories and, thus, library values are generally relied on. Compared with variation in chemical components, predictions of ME were less sensitive to variation in kd, and predictions of MP were more sensitive (Figure 3). Predictions of bacterial MP were most sensitive to the rate of starch digestion in both corn grain and corn silage (Figure 3C), whereas predictions of RUP were most sensitive to the PB1 kd in soybean meal, corn grain, and blood meal (Figure 3D) which agrees with the findings of Lanzas et al. (2007a, 2007b). These data demonstrate the importance of kd estimates in the feed library, particularly for the prediction of MP. To improve MP prediction, methods that are practical for commercial laboratories to routinely estimate the kd of starch and protein fractions are urgently needed.

Overall, the prediction of ME-allowable milk was more sensitive to variation in the chemical composition compared with MP-allowable milk, which was more sensitive to variation in kd (Figure 4). Model sensitivity to variation in forage inputs was generally higher than concentrates, which can be attributed to the variation of the feed itself (Table 4), but also the higher inclusion of forage feeds in the reference diet (Table 7). The exception was corn grain, which despite having lower variability had a high inclusion that inflated the effect of its variance. Therefore, the components the model is most sensitive to are not static and will vary depend on the diet fed. Both variability and dietary inclusion should be considered when deciding on laboratory analyses to request for input into the CNCPS. Regular laboratory analyses of samples taken on-farm remains the recommended approach to characterizing the components in a ration and reduce the likely variance in the outputs.

CONCLUSIONS

Chemical components of feeds in the CNCPS feed library have been evaluated and refined using a multistep process designed to pool data from various sources and optimize feeds to be both internally consistent, and consistent with current laboratory data. When predicting ME, the model is most sensitive to variation in chemical composition, whereas MP predictions are more sensitive to variation in kd. Methods that are practicable for commercial laboratories to routinely estimate the kd of starch and protein fraction are necessary to improve MP predictions. When using the CNCPS to formulate rations, the variation associated with environmental and management factors, both pre- and postharvest, should not be overlooked, as they can have marked effects on the composition of a feed. Regular laboratory analysis of samples taken on-farm, therefore, remains the recommended approach to characterizing the components in a ration. However, updates to CNCPS feed library provide a database of ingredients that are consistent with current laboratory data and can be used as a platform to both formulate rations and improve the biology within the model.

ACKNOWLEDGMENTS

The authors thank Cumberland Valley Analytical Services (Maugansville, MD) and Dairy One Cooperative (Ithaca, NY) for providing the feed chemistry data and Evonik Industries (Hanau, Germany) and Adisseo (Commentry, France) for providing the AA data. Financial support for R. J. Higgs was provided in partnership by DairyNZ (Hamilton, New Zealand) and Adisseo.

REFERENCES

Allred, M. C., and J. L. MacDonald. 1988. Determination of sulfur amino acids and tryptophan in foods and food and feed ingredients: collaborative study. J. Assoc. Off. Anal. Chem. 71:603–606.

AOAC International. 2005. Official Methods of Analysis of AOAC International. AOAC International, Gaithersburg, MD.

Armentano, L. E., S. J. Bertics, and G. A. Ducharme. 1997. Response of lactating cows to methionine or methionine plus lysine added to high protein diets based on alfalfa and heated soybeans. J. Dairy Sci. 80:1194–1199.

Broderick, G. A. 1987. Determination of protein degradation rates using a rumen in vitro system containing inhibitors of microbial nitrogen metabolism. Br. J. Nutr. 58:463–475.
Broderick, G. A., R. J. Wallace, E. R. Ørskov, and L. Hansen. 1988. Comparison of estimates of ruminal protein degradation by in vitro and in situ methods. J. Anim. Sci. 66:1739–1745.
Chen, Z. H., G. A. Broderick, N. D. Luchini, B. K. Sloan, and E. Devillard. 2011. Effect of feeding different sources of rumen-protected methionine on milk production and N-utilization in lactating dairy cows. J. Dairy Sci. 94:1978–1988.
Choi, C. W., S. Ahvenjarvi, A. Vanhatalo, V. Toivonen, and P. Huhtanen. 2002. Quantitation of the flow of soluble non-ammonia nitrogen entering the omasal canal of dairy cows fed grass silage based diets. Anim. Feed Sci. Technol. 96:203–220.
Cottrell, W. 1999. Simplified program evaluation and review technique (PERT). J. Constr. Eng. Manage. 125:16–22.
Fox, D. G., L. O. Tedeschi, T. P. Tylutki, J. B. Russell, M. E. Van Amburgh, L. E. Chase, A. N. Pell, and T. R. Overton. 2004. The Cornell Net Carbohydrate and Protein System model for evaluating herd nutrition and nutrient excretion. Anim. Feed Sci. Technol. 112:29–78.
Givens, D. I., and H. Rulquin. 2004. Utilisation by ruminants of nitrogen compounds in silage-based diets. Anim. Feed Sci. Technol. 114:1–18.
Haefner, J. W. 2005. Modeling Biological Systems Principles and Applications. 2nd ed. Springer, New York, NY.
Hall, M. B. 2014. Selection of an empirical detection method for determination of water-soluble carbohydrates in feedstuffs for application in ruminant nutrition. Anim. Feed Sci. Technol. 198:28–37.
Hall, M. B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. J. AOAC Int. 98:397–409.
Hall, M. B., and D. R. Mertens. 2012. A ring test of in vitro neutral detergent fiber digestibility: Analytical variability and sample ranking. J. Dairy Sci. 95:1992–2003.
Higgs, R. J., L. E. Chase, and M. E. Van Amburgh. 2012. Development and evaluation of equations in the Cornell Net Carbohydrate and Protein System to predict nitrogen excretion in lactating dairy cows. J. Dairy Sci. 95:2004–2014.
Johnson, D. 1997. The triangular distribution as a proxy for the beta distribution in risk analysis. J. Royal Stat. Soc. 46:387–398. http://dx.doi.org/10.1111/1467-9884.00091.
Kertz, A. F. 1998. Variability in delivery of nutrients to lactating dairy cows. J. Dairy Sci. 81:3075–3084.
Krishnamoorthy, U., T. V. Muscato, C. J. Sniffen, and P. J. Van Soest. 1982. Nitrogen fractions in selected feedstuffs. J. Dairy Sci. 65:217–225.
Landry, J., and S. Delhaye. 1992. Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. J. Agric. Food Chem. 40:776–779.
Lanzas, C., C. J. Sniffen, S. Seo, L. O. Tedeschi, and D. G. Fox. 2007a. A revised CNCPS feed carbohydrate fractionation scheme for formulating rations for ruminants. Anim. Feed Sci. Technol. 136:167–190.
Lanzas, C., L. O. Tedeschi, S. Seo, and D. G. Fox. 2007b. Evaluation of protein fractionation systems used in formulating rations for dairy cattle. J. Dairy Sci. 90:507–521.
Law, A. M., and W. D. Kelton. 2000. Simulation Modeling and Analysis. 3rd ed. Tata McGraw-Hill Publishing Company, New Delhi, India.
Licitra, G., T. M. Hernandez, and P. J. Van Soest. 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57:347–358.
Macgregor, C. A., C. J. Sniffen, and W. H. Hoover. 1978. Amino acid profiles of total and soluble protein in feedstuffs commonly fed to ruminants. J. Dairy Sci. 61:566–573.
Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217–1240.
Muck, R. E. 1988. Factors influencing silage quality and their implications for management. J. Dairy Sci. 71:2992–3002.
Muscato, T. V., C. J. Sniffen, U. Krishnamoorthy, and P. J. Van Soest. 1983. Amino acid content of noncell and cell wall fractions in feedstuffs. J. Dairy Sci. 66:2198–2207.
Nocek, J. E. 1988. In situ and other methods to estimate ruminal protein and energy digestibility: A review. J. Dairy Sci. 71:2051–2069.
NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.
O'Connor, J. D., C. J. Sniffen, D. G. Fox, and W. Chalupa. 1993. A net carbohydrate and protein system for evaluating cattle diets: IV. Predicting amino acid adequacy. J. Anim. Sci. 71:1298–1311.
Offner, A., A. Bach, and D. Sauvant. 2003. Quantitative review of in situ starch degradation in the rumen. Anim. Feed Sci. Technol. 106:81–93.
Offner, A., and D. Sauvant. 2004. Comparative evaluation of the Molly, CNCPS, and LES rumen models. Anim. Feed Sci. Technol. 112:107–130.
Pacheco, D., R. A. Patton, C. Parys, and H. Lapierre. 2012. Ability of commercially available dairy ration programs to predict duodenal flows of protein and essential amino acids in dairy cows. J. Dairy Sci. 95:937–963.
Palisade. 2010a. Guide to using @Risk version 5.7. Palisade Corporation, Ithaca, NY.
Palisade. 2010b. Guide to using RISKOptimizer version 5.7. Palisade Corporation, Ithaca, NY.
Raffrenato, E. 2011. Physical, chemical and kinetic factors associated with fiber digestibility in ruminants and models describing these relationships. PhD Diss. Cornell University, Ithaca, NY.
Raffrenato, E., and M. Van Amburgh. 2011. Technical note: Improved methodology for analyses of acid detergent fiber and acid detergent lignin. J. Dairy Sci. 94:3613–3617.
Reynal, S. M., I. R. Ipharraguerre, M. Lineiro, A. F. Brito, G. A. Broderick, and J. H. Clark. 2007. Omasal flow of soluble proteins, peptides, and free amino acids in dairy cows fed diets supplemented with proteins of varying ruminal degradabilities. J. Dairy Sci. 90:1887–1903.
Rulquin, H., and L. Delaby. 1997. Effects of the energy balance of dairy cows on lactational responses to rumen-protected methionine. J. Dairy Sci. 80:2513–2522.
Ruppel, K. A., R. E. Pitt, L. E. Chase, and D. M. Galton. 1995. Bunker silo management and its relationship to forage preservation on dairy farms. J. Dairy Sci. 78:141–153.
Russell, J. B., J. D. O'Connor, D. G. Fox, P. J. Van Soest, and C. J. Sniffen. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. J. Anim. Sci. 70:3551–3561.
SAS Institute. 2010. JMP v7. SAS Institute Inc., Cary, NC.
Schwab, C. G. 1996. Rumen-protected amino acids for dairy cattle: Progress towards determining lysine and methionine requirements. Anim. Feed Sci. Technol. 59:87–101.
Schwab, C. G., C. K. Bozak, N. L. Whitehouse, and M. M. A. Mesbah. 1992. Amino acid limitation and flow to duodenum at four stages of lactation. 1. Sequence of lysine and methionine limitation. J. Dairy Sci. 75:3486–3502.
Shapiro, A. 2003. Monte Carlo sampling methods. Pages 353–425 in Handbooks in Operations Research and Management Science. Vol. 10. A. Ruszczynski and A. Shapiro, ed. Elsevier, Amsterdam, the Netherlands.
Siegfried, V. R., H. Ruckemmann, and G. Stumpf. 1984. Method for the determination of organic acids in silage by high performance liquid chromatography. Landwirtschaftliche Forschung 37:298–304.
Sniffen, C. J., J. D. O'Connor, P. J. Van Soest, D. G. Fox, and J. B. Russell. 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J. Anim. Sci. 70:3562–3577.
Tedeschi, L. O., A. N. Pell, D. G. Fox, and C. R. Llames. 2001. The amino acid profiles of the whole plant and of four plant residues from temperate and tropical forages. J. Anim. Sci. 79:525–532.
Tylutki, T. P., D. G. Fox, V. M. Durbal, L. O. Tedeschi, J. B. Russell, M. E. Van Amburgh, T. R. Overton, L. E. Chase, and A. N. Pell.

2008. Cornell Net Carbohydrate and Protein System: A model for precision feeding of dairy cattle. Anim. Feed Sci. Technol. 143:174–202.

Van Amburgh, M. E., L. E. Chase, T. R. Overton, D. A. Ross, E. B. Recktenwald, R. J. Higgs, and T. P. Tylutki. 2010. Updates to the Cornell Net Carbohydrate and Protein System v6.1 and implications for ration formulation. Pages 144–159 in Proc. Cornell Nutr. Conf., Syracuse, NY. Cornell University, Ithaca, NY.

Van Amburgh, M. E., E. A. Colleo-Saenz, R. J. Higgs, D. A. Ross, E. B. Recktenwald, E. Raffrenato, L. E. Chase, T. R. Overton, J. K. Mills, and A. Foskolos. 2015. The Cornell Net Carbohydrate System: Updates to the model and evaluation of version 6.5. J. Dairy Sci. 98:6361–6380. http://dx.doi.org/10.3168/jds.2015-9378.

Van Amburgh, M. E., A. Foskolos, E. A. Collao-Saenz, R. J. Higgs, and D. A. Ross. 2013. Updating the CNCPS feed library with new amino acid profiles and efficiencies of use: Evaluation of model predictions - Version 6.5. Pages 59–76 in Proc. Cornell Nutr. Conf., Syracuse, NY. Cornell University, Ithaca, NY.

Van Amburgh, M. E., E. B. Recktenwald, D. A. Ross, T. R. Overton, and L. E. Chase. 2007. Achieving better nitrogen efficiency in lactating dairy cattle: Updating field usable tools to improve nitrogen efficiency. Pages 25–38 in Proc. Cornell Nutr. Conf., Syracuse, NY. Cornell University, Ithaca, NY.

Van Amburgh, M. E., P. J. Van Soest, J. B. Robertson, and W. F. Knaus. 2003. Corn silage neutral detergent fiber: Refining a mathematical approach for in vitro rates of digestion. Pages 99–108 in Proc. Cornell Nutr. Conf., Syracuse, NY. Cornell University, Ithaca, NY.

Van Soest, P. J. 1994. Nutritional Ecology of the Ruminant. 2nd ed. Cornell University Press, Ithaca, NY.

Van Soest, P. J. 2015. The Detergent System of Analysis for Foods and Feeds. M. E. Van Amburgh, P. Uden, and P. Robinson, ed. Cornell University Publishing Services, Ithaca, NY.

Van Soest, P. J., and V. C. Mason. 1991. The influence of the maillard reaction upon the nutritive-value of fibrous feeds. Anim. Feed Sci. Technol. 32:45–53.

Van Soest, P. J., D. R. Mertens, and B. Deinum. 1978. Preharvest factors influencing quality of conserved forage. J. Anim. Sci. 47:712–720.

Velle, W., Ø. V. Sjaastad, A. Aulie, D. Grønset, K. Feigenwinter, and T. Framstad. 1997. Rumen escape and apparent degradation of amino acids after individual intraruminal administration to cows. J. Dairy Sci. 80:3325–3332.

Volden, H., W. Velle, O. M. Harstad, A. Aulie, and O. V. Sjaastad. 1998. Apparent ruminal degradation and rumen escape of lysine, methionine, and threonine administered intraruminally in mixtures to high-yielding cows. J. Anim. Sci. 76:1232–1240.

Waldo, D. R., L. W. Smith, and E. L. Cox. 1972. Model of cellulose disappearance from the rumen. J. Dairy Sci. 55:125–129.

Weiss, W. P. 1994. Estimation of digestibility of forages by laboratory methods. Pages 644–681 in Forage Quality, Evaluation, and Utilization. G. C. Fahey, ed. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI.

AOAC Official Methods of Analysis (OMA) Method Use & Performance Feedback (for NON-SMPR approved O...

Submission Date	2016-08-17 11:49:50
Please indicate your perspective	Method End-User
AOAC Official Methods Number (XXXX.XX)	2014_10
Method Name or Manuscript Title	Dietary Starch in Animal Feed
Please include the manuscript publication reference1if available. (OMA methods have manuscripts unless otherI ise noted in the OMA)	J. AOAC Int. 98. 397(2015)
B. gn your e"perience usinQ the method1does the method perform accordinQ to the methodFs applicability as I ritten4	Yes
x pload documentation to support the Method Applicability gnformation	Dietary Starch in Animal Feed Method Applicability Comparison to Method group 012 from User lab.docx
?. gn your e"perience I ith the method1are there any safety concerns identified I hile usinQ or reQardinQ use of the method4	No
x pload documentation to support the Safety Concerns	Starch safety..docx
L. gn your e"perience I ith the method1are there available reference materials4 Were there any concerns identified I hile usinQ or reQardinQ use of the method4	No
k. gn your e"perience I ith the method1do you have data demonstratinQ linearity1 accuracy1repeatability1 YODzYOE4	Yes
x pload documentation to support the SinQle Yaboratory - alidation gnformation	starch qc.xls

5. Do you have any information that supports the reproducibility of this method as l ritten4 g so1please specify and submit information.	No
6. 3ased on your e"perience l ith the method1are there any recommended chanQes to the AOAC First Action method as l ritten4	Yes
g / es1please note any recommended chanQes to the AOAC First Action Method as l ritten. Please note that this information can be uploaded as a file attachment.	The method is very simple to complete and repeatability is very good across a large range of values from 1% to 85% on a range of agricultural products.
Would you li, e to provide comment(s) or feedbac, on additional methods4 This form contains feedbac, for three (L) methods. / ou may nol provide feedbac, on tl o (?) additional methods.	No, thank you!
Name	Brian Steinlicht
OrQaniwation	Dairyland Laboratories Inc
56mail Address	bsteinlicht@dairylandlabs.com

Dietary Starch in Animal Feed Method Applicability Comparison to Method group 012 from User lab

AAFCO Check Sample No.	Rob Mean	Value	Rob SD	R-Bar	#Labs
201430	30.06	30.04	2.13	.33269	36
201431	.41488	.55	.43966	.04918	21
201432	30.581	31.33	2.1870	.44907	36
201521	4.2488	3.89	.57905	.1469	27
201522	36.759	36.18	2.9984	.32589	27
201523	2.5539	1.81	1.6667	.10953	20
201524	18.906	18.185	1.6280	.25999	34
201525	18.441	17.585	1.6061	.30553	39
201526	6.1023	5.635	1.6242	.19636	34
201527	35.762	35.275	1.9284	.42745	32
201528	33.052	32.99	1.9750	.35037	32
201529	17.096	12.12	1.9859	.30663	33
201530	25.772	24.96	1.3459	.33156	31
201531	7.5558	7.145	1.4958	.21688	26
201591	8.3709	7.74	1.4240	.20552	23
201532	13.302	13.32	1.0062	.32238	30
201621	8.4036	8.135	2.2589	.19904	23
201622	19.985	19.1	2.0445	.29413	29
201623	32.036	30.2	2.0401	.43485	33
201624	15.22	14.675	1.5278	.30109	32
201625	33.688	32.300	2.0603	.32512	32
201626	7.8288	6.8850	1.5867	.20835	34

This data includes all methods that AAFCO includes under Method code 012. One important thing to note is that Free Glucose is not included in the calculation for values supplied by my laboratory to AAFCO and may be the reason for why our values are slightly lower than the Rob Mean. It is still important to note that this method was within one standard deviation from the Rob mean on all of the samples tested.

The Dietary Starch method uses a dilute acetic acid solution to do the hydrolysis for starch to glucose instead of strong acids or DMSO therefore it makes analyses very simple and safe.

FOOD COMPOSITION AND ADDITIVES

Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method: Collaborative Study

MARY BETH HALL
U. S. Department of Agriculture–Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Dr, Madison, WI 53706

Collaborators: J. Arbaugh; K. Binkerd; A. Carlson; T. Doan; T. Grant; C. Heuer; H. D. Inerowicz; B. Jean-Louis; R. Johnson; J. Jordan; D. Kondratko; E. Maciel; K. McCallum; D. Meyer; C. A. Odijk; A. Parganlija-Ramic; T. Potts; L. Ruiz; S. Snodgrass; D. Taysom; S. Trupia; B. Steinlicht; D. Welch

Starch, glycogen, maltooligosaccharides, and other α-1,4- and α-1,6-linked glucose carbohydrates, exclusive of resistant starch, are collectively termed "dietary starch". This nutritionally important fraction is increasingly measured for use in diet formulation for animals as it can have positive or negative effects on animal performance and health by affecting energy supply, glycemic index, and formation of fermentation products by gut microbes. AOAC Method 920.40 that was used for measuring dietary starch in animal feeds was invalidated due to discontinued production of a required enzyme. As a replacement, an enzymatic-colorimetric starch assay developed in 1997 that had advantages in ease of sample handling and accuracy compared to other methods was considered. The assay was further modified to improve utilization of laboratory resources and reduce time required for the assay. The assay is quasi-empirical: glucose is the analyte detected, but its release is determined by run conditions and specification of enzymes. The modified assay was tested in an AOAC collaborative study to evaluate its accuracy and reliability for determination of dietary starch in animal feedstuffs and pet foods. In the assay, samples are incubated in screw cap tubes with thermostable α-amylase in pH 5.0 sodium acetate buffer for 1 h at 100°C with periodic mixing to gelatinize and partially hydrolyze α-glucan. Amyloglucosidase is added, and the reaction mixture is incubated at 50°C for 2 h and mixed once. After subsequent addition of water, mixing, clarification, and dilution as needed, free + enzymatically released glucose are measured. Values from a separate determination of free glucose are subtracted to give values for enzymatically released glucose. Dietary starch equals enzymatically released glucose multiplied by 162/180 (or 0.9) divided by the weight of the as received sample. Fifteen laboratories that represented feed company, regulatory, research, and commercial feed testing laboratories analyzed 10 homogenous test materials representing animal feedstuffs and pet foods in duplicate using the dietary starch assay. The test samples ranged from 1 to 70% in dietary starch content and included moist canned dog food, alfalfa pellets, distillers grains, ground corn grain, poultry feed, low starch horse feed, dry dog kibbles, complete dairy cattle feed, soybean meal, and corn silage. The average within-laboratory repeatability SD (s_r) for percentage dietary starch in the test samples was 0.49 with a range of 0.03 to 1.56, and among-laboratory repeatability SDs (s_R) averaged 0.96 with a range of 0.09 to 2.69. The HorRat averaged 2.0 for all test samples and 1.9 for test samples containing greater than 2% dietary starch. The HorRat results are comparable to those found for AOAC Method 996.11, which measures starch in cereal products. It is recommended that the dietary starch method be accepted for Official First Action status.

Received August 5, 2014.
The method was approved by the Expert Review Panel for Dietary Starch.

The Expert Review Panel for Dietary Starch invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

Mention of any trademark or proprietary product in this paper does not constitute a guarantee or warranty of the product by the USDA or the Agricultural Research Service and does not imply its approval to the exclusion of other products that also may be suitable.

Corresponding author's e-mail: marybeth.hall@ars.usda.gov
DOI: 10.5740/jaoacint.15-012

Starch is an important, frequently analyzed component of animal feedstuffs. It can have substantial positive effects on animal performance and potential undesirable effects on glycemic response and animal health (1). AOAC *Official Method*^{SM} **920.40** for starch in animal feeds (2) is no longer valid because of discontinued production of the enzyme "Rhozyme-S" (Rohm and Haas, Philadelphia, PA) specified in the procedure. Accordingly, another approved method for starch in animal feeds is needed. Additionally, new terminology is needed to define "starch" to more accurately describe the nutritionally relevant fraction of interest, and the definition can be used to specify the analysis.

Starch has long been defined as a natural vegetable polymer consisting of long linear unbranched chains of α-1,4-linked D-glucose units (amylose) and/or long α-1,6-branched chains of α-1,4-linked glucose units (amylopectin; 3). However, the amylases and amyloglucosidases that specifically hydrolyze the linkages in plant starch also hydrolyze those same linkages in glycogen from animal (4) or microbial (5) sources and in maltooligosaccharides that are breakdown products of starch

but are not polysaccharides. Accordingly, enzymatic starch methods do not measure plant starch alone (6), unless animal and microbial ingredients and the feedstuffs that contain them are excluded from analysis. From a nutritional standpoint, inclusion of glycogen, starch, and maltooligosaccharides more completely describes the pool of carbohydrate that is potentially available to digestion by salivary or small intestinal amylases or amyloglucosidases (7), but the pool can not be called "starch" because that term is well established as referring to a plant polysaccharide.

Recognizing the aim of nutritional characterization, the Laboratory Methods & Services Committee of the Association of American Feed Control Officials with involvement of researchers and industry arrived at a definition for "Dietary Starch": An alpha-linked-glucose carbohydrate of or derived from plants, animals, or microbes from which glucose is released through the hydrolytic actions of purified α-amylases and amyloglucosidases that are specifically active only on α-(1-4) and α-(1-6) linkages in feed materials that have been gelatinized in heated, mildly acidic buffer. Its concentration in feed is determined by enzymatically converting the α-linked glucose carbohydrate to glucose and then measuring the liberated glucose. This definition encompasses plant starch, glycogen, maltooligosaccharides, and maltose/isomaltose. The use of mildly acidic buffer for the gelatinization excludes the use of alkali or dimethyl sulfoxide and, thus, excludes resistant starch from inclusion in the dietary starch fraction.

The proposed dietary starch method avoids known analytical defects and allows handling of diverse physical forms of samples. It is based on an assay published by Bach Knudsen (8) that was slightly modified to improve use of laboratory resources, reduce run time, and maintain starch recovery (9). It is similar in chemistry to AOAC Method **996.11** (10), but differs in the buffer used and in sample handling procedures and gave a greater recovery of starch (9). Specific to the dietary starch assay, all enzymatic reactions are carried out in an acidic buffer that improves recovery by limiting the production of maltulose, an isomerization product produced at more neutral pH (11). Maltulose is resistant to enzymatic hydrolysis and reduces starch recovery. The use of a screw cap tube as a reaction vessel allows for more vigorous mixing, which is useful for all types of feed materials but may be essential for those that clump, are moist, or do not behave like dry, ground powders. Although enzymes used in development of the method will be listed, learning from the loss of AOAC Method **920.40** (2), this assay will not be set to use specific commercial enzymes but rather enzymes with specific activity that give desired results under the conditions of the method. The detection method specified is a colorimetric glucose oxidase-peroxidase method based on an assay developed by Karkalas (12), but recommendations are made to use other approved chromatographic analyses if interferences such as antioxidants are present.

Collaborative Study

Method Performance Parameters and Optimization

The performance parameters of the dietary starch procedure were investigated by the Study Director, who developed the method evaluated in this study. The following factors were evaluated:

(*1*) *Repeatability.*—As tested previously in a single laboratory, the SDs of within laboratory replicates for dietary starch analysis of food and feed substrates were low (dietary starch mean = 46.9%, s_r = 0.48%; dry matter basis; 9).

(*2*) *LOD.*—LOD for the dietary starch assay was calculated from absorbance values as the mean reagent blank value + 3 × SD (13). The means and SD were calculated for the absorbances of duplicate readings for seven undiluted with-enzyme reagent blanks from six separate assay runs. For each reagent blank, the value of the mean absorbance + 3 SD was used in the glucose standard curve determined for that run to calculate the detected glucose value. This value was multiplied by the final reaction volume (51.1 mL), by 162/180 to convert glucose to a starch basis, and converted to g. The calculated dietary starch LOD are 0.3% of sample weight based on analysis of a 100 mg test portion.

(*3*) *Accuracy/recovery.*—Recovery of pure corn starch was determined on samples analyzed singly in five separate analytical runs and in duplicate in an additional run. The average recovery ± SD was 99.3 ± 0.8% on a dry matter basis. In the collaborative study, the average dietary starch value for the control corn starch sample was 89.9 ± 3.7% on an as received basis with an estimated actual value of 89.4%.

(*4*) *Linearity.*—Linearity of the dietary starch assay was evaluated on a dry matter basis using purified corn starch samples weighing 25, 50, 75, and 100 mg analyzed on 3 separate days. The effect of starch amount tended to have a linear effect on recovery (P = 0.07), but the difference was small at a maximum of 2 percentage units between the highest and lowest recoveries. The least squares means ± SD for recovery were 101.9 ± 1.7, 99.9 ± 0.2, 100.3 ± 0.4, and 100.0 ± 0.7% for 25, 50, 75, and 100 mg of corn starch, respectively.

(*5*) *Specificity.*—The dietary starch method gave very low values (mean ± SD) for sucrose (0.17 ± 0.00% of sample dry matter), α-cellulose (0.03 ± .02% of air dried sample), and isolated oat beta-glucan (0.31 ± 0.09% of air dried sample), indicating that run conditions and enzyme preparations used did not appreciably hydrolyze these feed components. Sucrose, in particular, has been shown to interfere with starch analysis (14), likely due to side activity of the enzyme preparations used. Use of separate free glucose determinations allows correction for free glucose and background absorbance associated with each sample. The final detection method, the glucose oxidase – peroxidase (GOPOD) method, is specific for glucose, which limits interference from other carbohydrates.

(*6*) *Interference.*—Antioxidants can depress glucose detection in the GOPOD assay. Addition of ascorbic acid as a model antioxidant gave a linear decrease in absorbance at additions of greater than 10 μmoles of ascorbic acid (15). The effect was relatively small up to 10 μmol of ascorbic acid. Investigations into the antioxidant content of foodstuffs (16) showed that most of the high starch or leafy vegetable foods had hydrophilic antioxidant values that would be equivalent to less than 10 μmoles of ascorbic acid/0.1 g of dry matter. Exceptions included foods high in phenolic compounds (e.g., beets and red sorghum grain with antioxidant content approximately equivalent to 23 and 14 μmol ascorbic acid, respectively). Because of the interference in the GOPOD assay, another method for measuring glucose should be considered for feeds or foods exceeding 10 to 20 μmol of hydrophilic antioxidant/0.1 g of test sample dry matter.

(*7*) *Use of quadratic standard curves.*—The standard curves in the GOPOD assay are slightly nonlinear, and this is normal for this assay within the glucose concentrations commonly used (15). The linear equations describing glucose standard curves had R^2 of nearly 1.0 (0.9998 to 1.0) suggesting a very good fit to the linear form but the intercepts were not 0. Thus, when the standard curves were used to predict glucose concentrations of the standard solutions used to produce them, the predicted values frequently differed slightly from the expected values. It was determined that a quadratic form fit the standard curves better than a linear form based on significance of the quadratic term in the regression equation, the reduction in the root mean squared error of the standard curve and the relative decrease in residual sums of squares (residual = observed minus predicted) between the linear and quadratic equations, and evaluation of the residual versus predicted value plots (15). Other nonlinear forms were not explored.

(*8*) *Determination of final volume by summation of liquid additions.*—The method uses summing of added reagent volumes or use of volumetric flasks to give the final volume of test solutions before dilution. Total volumes of test solutions and dilutions can be determined by summing of added volumes if accurately quantitative volumetric pipets and dispensers are used to add reagents. An evaluation of summation of volumes and determination of final volume by weight and density showed no difference in recovery of glucose ($P = 0.21$) or of corn starch ($P = 0.62$) analyzed with the dietary starch assay. The density of test sample solutions and reagent blanks appears to be quite consistent (0.999 g/mL, SD = 0.002, $n = 120$ from 16 analysis runs over 16 months). Accuracy of reagent additions can be determined by the final weight of total added liquid [(weight of tube + test sample + liquid) minus (weight of tube + test sample)]. The weights of total added liquid are 49.9 and 51.0 g for the portions of the assay run without or with enzyme additions, respectively. The deviations from these values should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. Alternatively, after the addition of water, test solutions can be quantitatively transferred with filtration through Whatman (Florham Park, NJ) 54 or equivalent paper into 100 mL volumetric flasks and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

(*9*) *Ease of use/efficiency.*—The method has the advantage that all reagent additions are made to samples in tubes that can be handled in racks. It does not require transfer of sample until the final dilution and measurement of glucose. Vortexing of the sealed tubes rinses the entire interior of the tube with solution, thus minimizing the possibility that test samples will escape contact with reagents. Studies verified the acceptability of using the same temperature for the amyloglucosidase digestion and glucose analysis incubations (15), which allowed more economic use of laboratory resources.

(*10*) *Use of control samples.*—The use of glucose and corn starch as control samples allows evaluation of quantitative recovery, and starch allows evaluation of quantitative recovery and efficacy of the assay.

(*11*) *Evaluation of enzymes for suitability.*—It is essential that the enzymes and run conditions used release only glucose bound by α-1,6- and α-1,4-linkages and give close to 100% recovery of corn starch. Sucrose is the most common interfering carbohydrate encountered in feedstuffs (14) typically due to its hydrolysis through side activity of the enzyme preparations used. Though the run conditions used will not hydrolyze sucrose, commonly available enzyme preparations have activity that can and are thus unsuitable for this assay. Analysis of glucose, corn starch, and sucrose with candidate enzymes should give values (mean ± SD) of glucose 90 ± 2%, starch 100 ± 2%, and sucrose 0.7 ± 0.3% on a dry matter basis. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%) or background absorbance readings will interfere with test sample measurements.

(*12*) *Method of glucose detection.*—The dietary starch protocol specifies use of an enzymatic-colorimetric assay that has been found to be very precise (15). However, it also allows use of other AOAC-approved glucose-specific assays that have been proven in laboratory validation to be appropriate for the dietary starch assay. On this basis, qualifying assays that are devoid of interference and are, thus, more suitable for use on specific matrixes, or are preferred in a given laboratory may be used.

Collaborating Laboratories

The 15 laboratories that participated in the study represented eight regulatory laboratories, three commercial feed testing laboratories, two feed company laboratories, and two research laboratories. One each of research, commercial feed testing, and regulatory laboratories that expressed interest in participating did not complete the study. Participating laboratories received no compensation. Collaborators were provided with blind test samples, control glucose and corn starch, thermostable α-amylase (Multifect AA 21L, Genencor International, Rochester, NY), amyloglucosidase (E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland), glucose standards, electronic data sheets, and larger reaction tubes if needed. They were required to prepare the GOPOD reagent, perform the dietary starch assay as written, analyze test samples in duplicate, and provide comments and detailed result forms containing both raw and calculated data describing their analyses of three blind familiarization test materials, 10 blind collaborative study test materials, and control samples for dietary starch.

Materials

Test materials selected for the collaborative study covered a wide range of dietary starch contents, ranging from 1 to 69% on an as-received basis and derived from single batches of manufactured and commodity feedstuffs used with different animal species. The test sample grinding and homogenizing methods used were designed to produce materials that would pass a 40 mesh screen. By virtue of their diverse handling characteristics, a number of different methods were used to prepare the samples for analysis. Corn silage, poultry feed, low starch horse feed, and alfalfa pellets were ground through the 6 mm screen of a cutting mill (Pulverisette 19, Fritsch GmbH, Idar-Oberstein, Germany) and then processed through the 0.5 mm screen of a centrifugal mill (ZM200 with 12 blade knife, Retsch GmbH, Haan, Germany). Dry corn, soybean meal, and distillers grains were ground to pass the 0.5 mm screen of a centrifugal mill (ZM200 with 12 blade knife), as

Table 1. Homogeneity of dietary starch for four sample sets of each test material[a]

Material	*n*	Mean, %	s_r	s_R	RSD_r, %	RSD_R,%	2.8 × s_r	2.8 × s_R	HorRat
Moist canned dog food	4	1.58	0.01	0.02	0.86	1.06	0.04	0.05	0.29
Low starch horse feed	4	7.17	0.06	0.11	0.85	1.56	0.17	0.31	0.53
Dry ground corn	4	72.70	0.34	0.34	0.46	0.46	0.95	0.95	0.22
Complete dairy feed	4	28.38	0.10	0.40	0.34	1.40	0.27	1.11	0.58
Soybean meal	4	1.17	0.05	0.05	3.94	3.94	0.13	0.13	1.01
Distillers grains	4	4.23	0.06	0.06	1.37	1.37	0.16	0.16	0.43
Pelleted poultry feed	4	28.50	0.32	0.32	1.14	1.14	0.91	0.91	0.47
Corn silage	4	41.15	1.06	1.06	2.58	2.58	2.98	2.98	1.13
Dog kibble, dry	4	27.82	0.95	1.01	3.43	3.64	2.67	2.83	1.50
Alfalfa pellets	4	1.46	0.04	0.05	3.06	3.18	0.12	0.13	0.84

[a] s_r = SD of repeatability within sample; s_R = SD within and among sample sets; RSD_r = repeatability SD; RSD_R = reproducibility SD.

was the textured dairy complete feed but with dry ice used in the grinding of this sample. Dog kibble was ground with a kitchen processing mill (Assistent, MagicMill, Upper Saddle River, NJ) and further processed through a blending mill (1095 Knifetec sample mill, Foss Tecator, Höganäs, Sweden). The moist, canned dog food was homogenized with a commercial blender (Waring laboratory blender, 14-509-66, Fisher Scientific, Pittsburgh, PA). Dry ground test samples were subsampled using a rotary splitter (Laborette 27, Fritsch GmbH) and stored at –20°C in vacuum sealed bags (3.5 mil nylon polyethylene standard barrier vacuum bag, DCE, Inc., Springville, CA) until shipment. Homogenized moist dog food was transferred to individual sealed plastic bags (Whirl-Pak 58 mL, B01009WA, Nasco, Fort Atkinson, WI) and stored at –20°C. Test sample weights/bag were approximately 20 g for dried ground samples and 25 g of homogenized moist dog food.

For the collaborative study, individual test samples were labeled with a letter. Dry test samples and control samples were packed together in a sealed plastic bag. The homogenized moist dog food test sample and enzymes were packaged in an insulated container with a frozen ice pack. Materials were shipped overnight to the laboratories with directions to place the homogenized moist dog food test sample in the freezer until analysis. That sample was to be thawed overnight at 4°C, and all analyses in the dietary starch procedure were to be performed on it on the following day; no such limitations were placed on analyses of the dry test samples.

As per the example of Mertens (17), dietary starch analyses in duplicate of four randomly selected samples of each test material were used to evaluate random variation within and among samples. In this application, the SD of repeatability within sample (s_r) and SD of reproducibility among laboratories (s_R) calculated using the AOAC spreadsheet designed for evaluating collaborative studies represent the variation within and between separate samples of test materials as tested in the Study Director's laboratory. The s_r and s_R were similar within each sample, indicating that the prepared test samples were homogenous (Table 1). The HorRat values for corn silage and dog kibble were greater than 1.1. As concluded in a similar evaluation (17), these results suggest that these samples were less homogenous or for some reason more difficult to analyze for dietary starch than the other samples. For the dog kibble test sample, small dark particles that did not dissolve or degrade and had the coloration of one form of kibble present in the original unground material were visible in the acetate buffer during incubations.

Statistical Analyses

Data from all laboratories were reviewed for data entry and calculation errors before statistical evaluation, and results were reverified if values were identified as outliers. Ranking scores (18) were used to identify laboratories that were outliers across all materials. Data from the one such identified laboratory were excluded from further data analysis.

The AOAC INTERNATIONAL Interlaboratory Study Workbook for Evaluation of Blind Duplicates (Version 2.0, 2006) spreadsheet was used to evaluate data from the collaborative study and from the homogeneity test performed in the Study Director's laboratory.

AOAC Official Method 2014.10
Dietary Starch in Animal Feeds and Pet Food
Enzymatic-Colorimetric Method
First Action 2014

(Applicable for the determination of dietary starch in forages, grains, grain by-products, dry, semi-moist, and moist pet food products, and mixed feeds that range in concentration from 1 to 100%.)

Caution: Acetic acid is flammable in both liquid and vapor forms. It can cause severe skin burns and eye damage and is toxic if inhaled. Avoid breathing fumes. Wear protective gloves, clothing, and eye and face protection.

α-Amylase and glucose oxidase are respiratory sensitizers, which may cause allergy or asthma symptoms. Avoid breathing dust. Amylase preparations can cause allergic reactions in hypersensitive individuals. Avoid inhaling aerosols or dusts.

Benzoic acid causes serious eye damage and respiratory irritation. Avoid breathing dust and mist. Wear eye protection.

Phenol can be toxic and cause severe burns and eye damage. It is suspected of causing genetic defects and may cause damage to organs. Do not breathe dust or fumes. Wear protective gloves, clothing, eye protection, and face protection.

See Table **2014.10** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Ground or homogenized animal feed and pet food test portions are mixed with acetic acid buffer and heat-stable α-amylase and incubated at 100°C for 1 h with periodic mixing to gelatinize and partially hydrolyze the starch. After cooling, amyloglucosidase is added and the test mixture is incubated for 2 h at 50°C. The digested mixture is clarified, diluted as needed, and glucose detected in the resulting test solution using a colorimetric glucose oxidase-peroxidase (GOPOD) method that is sensitive and specific to glucose. Free glucose is measured simultaneously or in a separate analytical run for each test sample by carrying a second test portion of each test sample through the procedure omitting enzymes and incubating at 100°C for 1 h with periodic mixing. Dietary starch is determined as 0.9 times the difference of glucose in the digested test portion minus free glucose in the undigested test portion.

B. Apparatus

(**a**) *Grinding mill*.—Mills such as an abrasion mill equipped with a 1.0 to 0.5 mm screen, or a cutting mill with 0.5 mm screen, or other appropriate device to grind test samples to pass a 40 mesh screen.

(**b**) *Homogenizer, blender, or mixer*.—To provide homogenous suspension of canned pet food, liquid animal feed, semi-moist pet food, and other materials containing less than 85% dry matter.

(**c**) *Bench centrifuge or microcentrifuge.*—Capable of centrifuging at 1000 × *g* to 10 000 × *g*.

(**d**) *Water bath.*—Capable of maintaining 50 ± 1°C.

(**e**) *Vortex mixer.*

(**f**) *pH meter.*

(**g**) *Stop clock timer (digital).*

(**h**) *Top-loading balance*.—Capable of weighing accurately to ±0.01 g.

(**i**) *Analytical balance.*—Capable of weighing accurately to ±0.0001 g.

(**j**) *Laboratory ovens.*—With forced-convection; capable of maintaining 100 ± 1°C for carrying out incubations.

(**k**) *Spectrophotometer*.—Capable of operating at absorbances of 505 nm.

(**l**) *Pipets.*—Capable of delivering 0.1 and 1.0 mL; with disposable tips.

(**m**) *Positive-displacement repeating pipet.*—Capable of accurately delivering 0.1, 1.0, and 3.0 mL.

(**n**) *Dispenser.*—1000 mL or greater capacity; capable of accurately delivering 20 and 30 mL.

(**o**) *Glass test tubes*.—16 × 100 mm.

(**p**) *Glass tubes.*—25 × 200 mm, with polytetrafluoroethylene (PTFE)-lined screw caps or comparable tubes to hold 51.1 mL and allow for adequate mixing when sealed.

(**q**) *Plastic film.*—Or similarly nonreactive material.

(**r**) *Magnetic stir plate.*

(**s**) *Glass fiber filter.*—With 1.6 µm retention.

(**t**) *Hardened filter paper.*—With 22 µm retention.

C. Reagents

Note: Use high-quality distilled or deionized water for all water additions.

(**a**) *Acetate buffer (100 mM, pH 5.0)*.—Weigh 6.0 g or pipet 5.71 mL glacial acetic acid and transfer immediately to a flask; quantitatively transfer weighed acid with H_2O rinses. Bring volume to ca 850 mL. While stirring solution on a magnetic stir plate, adjust pH to 5.0 ± 0.1 with 1 M NaOH solution. Dilute to 1 L with H_2O. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**b**) Heat-stable α-amylase solution.—Liquid, heat-stable, α-amylase (examples: Product Termamyl 120 L, Novozymes North America, Franklinton, NC; Product Multifect AA 21L, Genencor International, Rochester, NY; origin: Bacillus licheniformis, or equivalent). Should not contain greater than 0.5% glucose. pH optima must include 5.5–5.8.

Based on Bacterial Amylase Unit (BAU) method.—Approximately 83000 BAU/mL of concentrated enzyme (1 BAU is defined as the amount of enzyme that will dextrinize starch at the rate of 1 mg/min at pH 6.6 and 30 ± 0.1°C; 19). If modifications of volume delivered are necessary due to enzymatic activity of the enzyme used, the volume used per test portion should deliver approximately 8300 ± 20 BAU (19).

Table 2014.10. Method performance for determination of dietary starch in feeds

Material	No. of labs	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	r[a]	R[b]
Moist canned dog food	11	1.54	0.03	0.09	2.21	5.99	0.10	0.26
Low starch horse feed	13	7.02	0.23	0.36	3.32	5.19	0.65	1.02
Dry ground corn	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54
Complete dairy feed	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48
Soybean meal	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31
Distillers grains	13	4.11	0.11	0.20	2.67	4.94	0.31	0.57
Pelleted poultry feed	13	28.24	0.73	1.34	2.58	4.76	2.04	3.76
Corn silage	13	39.04	0.80	1.88	2.05	4.82	2.24	5.27
Dog kibble, dry	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46
Alfalfa pellets	13	1.38	0.12	0.13	8.61	9.69	0.33	0.38

[a] $r = 2.8 \times s_r$.

[b] $R = 2.8 \times s_R$.

The enzymes should be of a purity meeting the specifications listed in *Official Method*SM **991.43** (20), but as modified below for application in the assay for dietary starch. The enzyme preparation used must be validated within laboratory to verify efficacy, as well as lack of interference. Recommended validation: analyze 0.1 g test portions of purified glucose, sucrose, and purified corn starch with the enzymatic portion of the dietary starch assay and using a free glucose value of zero in calculations. Analyses with candidate enzyme should give values of [mean ± standard deviation (SD)] glucose: 90 ± 2%, starch: 100 ± 2%, and sucrose: 0.7 ± 0.3% on a dry matter basis. To test for interference from release of glucose from fiber carbohydrates, analyze 0.1 g test portions of α-cellulose and barley β-glucan that are not contaminated with free glucose with the enzymatic portion of the dietary starch assay. Recovery of these substrates should be less than 0.5% on a dry matter basis (20). Use AOAC approved methods for determination of dry matters of the samples. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%), or background absorbance readings will interfere with test sample measurements.

(**c**) *Diluted amyloglucosidase solution.*—Dilute concentrated amyloglucosidase with 100 mM sodium acetate buffer, **C**(**a**), to give 1 mL of solution per test portion with 2 to 5 mL excess. Add 1/3 of needed buffer to an appropriately sized graduated cylinder. Pipet concentrated amyloglucosidase into buffer, rinsing tip by taking up and expelling buffer in the graduated cylinder. Bring to desired volume with additional buffer. Cap cylinder with plastic film and invert cylinder repeatedly to mix. The concentrated amyloglucosidase used should not contain greater than 0.5% glucose, and should have a pH optimum of 4.0 and pH stability between 4.0–5.5 (example of concentrated amyloglucosidase: Product E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland; origin: *Aspergillus niger*, or equivalent).

(*1*) *Based on release of glucose from soluble starch or glycogen.*—200 U/mL (1 unit of enzyme activity is defined as the amount of enzyme required to release 1 μmole glucose/min at pH 4.5 and 40°C; 21).

(*2*) *Based on p-nitrophenyl-β-maltoside method.*—13 units/mL (1 unit is defined as the amount of enzyme required to release 1 μmole *p*-nitrophenol from *p*-nitrophenyl-β-maltoside/min at pH 4.5 and 40°C; 22).

The enzyme used must be validated within laboratory to verify efficacy as well as lack of interference. Use the same validation procedure as described for heat-stable α-amylase, **C**(**b**).

(**d**) *Benzoic acid solution (0.2%).*—Weigh 2.0 g benzoic acid (solid, ACS reagent, >99.5% purity) and add to a flask. Bring flask to 1 L volume with H_2O. Add magnetic stir bar, stopper flask, and allow to stir overnight to dissolve benzoic acid. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**e**) *GOPOD reagent.*—(*1*) *Mixture of glucose oxidase, 7000 U/L, free from catalase activity; peroxidase, 7000 U/L; and 4-aminoantipyrine, 0.74 mM.*—Prepare by dissolving 9.1 g Na_2HPO_4 (dibasic, anhydrous) and 5.0 g KH_2PO_4 in ca 300 mL H_2O in a 1 L volumetric flask. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add 1.0 g phenol (ACS grade) and 0.15 g 4-aminoantipyrine. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add glucose oxidase (7000 U) and peroxidase (7000 U), rinse enzymes into flask with H_2O, and swirl gently to dissolve without causing excessive foaming. Bring to 1 L volume with H_2O. Seal and invert repeatedly to mix. Filter solution through a glass fiber filter with 1.6 μm retention, **B**(**s**). Store in a sealed amber bottle at ca 4°C. Reagent life: 1 month. Before use in test sample determinations, determine a standard curve for the reagent using a 5-point standard curve using **C**(**e**) and **C**(**f**) according to **D**(**b**).

(*2*) Alternatively, use another AOAC-approved glucose-specific assay that has passed in laboratory validation to accurately determine glucose concentrations of glucose standard solutions and give values equivalent to the values listed for determination of efficacy of enzymes. Recommended validation: analyze all five glucose working standard solutions and 100 mg test portions of purified glucose, purified sucrose, and purified corn starch that have been processed through the enzymatic hydrolysis portion of the dietary starch procedure and using a free glucose value of zero in calculations. The glucose values of the working standard solutions should be predicted ±6 μg glucose/mL. On a dry matter basis, the control sample glucose should give a dietary starch value (mean ± SD) of 90 ± 2%, corn starch at 100 ± 2%, and sucrose 0.7 ± 0.3%.

(**f**) *Glucose working standard solutions.*—0, 250, 500, 750, and 1000 μg/mL. Determine the dry matter of powdered crystalline glucose (purity ≥99.5%) by an AOAC-approved method. Weigh approximately 62.5, 125, 187.5, and 250 mg portions of glucose and record weight to 0.0001 g. Rinse each portion of glucose from weigh paper into a separate 250 mL volumetric flask with 0.2% benzoic acid solution, **C**(**d**), and swirl to dissolve. Bring each standard to 250 mL volume with 0.2% benzoic acid solution, **C**(**d**), to give four independent glucose standard solutions. The 0.2% benzoic acid solution, **C**(**d**), serves as the 0 μg/mL standard solution. Multiply weight of glucose by dry matter percentage and percentage purity as provided by the manufacturer in the certificate of analysis and divide by 250 mL to calculate actual glucose concentrations of the solutions. Prepare solutions at least one day before use to allow equilibration of α- and β- forms of the glucose. Standard solutions may be stored at room temperature for 6 months.

(**g**) *Internal quality control samples*.—Powdered crystalline glucose (purity ≥99.5%) and isolated corn starch. For the corn starch sample, crude protein as nitrogen content × 6.25 and ash should be determined to determine the nonprotein organic matter content of the sample. For use in recovery calculations, actual starch content of the corn starch control sample is estimated as 100% minus ash% and minus crude protein%, all on a dry matter basis. Analyze 100 mg of each sample with each batch of test samples. Glucose will allow evaluation of quantitative recovery, and starch will allow evaluation of quantitative recovery and efficacy of the assay.

(**h**) *Determination of accuracy of volume additions for use of summative volume approach*.—The method as described relies on accurate volumetric additions in order to use the sum of volumes to describe test solution volume. Accuracy of volume additions can be evaluated before the assay by the following procedure: Using 1–2 L distilled water at ambient temperature, determine the g/mL density of the water by recording the weight of three empty volumetric flasks (volumes between 50 and

100 mL), add the water to bring to volume, and weigh the flasks + water. Calculate water density g/mL as:

$$\text{Water density g/mL} = [(\text{flask + water, g}) - (\text{flask, g})]/\text{water volume mL}$$

Record the weights of five empty tubes used for the dietary starch assay. Using the ambient temperature water and the devices used to deliver the liquid volumes for the enzymatic hydrolysis portion of the assay, deliver the 30, 0.1, 1, and 20 mL volumes to each tube (total of 51.1 mL in each tube). Record the weight of each tube + water. Calculate the grams of water in each tube as:

$$\text{Water in each tube, g} = (\text{tube + water, g}) - (\text{tube, g})$$

Divide the weight of water in each tube by the determined average density of water to give the volume of water in each tube. The deviation should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. If the deviations are greater than these, after the addition of 20 mL water during the dietary starch assay, individual samples should be quantitatively transferred with filtration through hardened filter paper with a 22 µm retention, **B**(**t**), into a 100 mL volumetric flask and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

D. Preparation of Reagent Blanks, Standard Curves, and Test Samples

(**a**) *Reagent blank.*—For each assay, two reaction tubes containing only the reagents added for each method are carried through the entire procedure. Reagent blanks diluted to the same degree as samples (no dilution or diluted to the same degree as control and test samples) are analyzed. Absorbance values for the reagent blanks are subtracted from absorbance values of the test solutions prepared from test and control samples.

(**b**) *Standard curves.*—Pipet 0.1 mL of 0.2% benzoic acid solution, **C**(**d**), and nominal 250, 500, 750, and 1000 µg/mL working standard glucose solutions, **C**(**f**), in duplicate into the bottoms of 16 × 100 mm glass culture tubes. Add 3.0 mL GOPOD reagent, **C**(**e**), to each tube using a positive displacement repeating pipet aimed against wall of tube, so it will mix well with the sample. Vortex tubes. Cover tops of tubes with plastic film. Incubate in a 50°C water bath for 20 min. Read absorbance at 505 nm using the 0 µg glucose/mL standard to zero the spectrophotometer. All readings should be completed within 30 min of the end of incubation; avoid subjecting solutions to sunlight as this degrades the chromogen. Calculate the quadratic equation describing the relationship of glucose µg/mL (response variable) and absorbance (abs) at 505 nm (independent variable) using all individual absorbances (do not average within standard). The equation will have the form:

$$\text{Glucose, µg/mL} = \text{abs x quadratic coefficient} + \text{abs} \times \text{linear coefficient} + \text{intercept}$$

Use this standard curve to calculate glucose µg/mL in test solutions. A new standard curve should be run with each glucose determination run.

(**c**) *Test samples.*—Feed and pet food amenable to drying should be dried at 55°C in a forced-air oven. Dried materials are then ground to pass the 0.5 or 1.0 mm screen of an abrasion mill or the 0.5 mm screen of a cutting mill or other mill to give an equivalent fineness of grind (to pass a 40 mesh screen). Ground, dried materials are transferred into a wide mouthed jar and mixed well by inversion and tumbling before subsampling. Semi-moist, moist, or liquid products may be homogenized, blended, or mixed to ensure homogeneity and reduced particle size (23).

E. Determination of Dietary Starch

The analyses for free glucose and enzymatically released glucose + free glucose may be performed in separate analytical runs. For flow of assay, *see* Figure **2014.10**.

(*1*) Accurately weigh two test portions (W_E, W_F) of 100 to 500 mg each of dried test samples or 500 mg semi-moist, moist, or liquid samples (for all samples, use ≤500 mg, containing ≤100 mg dietary starch; use 500 mg for samples containing <2% dietary starch) into screw-cap glass tubes. Test portion W_E is for the analysis of enzymatically released glucose and W_F is for the determination of free glucose. In addition to unknowns, weigh test portions (W_E, W_F) of D-glucose and purified corn starch, which serve as quality control samples **C**(**g**). Also include two tubes with no test portion to serve as reagent blanks per each analytical run for free glucose or enzymatically released glucose + free glucose.

(*2*) Dispense 30 mL of 0.1 M sodium acetate buffer, **C**(**a**), into each tube.

(*3*) To tubes with test portions designated W_E and to each of the reagent blanks to be used with analysis of enzymatically released glucose + free glucose, add a volume of heat-stable, α-amylase, **C**(**b**), to deliver ca 1800 to 2100 liquefon units or 8200 to 8300 BAU of enzyme activity (typically 0.1 mL enzyme as purchased); do not add the amylase to W_F and to the reagent blanks to be used with free glucose determinations. Cap tubes and vortex to mix.

Note: Vortex tube so that the solution column extends to the cap, washing the entire interior of the tube and dispersing the test portion.

(*4*) Incubate all tubes for 1 h at 100°C in a forced-air oven, vortexing tubes at 10, 30, and 50 min of incubation.

(*5*) Cool tubes at ambient temperature on bench for 0.5 h. At this point, separate tubes designated for free glucose analysis (tubes containing W_F test portions and reagent blanks with no enzyme) from the rest of the run. Those designated for free glucose should skip steps (*6*) and (*7*) and continue with steps (*8*)–(*13*).

(*6*) Add 1 mL of diluted amyloglucosidase solution, **C**(**c**), to W_E test and quality control samples and reagent blanks. Vortex tubes.

(*7*) Incubate tubes for 2 h in a water bath at 50°C, vortexing at 1 h of incubation.

(*8*) Add 20 mL water to each tube. Cap and invert at least 4 times to mix completely. Proceed immediately through steps (*9*)–(*13*).

(*9*) (*a*) *Volume by sum of volume additions*.—Transfer ca 1.5 mL test sample solutions to microcentrifuge tubes, and centrifuge at 1000 x *g* for 10 min. If the sample remains cloudy after centrifugation, centrifuge an additional 10 min at

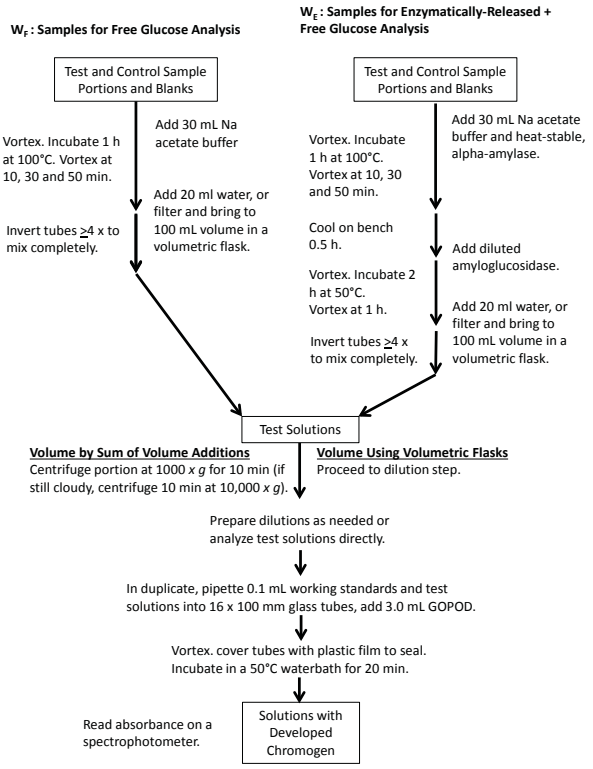


Figure 2014.10. Flow chart of the dietary starch assay.

10 000 × *g* to clarify the solution before proceeding. Solutions may increase in temperature during centrifugation; allow centrifuged solutions to come to room temperature before preparing dilution.

(*b*) *Volume using volumetric flasks*.—Quantitatively transfer test sample solutions with filtration through a hardened paper filter with 22 µm retention and rinses with water to 100 mL volumetric flasks.

(*10*) Prepare dilutions as needed with distilled or deionized water. Solutions from control samples and test samples estimated to give greater than 1000 µg glucose/mL concentrations of free and released glucose should be diluted 1 in 10 if processed as in (*9*)(*a*) or 1 in 5 if processed as in (*9*)(*b*). Reagent blanks should be diluted to provide solutions with the same dilutions as used with the test solutions, so that the diluted reagent blank solutions can be used to make corrections for similarly diluted test solutions. Dilutions may be prepared using volumetric flasks or by accurate pipetting. If done by pipetting, use a minimum of 0.5 mL test sample or control solution to minimize the impact of variation in pipetting small volumes.

(*11*) Pipet 0.1 mL in duplicate of glucose working standard solutions (0, 250, 500, 750, and 1000 µg/mL glucose), **C**(**f**), and reagent blank, quality control sample, and test sample solutions into the bottoms of 16 × 100 mm glass test tubes using two tubes/solution. Add 3.0 mL GOPOD reagent, **C**(**e**)(*1*), to each tube. Vortex tubes. Place tubes in a rack and cover with plastic film to seal.

Note: Alternative to the use of the GOPOD method, proceed with alternate glucose determination method, **C**(**e**)(*2*), for measurement of glucose in working standards, reagent blank, control sample, and test sample solutions.

(*12*) Incubate in a 50°C water bath for 20 min.

(*13*) Set spectrophotometer to measure absorbance at 505 nm. After the incubation is complete, zero the spectrophotometer with the GOPOD-reacted 0 µg/mL working standard solution. Read absorbances of remaining GOPOD-reacted working standard solutions, and reagent blank, control sample, and test sample solutions. All reacted solutions must be read within 30 min of the end of the GOPOD incubation. The duplicate absorbance values are averaged for each reagent blank, test sample, and control sample solution and used in *Calculations*.

F. Calculations

Determine the quadratic equation that fits the absorbances of the working standard solutions. The absorbance values, A_{CF} or A_{CE}, are the independent variables (X), and actual glucose concentrations are the dependent variables (Y). Individual absorbance values of the working standard solutions, not averages, are used. The equation has the form:

$$\mu g\ Glucose/mL = (A_{CF\ or\ CE}^{2} \times Q + A_{CF\ or\ CE} \times S + I)$$

Calculate dietary starch content in test sample as received as follows:

$$Free\ glucose,\ \% = (A_{CF}^{2} \times Q + A_{CF} \times S + I) \times V_F \times DF_F \times 1/1\,000\,000 \times 1/W_F \times 162/180 \times 100$$

$$Dietary\ starch,\ \% = [(A_{CE}^{2} \times Q + A_{CE} \times S + I) \times V_E \times DF_E \times 1/1\,000\,000 \times 1/W_E \times 162/180 \times 100] - free\ glucose\ \%$$

where subscript *F* represents values for samples analyzed for free glucose and subscript *E* represents values for samples treated with amylase and amyloglucosidase; A_{CF}, A_{CE} = absorbance of reaction solutions minus the absorbance of the appropriately diluted reagent blank, values are averages of the two replicates for each test solution; Q = quadratic slope term, S = linear slope term, and I = intercept of the standard curve to convert absorbance values to µg glucose/mL; V_F, V_E = final sample solution volume, ca 50.0 mL for V_F and 51.1 mL for V_E if done by summation of volumetric additions, otherwise, by size of volumetric flask used; DF = dilution factor, e.g., 0.5 mL sample solution diluted into 5.0 mL = 5.0/0.5 = 10; 1 g/1 000 000 µg = conversion from µg to g; W_E, W_F = test portion weight, as received; 162/180 = factor to convert from measured glucose as determined, to anhydroglucose, as occurs in starch.

If test samples are run in duplicate portions, the free glucose % in the dietary starch equation is the average free glucose % value determined for the test sample.

Results and Discussion

Evaluation of the Dietary Starch Method

Initial evaluation of data from all laboratories showed that most outliers occurred in two laboratories (Table 2). Laboratory 14 had significant Cochran's tests for five of the test materials, indicating suspect replicate results within this laboratory. Unlike the other laboratories, Laboratory 14 ran duplicate portions of test materials on separate days, rather than together within the same run. Based on laboratory ranking scores (18), this laboratory was designated as an outlier and its data were

Table 2. Results of collaborating laboratories for dietary starch individual replicate values on an as-received basis

		Collaborating laboratory														
Material	Duplicate	0	1	2	3	4	5	6	7	8	9	10	11[a]	12	13	14[b]
Moist canned dog food	1	1.58	1.42	1.34	1.56	1.58	1.58	1.57	1.64	1.59	1.44	**2.47**[c]	**1.94**[d]	1.55	**1.84**[c]	**0.22**[c]
	2	1.57	1.46	1.36	1.67	1.62	1.59	1.47	1.62	1.60	1.44	**1.59**[c]	**1.94**[d]	1.53	**1.61**[c]	**0.32**[c]
Low starch horse feed	1	7.03	6.29	7.01	7.30	6.78	7.21	6.88	7.33	7.27	6.47	6.68	8.32	7.15	7.02	**5.76**[c]
	2	7.21	6.50	7.44	7.60	6.43	7.61	7.02	7.33	6.98	6.74	7.37	7.87	7.08	6.68	**6.50**[c]
Dry ground corn	1	70.80	63.08	71.80	**58.85**[c]	71.27	68.13	70.18	71.22	71.52	71.25	67.97	**5.84**[d]	70.39	68.98	**60.19**[c]
	2	69.24	63.14	72.89	**26.64**[c]	70.23	67.33	71.47	73.29	71.08	70.04	65.82	**5.93**[d]	70.53	68.74	**65.42**[c]
Complete dairy feed	1	29.19	26.86	28.53	28.90	26.88	28.27	28.39	29.33	29.07	27.59	**26.89**[c]	**37.21**[d]	28.41	25.42	27.85
	2	29.79	26.69	28.49	30.02	26.11	28.70	28.19	29.10	28.89	27.49	**30.90**[c]	**35.45**[d]	28.01	26.10	27.28
Soybean meal	1	1.01	1.04	**1.09**[c]	1.10	0.97	1.13	0.94	1.04	1.06	0.87	1.02	**2.35**[d]	0.82	1.00	**0.02**[c]
	2	1.03	1.11	**1.42**[c]	1.19	0.93	1.11	0.90	0.93	1.09	0.78	1.16	**2.38**[d]	0.84	1.02	**0.82**[c]
Distillers grains	1	4.02	3.90	4.23	4.27	4.05	4.55	4.05	4.16	3.99	4.10	3.81	**4.82**[e]	4.19	3.98	**3.16**[c]
	2	4.07	3.90	4.09	4.30	4.08	4.49	3.94	4.14	4.06	4.06	4.09	**4.85**[e]	4.58	3.79	**3.00**[c]
Poultry feed	1	28.67	28.12	28.57	28.71	26.47	27.99	27.44	29.59	28.78	27.67	27.9	26.50	29.07	25.06	27.51
	2	29.25	27.35	27.95	30.26	28.00	28.27	28.52	29.43	28.83	27.65	30.39	25.18	29.45	24.80	26.56
Corn silage	1	41.10	37.44	39.20	40.92	37.54	39.18	38.08	39.17	40.91	37.00	37.26	36.03	43.50	36.59	37.99
	2	40.34	36.84	39.02	41.59	37.71	38.58	37.65	39.83	40.22	37.34	40.23	35.72	41.31	36.40	36.55
Dog kibble, dry	1	29.87	25.50	24.58	27.73	29.23	27.53	27.37	24.10	27.32	**17.99**[f]	25.73	27.55	28.68	26.30	24.31
	2	27.92	26.45	27.52	24.21	26.57	27.33	25.64	28.00	25.19	**18.35**[f]	27.25	26.93	29.34	25.70	26.25
Alfalfa pellets	1	1.29	1.17	1.56	1.32	1.56	1.59	1.61	1.35	1.33	1.58	1.42	1.31	1.13	1.25	0.60[c]
	2	1.36	1.43	1.43	1.41	1.32	1.61	1.31	1.24	1.34	1.38	1.35	1.13	1.38	1.27	**1.01**[c]

[a] Data for this laboratory was omitted from analysis based on a 7% change in glucose standard absorbances between runs for detection of free glucose and free + enzymatically released glucose. When data were included, four of 10 samples were identified as outliers by the single Grubbs' test, and one by the double Grubbs' test.

[b] Outlier laboratory detected by laboratory ranking.

[c] Outlier detected by the Cochran's test.

[d] Outlier detected by the single Grubbs' test.

[e] Outlier detected by the double Grubbs' test.

[f] Data omitted from analysis because the large test portion used (0.5 g) exceeded the 100 mg α-glucan limit for this assay.

not used in calculation of the study statistics. Laboratory 11 had four outlier values detected by the single Grubbs' test, which would indicate that this laboratory's values for these test samples were substantially higher or lower than those generated by the other laboratories. The very low value for dry ground corn appeared to be a possible error in recording the dilution of the sample, but laboratory records indicated that that was not the case. The basis for the high values for dairy feed, soybean meal, and moist canned dog food was not immediately obvious. The distillers grains results for Laboratory 11 was designated as an outlier based on results of the double Grubbs' test.

Laboratory 11 was not designated as an outlier by the ranking procedure, but test material results were generally higher for this laboratory. A likely basis for the higher dietary starch values was that the absorbances of the glucose standards were lower in the analytical run with the test samples treated with enzyme than were those reported for two other standard curves run for the dietary starch assay in that laboratory. The decrease in absorbance was on the order of 0.029 to 0.089 for 500 and 1000 mg glucose/mL standard solutions. To put this in perspective, the difference in absorbance values between runs represents an almost 8% lower absorbance value for the 1000 mg glucose/mL standard in the assay with enzyme-treated test samples. Standard curves produced from lower absorbance values will give higher calculated glucose and dietary starch values if the absorbances of the test samples are not similarly depressed. Absorbance values for glucose standards are not expected to be identical among analytical runs. However, the glucose oxidase-peroxidase assay used tends to be very consistent. For example, in the Study Director's laboratory, eight glucose standard curves run with dietary starch assays on 4 separate days showed RSD values (SD/mean) of less than 0.8% for absorbance values determined across runs within glucose standard (Table 3). Data from 12 collaborating laboratories that provided absorbance data for more than one standard curve showed the RSD of the absorbances calculated for individual glucose standards and then averaged across all standards were less than 1% for five laboratories, less than 2% for eight,and more than 2% for four (Table 4). Replicate absorbance readings for glucose standards within analytical run showed overall good repeatability for all laboratories. Laboratory 14, which was excluded from the study based on a ranking test, had the

Table 3. Absorbance values for glucose standards analyzed in repeated runs and in the collaborative study

Repeated analyses of glucose standard solutions: values by standard[a]						
Glucose standard, µg/mL	Runs[b]	Mean[c]	SD[d]	CV%[d]	Minimum value	Maximum value
249.4	8	0.285	0.0020	0.69	0.282	0.289
499.4	8	0.568	0.0028	0.49	0.563	0.574
748.7	8	0.848	0.0031	0.36	0.841	0.852
998.7	8	1.125	0.0045	0.40	1.116	1.133
Collaborative study: means across standards of values calculated for individual standards						
Laboratory	Runs	Overall mean[e]	Mean SD[f]	Mean CV, %[g]	Replicate SD[h]	
Study Director	3	0.704	0.0023	0.35	0.001	
7	2	0.688	0.0031	0.46	0.002	
8	4	0.712	0.0040	0.62	0.003	
13	2	0.658	0.0034	0.68	0.003	
2	2	0.855	0.0068	0.79	0.007	
1	6	0.827	0.0083	1.41	0.004	
12	3	0.684	0.0092	1.47	0.004	
3	2	0.736	0.0073	1.49	0.005	
6	2	0.723	0.0121	1.56	0.009	
4	3	0.682	0.0143	2.22	0.008	
5	4	0.727	0.0160	2.28	0.007	
11	3	0.709	0.0287	3.55	0.009	
14	2	0.667	0.0531	8.78	0.004	

[a] Glucose standards were analyzed in the Study Director's laboratory in eight separate analytical runs for the dietary starch assay. Glucose standards were analyzed in duplicate in two separate runs/day on 4 days. Two separate batches of GOPOD reagent were each used for four runs. The same preparations of glucose standards were used for all eight runs.

[b] Number of separate analytical runs in which the glucose standards were analyzed in duplicate.

[c] The mean value of the 16 replicates for each glucose standard.

[d] SD = standard deviation; RSD = 100 × (SD/mean).

[e] Mean of all absorbance values generated by the laboratory.

[f] The mean of all SD of absorbance values calculated for individual glucose standards.

[g] The mean of all RSD of absorbance values calculated for individual glucose standards.

[h] The mean of all SD of absorbance values for replicate pairs of glucose standards.

largest average RSD for absorbances of the glucose standards. Given the good replication for duplicates in this laboratory, the large RSD reflects differences in glucose standard absorbances between analytical runs. The difference this variation would generate in the standard curves could explain the variation detected in test sample replicates for this laboratory, because test sample duplicates were analyzed singly in separate runs, each of which used a different standard curve. Laboratory 11 had the second highest average RSD for absorbances of the standards. Discussions with Laboratory 11 did not uncover the basis for the variation between analytical runs. The dietary starch assay relies on the soundness of the standard curves to give reliable results. For Laboratory 11, because the glucose standard results used with the enzyme-treated samples deviated from two other standard curves they performed, and because the lower absorbances gave a standard curve that appears to have inflated the dietary starch values, the data are suspect and has been omitted from the statistical analysis of this study.

It is important to control the run to run and between replicate variation in analysis of the glucose standards because of the impact these have on accuracy of results. This GOPOD glucose detection assay is highly sensitive to pipetting accuracy. Samples should be read within 30 min of the end of incubation with GOPOD. It is also recommended that the incubated GOPOD-reacted samples be kept out of sunlight as this can degrade the chromagen. In addition to evaluating standard curve data for obvious changes in response, it is recommended that for each batch of GOPOD a log be kept of absorbance data for glucose standards from all runs. Within a glucose standard, calculate the SD of all absorbances. The mean of these SDs across all standards should not be greater than 0.016. Even lower levels of variability in absorbances can be readily achieved with this assay.

Another factor that likely affected accuracy was exceeding the 100 mg of starch limit/test portion in the assay, which was the case for Laboratory 9 when dry dog kibble was analyzed using 0.5 g test portions. The resulting low dietary starch values were likely the result of the enzyme no longer being in the excess required for complete hydrolysis of the dietary starch.

Variability of results may also have been affected by the approach to sample dilution. Laboratory 3 used 0.1 mL of test sample solution and 0.9 mL of water to make a 1 in 10 dilution

Table 4. Statistical data for dietary starch results

Material	Outlier	*n*	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	2.8 × s_r	2.8 × s_R	HorRat	Largest within-lab variance	Largest average lab result	Smallest average lab result
Moist canned dog food	10, 13	11	1.53	0.03	0.09	2.21	5.99	0.10	0.26	1.60	0.01	1.63	1.35
Low starch horse feed		13	7.02	0.23	0.36	3.32	5.19	0.65	1.02	1.74	0.24	7.45	6.40
Dry ground corn	3	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54	1.83	2.31	72.34	63.11
Complete dairy feed	10	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48	1.83	0.64	29.49	25.76
Soybean meal	2	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31	2.79	0.01	1.15	0.83
Distillers grains		13	4.11	0.11	0.20	2.67	4.94	0.31	0.57	1.53	0.08	4.52	3.88
Pelleted poultry feed		13	28.24	0.73	1.34	2.58	4.76	2.04	3.76	1.97	3.10	29.51	24.93
Corn silage		13	39.04	0.80	1.88	2.05	4.82	2.24	5.27	2.09	4.41	42.40	36.49
Dog kibble, dry	9	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46	2.43	7.61	29.01	25.97
Alfalfa pellets		13	1.38	0.12	0.13	8.61	9.69	0.33	0.38	2.54	0.05	1.60	1.25

for the ground corn sample. Even with small differences in pipetted amounts, such an approach could result in the between duplicate difference noted for that sample. Test solutions from the enzymatic hydrolysis procedure can be "sticky", i.e., they do not pipet exactly like water, and require care to pipet accurately. If dilutions are made by pipetting, prewetting of pipet tips and use of larger volumes, such as 0.5 mL of test solution and 4.5 mL of water, are recommended.

The quantity of test material used also may have affected assay variability. Test samples with starch contents of less than 2% generally showed greater variability than test samples that contained more starch (Figure 1 and Table 4) in a pattern nearly identical to that described by Wehling and DeVries (24) for dietary fiber assays. However, among the low starch materials, the moist dog food had RSD values for repeatability and reproducibility that were approximately half those of soybean meal and alfalfa pellets (Table 4); these latter two samples also had the highest HorRat values in the study. In addition to being the only moist, homogenized sample, laboratories were directed to use 0.5 g of the moist dog food as compared to 0.1 g of other samples. The one case in which dietary starch values for the moist dog food were identified by the Cochran test as suspect replicates within laboratory was where Laboratory 10 reported values determined on 0.10 g test samples for this material (Table 2). In the collaborative study, the 0.1 g sample size was used for most samples to minimize the likelihood that the 100 mg limit of dietary starch/test portion would be exceeded, based on the laboratories' prestudy results with the assay; however, it also greatly reduced the concentration of glucose to be detected in low starch test samples. Final glucose concentrations of test sample solutions for 0.1 g enzyme-treated test portions of soybean meal and alfalfa pellets were 22 and 30 µg/mL, respectively as compared to 167 µg/mL for the moist dog food using 0.5 g test portions. These glucose concentrations of the low starch feeds equate to absorbance values of 0.035, 0.054, and 0.221, respectively, as determined in the Study Director's laboratory. Although the glucose detection assay is sensitive and precise, small variations in absorbances of test solutions with very low glucose concentrations will give more variability in calculated glucose values than the same amount of variation will with test solutions with higher glucose concentrations. This can result in greater within and between laboratory variability for low starch test samples for which smaller test portions are used. In the case of the dietary starch assay, as with gravimetric dietary fiber analyses, the increase in RSD as concentrations of the analyte approaches zero may be related to limits of precision of the detection methods themselves. The absorbances and glucose concentrations noted for soybean meal, alfalfa pellets, and moist dog food represent 1.0, 1.4, and 7.7 mg of dietary starch in the respective test portions. It is notable that the distillers grains, for which the 0.1 g test portion would provide approximately 4 mg of dietary starch, had a HorRat value below 2, possibly suggesting a level of dietary starch at and above which precision is improved.

A viable approach to decreasing RSD values for low starch test samples analyzed with the dietary starch method is to increase the size of the test portion in order to increase the amount of analyte to be detected. The idea of increasing the amount of test sample analyzed in order to improve precision by having a greater amount of analyte to measure has been raised (25). Unlike the dietary fiber analyses that may need to restrict test portion size to assure that the extractant remains in excess, starch assays will primarily be restricted by the need to maintain an excess of enzyme to assure complete hydrolysis of the α-glucan. The approach of allowing a range of test portions but a limit on the amount of starch added to the reaction vessel is used by two current AOAC starch methods: AOAC Method **948.02** for starch in plants (26) specifies a use of 0.1–1.0 g of test portion containing approximately 20 mg of starch, and AOAC Method **979.10** for starch in cereals (27) indicates use of a 0.5 g test portion and then specifies "≤1.0 g containing ≤0.5 g starch". In the present method, a limit of 100 mg of dietary starch in each reaction vessel leaves latitude to increase the size of the test portion to that upper limit. Although 0.1 g test portions may be generally adequate, increasing the amount of substrate within the bounds of the assay for feedstuffs with low starch contents may reduce variability of results. The remaining caveat is that as sample quantity is increased, attention must be paid to increasing amounts of interfering substances also brought into the reaction (e.g., antioxidants if the GOPOD assay is used).

With the exceptions of dry ground corn, dairy feed, poultry feed, and corn silage, s_r and s_R were similar within materials (Table 3). The HorRat values obtained in the present study compared favorably to those obtained with AOAC Method **996.11** (10; Table 3). In the collaborative study for that method, starch analyses performed without dimethyl sulfoxide (DMSO)

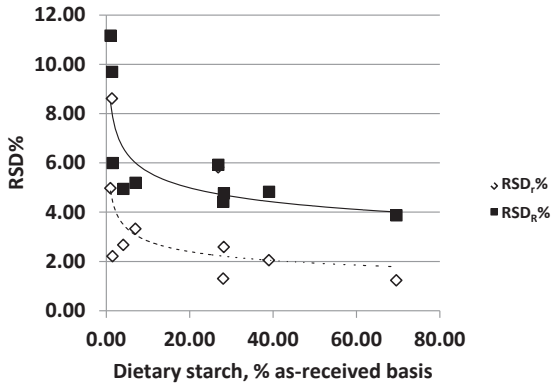


Figure 1. Relationship of dietary starch concentration and RSD values for repeatability within laboratory (RSD_r) and reproducibility between laboratories (RSD_R) obtained in the collaborative study. Equations for the regression lines are $RSD_r, \% = 4.8616x^{-0.236}$ ($R^2 = 0.35$; dashed line), and $RSD_R, \% = 8.4397x^{-0.176}$ ($R^2 = 0.66$; solid line), where x = dietary starch concentration.

had a starch content of 59.8% as received, and an average HorRat of 2.1 with one value below 2. For the dietary starch collaborative study, the HorRat was less than 2 for six of 10 materials, with an overall average of 2.0 on test materials that averaged 20.7% dietary starch on an as-received basis. Alfalfa pellets and soybean meal had HorRat values of greater than 2.5. As previously discussed, the high RSD_R for these test materials may relate to the combination of their low starch content and the small test portion amount used. Test samples with very low concentrations of the analyte have been reported to give elevated HorRat values (17). The high HorRat value for the dry dog kibble may reflect an issue with homogeneity of the sample, as described previously.

Collaborators' Comments

The collaborators all reported that the assay was not very complicated and was easy to do. They particularly liked additions of all reagents to a single vessel, performing reactions in screw cap tubes, determining total liquid volume as the sum of quantitative volume additions, and making sample solution dilutions by accurate pipetting of volumes. They indicated that they had to work within their laboratories to find tools of acceptable accuracy to make the volume additions, as some of the tools they worked with for other purposes were not adequate. They did report issues with screw cap tube adequacy to hold the needed volume; this was apparently related to differing amounts of glass used by the manufacturers while maintaining the same exterior dimensions of the tubes. That was addressed by describing the screw cap tubes by the volume they needed to contain while allowing adequate room for mixing. With the number of sodium phosphate chemicals available, it was noted that it was crucial to verify and use the exact chemicals specified for the GOPOD reagent. It was also raised that the only extended period to take a break from the assay was during the amyloglucosidase incubation; taking a break after adding water to the fully digested samples resulted in reduced recovery. Development of an approved assay for glucose detection that could be used on a plate reader or automated system was recommended as a way to increase throughput of the assay, which is currently limited by the 30 min period within which samples must be read after incubation in the GOPOD glucose detection assay. Some laboratories had issues with calculating quadratic glucose standard curves; this was resolved by graphing all individual glucose standard solution absorbances data with absorbance on the X-axis and glucose concentration on the Y-axis. Then, a quadratic or second order polynomial regression or "trend" line was graphed through the data. The regression line equation was used for calculation of glucose in test solutions. Collaborators gave extensive input on the method protocol writeup and recommended development of a flow chart for the assay

Recommendations

Based on the results of the collaborative study, the Study Director recommends that the enzymatic-colorimetric method for measurement of dietary starch in animal feeds and pet foods be adopted as Official First Action.

Acknowledgments

I thank Jan Pitas (U.S. Dairy Forage Research Center) for assistance in developing the dietary starch method and assistance with preparing and distributing materials for the study. I thank the Laboratory Methods & Services Committee of the Association of American Feed Control Officials for their orchestration of the effort for defining dietary starch and their support and input in this project. I thank Nancy Thiex, Larry Novotny, and the staff of the Olsen Biochemistry Laboratory at South Dakota State University for assistance in preparing the test samples. Special thanks go to Nancy Thiex for her invaluable guidance and assistance throughout the study. The U.S. Department of Agriculture, Agricultural Research Service provided funding for the materials used in the study. I also thank the following collaborators for their participation in this study:

Robin Johnson, Montana Department of Agriculture and Analytical Laboratory, Bozeman, MT

Brian Steinlicht and David Taysom, Dairyland Laboratories, Arcadia, WI

Courtney Heuer, Don Meyer, Zach Meyer, Lauren Meyer, and John Goeser, Rock River Laboratories, Watertown, WI

Kristi McCallum, Dominika Kondratko, and Tyler Potts, Colorado Dept. of Agriculture/I&CS Biochemistry Laboratory, Denver, CO

Lisa Ruiz, John Jordan, and Tuyen Thi Doan, Eurofins Scientific–Nutritional Analysis Center, Des Moines, IA

Kathryn S. Phillips, C. Andre Odijk, and Kenneth Hodel, NP Analytical Laboratories, St. Louis, MO

Lisa Means, Teresa Grant, and Steven Pleasants, North Carolina Department of Agriculture/Food and Drug Protection Division, Raleigh, NC

Christa Willaredt and Sabrina Trupia, NCERC Analytical Laboratory, Edwardsville, IL

Angela Carlson, SGS Brookings, Brookings, SD

Adela Parganlija-Ramic and Michele Swarbrick, Minnesota Department of Agriculture, St. Paul, MN

Kiley Schwartz, Berthier Jean-Louis, Eduardo Maciel, and Kiley Mulholland, Idaho State Department of Agriculture, Twin Falls, ID

Darren Welch and Audra Gile, Kansas Department of Agriculture Laboratory, Topeka, KS

Sarah Snodgrass and Joshua Arbaugh, West Virginia Department of Agriculture, Charleston, WV

H. Dorota Inerowicz and Keith Binkerd, Office of Indiana State Chemist/Feed Chromatography Laboratory Section, West Lafayette, IN

References

(1) Huntington, G.B. (1997) *J. Anim. Sci.* **75**, 852–867
(2) *Official Methods of Analysis* (2006) 18th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **920.40**
(3) ISO 15914 (E) Animal feeding stuffs – Enzymatic determination of total starch content, 1st Ed. 2004-02-01. International Organization for Standardization, Geneva, Switzerland
(4) Bernal-Santos, G., Perfield II, J.W., Barbano, D.M., Bauman, D.E., & Overton, T.R. (2003) *J. Dairy Sci.* **86**, 3218–3228
(5) Pham, T.-H., Mauvais, G., Vergoignan, C., De Coninck, J., Cachon, R., & Feron, G. (2008) *Biotechnol. Lett.* **30**, 287–294
(6) Approved Methods of the American Association of Cereal Chemists (2000) 10th Ed., AACC International, St. Paul, MN, Method **776-11**
(7) Hodge, J.E., & Osman, E.M. (1976) in *Principles of Food Science, Part 1, Food Chemistry*, O.R. Fennema (Ed.), Marcel Dekker, Inc., New York, NY, p. 58
(8) Bach Knudsen, K.E. (1997) *Anim. Feed Sci. Technol.* **67**, 319–338
(9) Hall, M.B. (2009) *J. AOAC Int.* **92**, 42–49
(10) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **996.11**
(11) Dias, F.F., & Panchal, D.C. (1987) *Starch/Stärke* **39**, 64–66
(12) Karkalas, J. (1985) *J. Sci. Food Agric.* **36**, 1019–1027
(13) *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* (2002) AOAC INTERNATIONAL, Rockville, MD
(14) Hall, M.B., Jennings, J.P., Lewis, B.A., & Robertson, J.B. (2001) *J. Sci. Food Agric.* **81**, 17–21
(15) Hall, M.B., & Keuler, N.S. (2009) *J. AOAC Int.* **92**, 50–60
(16) Wu, X., Beecher, G.R., Holden, J.M., Haytowitz, D.B., Gebhardt, S.E., & Prior, R.L. (2004) *J. Agric. Food Chem.* **52**, 4026–4037
(17) Mertens, D.R. (2002) *J. AOAC Int.* **85**, 1217–1240
(18) Wernimont, G.T. (1990) *Use of Statistics to Develop and Evaluate Analytical Methods*, W. Spendley (Ed.), AOAC INTERNATIONAL, Rockville, MD
(19) Food Chemicals Codex (2014) 9th Ed., The United States Pharmacopeial Convention, Rockville, MD, Appendix V, Enzyme Assays, α-Amylase Activity (Bacterial),1392–1393
(20) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **991.43**
(21) McCleary, B.V. (1999) *Cereal Foods World* **44**, 590–596
(22) McCleary, B.V., Bouhet, G., & Driguez, H. (1991) *Biotechnol. Tech.* **5**, 255–258
(23) Thiex, N., Novotny, L., Ramsey, C., Latimer, G., Torma, L., & Beine, R. (2000) *Guidelines for Preparing Laboratory Samples*, Association of American Feed Control Officials, Inc., Champaign, IL
(24) Wehling, P., & DeVries, J.W. (2012) *J. AOAC Int.* **95**, 1541–1546
(25) Horwitz, W., Albert, R., Deutsch, M., & Thompson, M. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 661–680
(26) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **948.02**
(27) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **979.10**

AOAC RESEARCH INSTITUTE

Expert Review Panel on Dietary Starches
OFFICIAL CHAIR'S EXPERT REVIEW PANEL REPORT

ACKNOWLEDGMENT

The undersigned chair hereby confirms that the following document has been reviewed and constitutes the final version of the Official Chair's Report for the Expert Review Panel on Dietary Starches held on Wednesday, September 10, 2014 during the AOAC Annual Meeting and Exposition at the Boca Raton Resort and Club, 501 East Camino Reel, Boca Raton, Florida 33432.

Lars Reimann

LARS REIMANN, EUROFINS

Expert Review Panel Chair

Oct 02, 2014

Date

AOAC RESEARCH INSTITUTE
2275 Research Blvd, Suite 300
Rockville, Maryland 20850
UNITED STATES

Contact:
La'Kia Phillips, Conformity Assessment Coordinator at lphillips@aoac.org
Deborah McKenzie, Sr. Director, DMcKenzie@aoac.org

EXPERT REVIEW PANEL MEETING ATTENDEES

Expert Review Panel Chair (s)
Lars Reimann, Eurofins

Expert Review Panel Members
Sean Austin, Nestle Research Centre
Sneh Bhandari, Silliker, Inc.
Kommer Brunt, Rotating Disc BV
Jon DeVries, Medallion Laboratories
Kai Liu, Eurofins
Barry McCleary, Megazyme International Ireland
Tom Phillips, MD Department of Agriculture
John Szpylka, Silliker, Inc. (Alternate)

Method Authors
Mary Beth Hall, U. S. Department of Agriculture

AOAC Staff
Deborah McKenzie
La'Kia Phillips

Observers
Jason Kong, Ohio Department of Agriculture
Maria Nelson, AOAC Technical Consultant
Ioannis Vrasidas, Eurofins Analytico

EXPERT REVIEW PANEL, METHOD BACKGROUND, AND CONCLUSIONS

Criteria for Vetting Methods to be considered:
AOAC convened the Official Methods of Analysis℠ (OMA) Expert Review Panel for Dietary Starches on Wednesday, September 10, 2014 from 8:00am to 10:00am during the AOAC Annual Meeting and Exposition in Boca Raton, Florida from September 7-10, 2014. The purpose of the meeting will be to 1) Review the Collaborative Study Manuscript/ OMAMAN-13: Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method Collaborative Study (Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA) and to 2) discuss First to Final Action requirements and Feedback mechanisms. The candidate method was reviewed against the approved collaborative study protocol. Supplemental information was also provided to the reviewers which included the collaborative study manuscript, Method Safety Checklist, Collaborative Study Tables, Collaborative Study Figures and Captions, and the Collaborative Study Protocol.

Criteria for Vetting Experts and Selection Process:
The following eight (8) candidates and one (1) alternate member were submitted for consideration by the Official Methods Board to evaluate candidate methods for Dietary Starches methods as per the Expert Review Panel (ERP) Policies and Procedures. The candidates were highly recommended by the Agricultural Materials Community, have participated in various AOAC activities, including but limited to, Method Centric Committees that were formed under the legacy OMA pathway, and were vetted by the Official Methods Board. The experts are Sean Austin, Sneh Bhandari, Kommer Brunt, Jon DeVries, Kai Liu, Barry McCleary, Tom Phillips, John Szpylka, and the Chair, Lars Reimann.

ERP Orientation:
The ERP members have completed the mandatory AOAC Expert Review Panel Orientation Webinar on Wednesday, July 16, 2014.

Expert Review Panel Meeting Quorum
The meeting of the Expert Review Panel was held in person. A quorum is the presence of seven (7) members or 2/3 of the total vetted ERP, whichever is greater. Eight (8) out of the eight (8) voting members were present and therefore met a quorum to conduct the meeting.

Standard Method Performance Requirements (SMPRs): N/A

Conclusion:
The Expert Review Panel reviewed OMAMAN-13: Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method and adopted this method for First Action Official Method status by a unanimous decision with additional revisions as noted in the meeting minutes.

Subsequent ERP Activities:
ERP members have stated that no additional data is requested to move from First to Final Action. User Feedback and supporting documentation in support of the need for quadratic standard curve is expected for this method to move forward to Final Action Official Method status. ERP members will continue to evaluate the method for 2 years.

MEETING MINUTES

I. Welcome and Introductions

The Expert Review Panel Chair, Lars Reimann welcomed Expert Review Panel members and initiated introductions. The Chair discussed with the panel the goal of the meeting.

II. Review of AOAC Volunteer Policies

Deborah McKenzie presented an overview of AOAC Volunteer Policies, Volunteer Acceptance Agreement and and Expert Review Panel Policies and Procedures which included Volunteer Conflicts of Interest, Policy on the Use of the Association, Name, Initials, Identifying Insignia, Letterhead, and Business Cards, Antitrust Policy Statement and Guidelines, and the Volunteer Acceptance Form (VAF). All members of the ERP were required to submit and sign the Volunteer Acceptance Form. The Expert Review panel openly discussed any potential conflicts of interest. The group approved all of the members after disclaimers were noted.

III. Expert Review Panel Process Overview and Guidelines

Deborah McKenzie presented an overview of the Expert Review panel process. The presentation included information regarding method submission, recruitment of ERP members, composition and vetting expertise, method assignments, meeting logistics, consensus, First Action to Final Action requirements, method modifications, publications, and documentation.

IV. Review of Methods

All members of the ERP presented a review and discussed the proposed collaborative study manuscript for Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method. The method author, Mary Beth Hall of the U. S. Department of Agriculture, was not present to address the concerns of the ERP members. A summary of comments was provided to the ERP members.[1]

MOTION:

Motion by DeVries; Second by Szpylka to adopt this method for First Action Official Methods Status with the requested revisions.

OMA METHOD: Line 376: Include “free from catalase activity”.
EDITORIAL: Line 351: Include “the enzymes should be of a purity meeting the specifications listed in OMA methods 985.29 and 991.43
Line 366: The “amylase” should be listed as “amyloglucosidase”
Line 344: Include activity definitions and assay procedures.
Line 118-122: Please clarify section.

The Expert Review Panel would like to know if GOPOD blank is used as instrument blank will the intercept disappear and negate the need for a quadratic standard curve?

Consensus demonstrated by: 8 in favor, 0 opposed, and 0 abstentions (Unanimous). *Motion Passed.*

[1] Attachment 1: Summary of Expert Reviewer Comments for OMAMAN-13

V. Discuss Final Action Requirements for First Action Official Methods (if applicable)

MOTION:
Motion by DeVries, Second by Liu that no additional data is requested to move from First to Final Action. User Feedback and supporting documentation in support of the need for quadratic standard curve is expected.
Consensus demonstrated by: 8 in favor, 0 opposed, and 0 abstentions (Unanimous). ***Motion Passed.***

VI. Adjournment

AOAC RESEARCH INSTITUTE

AOAC *OFFICIAL METHODS OF ANALYSIS* (OMA)

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

	Summary of Method
ER 1	Acceptable
ER 2	It consists of incubation of an aliquot of the sample with thermostable alpha-amylase in pH 5.0 acetate buffer for 1 hr at 100°C with periodic mixing to gelatinize and partially hydrolyze alpha-glucan. Amyloglucosidase is added and mixture is incubated at 50°C for 2 h and mixed. After subsequent addition of water, mixing, clarification, and dilution as needed, free + ezymetically released glucose are measured using a colorimetric glucose oxidase-peroxidase method. Values from a separate determination of free glucose are subtracted to give values of enzymatically-released glucose. Dietary starch = Enzymatically- released glucose multiplied by (162/180) or 0.9 and divided by the as received sample weight (g) used in the assay.
ER 3	Dietary starch is digested to glucose and the increase in glucose level is used to calculate %dietary starch. Potential interferences are either accounted for (inherent glucose) or excluded (deter inherent sucrose digestion and deter maltulose formation).
ER 4	Starch is digested by traditional amylase/amyloglucosidase using gelatinization conditions. Glucose released is measured colorimetrically with adjustment for free glucose in the sample.
ER 5	Ground or homogenized samples are digested with α-amylase and amyloglucosidate in acetate buffer to release glucose from dietary starch. The digestate, after optional dilution, is analyzed for its glucose content. A second sample portion is also assessed for free glucose by treatment with all reagents but the enzymes. The difference of the two glucose result is used to calculate dietary starch content in the sample.
ER 6	Sample (containing up to 100mg of starch) is weighed in duplicate. sodium acetate buffer (pH 5.0) is added to both tubes. Then to one tube alpha-amylase and amylglucosidase are added to hydrolyse the starch. To the other tube no enzymes are added. Samples are then clarified (centrifugation or filtration) and diluted. Aliquots from each tube are then taken for analysis of glucose using the glucose oxidase peroxidase (GOPOD) method, or other suitable validated method for glucose determination. Glucose determined in the untreated sample is subtracted from the glucose determined in the enzyme-treated sample. The result is then multiplied by 0.9 to correct for water uptake during hydrolysis to calculate starch content.
ER 7	good
ER 8	A well performed study. However, the advantages over AOAC Method 996.11 need to be more clearly identified. A significant contribution is the application to samples more relevant to the particular study, but some of the stated general advantages are not substantiated.

	Method Scope/Applicability
ER 1	Animal Feeds and pet foods. 1%-70% starch
ER 2	Animal feedstuffs and pet foods. Limitation in application: The method underestimates dietary starch in feeds and foods whose antioxidant content is known to exceed 10-20 micromol of hydrophilic antioxidant (as ascorbic acid) per 0.1 g of test dry matter. The method in the current format may not be easily applicable to foods/feeds high in phenolic compounds (e.g. beets, red sorghum grain).
ER 3	A wide range of animal and pet feeds were covered in the study. Dry and wet products were included along with a variety of grains as the base material.

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

ER 4	See method scope and applicability statement.
ER 5	Applicable to pet foods (wet and dry), animal feed, forage, as well as grains.
ER 6	method has been applied to a range of different animal feeds; canned dog food, low starch horse feed, ground corn, complete dairy feed, soybean meal, distillers grains, poultry feed, corn silage, dry dog kibble, alfaalfa pellets. It is applicable for the analysis of "dietary starch" as defined in the introduction of the paper.
ER 7	good
ER 8	Non-resistant starch in animal feeds

	General Comments
ER 1	Positive feedback from collaborators
ER 2	The manuscript describes a method and SLV and its performance in multilaboratory study for dietary Starch (glycogen, maltooligosaccharides, and other alpha-1,6-linked glucose carbohydrates, exclusive of resistant starch). This method is replacement of invalidated AOAC 920.14 due to unavailability of one of the enzyme required in the assay. The described method is more efficient than other methods considered.
ER 3	Measurement of carbohydrates by enzyme-digestion and analysis of the liberated mono-saccharides is an established approach which has worked well for a range of carbohydrates. The collaborative data from this study demonstrates this approach works well for dietary starches due to properly accounting for sucrose & inherent glucose interferences, and in deterring formation of maltulose.
ER 4	Excellent approach
ER 5	This method is similar to older, but now obsolete methods in principal, with better description in choice of enzymes and analysis approach of the glucose contents. This method also simplifies experimental procedures by adding reagents into the same tube until the final dilution step.
ER 6	The principles of the method are good. Enzymes are used to specifically hydrolyse the relevant alpha-glucans in feeds (i.e. starch, maltooligosaccharides, etc) composed of alpha-1,4 and alpha-1,6 linked glucose. Other poly- or oligosaccharides should not be hydrolysed. Resulting glucose is determined using a well established procedure (GOPOD) and free glucose which would interfere is accounted for by running a sample without enzymatic hydrolysis. I don't know if the concept of resistant starch is used in the animal feed world. If yes, it would be good to clarify if the methodology is expected to account for all the starch or only the available starch.
ER 7	none
ER 8	Page 2, line 25. In reference to AOAC Method 996.11, the author refers to the method being “quasi-empirical” and justifies this by stating that “glucose is the analyte detected, but its release is determined by run conditions and specification of enzymes.” The term “quasi-empirical” is unacceptable. This method was run through a full AOAC International interlaboratory evaluation involving 31 laboratories and over this number, the RSDr and RSDR values were similar to those reported in this paper. In reference to the comments about the run conditions and specification of enzymes, of course the method was defined. This is a requirement of any method. It is especially important to specify details of enzymes and particularly purity. This is the reason why so many enzyme based methods have failed in the past. It is dangerous to recommend industrial, or in fact any, enzymes that have not been analysed for activity and purity (contamination with other

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

interfering enzymes or sugars etc).

AOAC Method 996.11 has also been adapted to run at pH 5. This was evaluated after I had discussions with Mary Beth Hall in 2007 (or 2008). The method works fine at pH 5 and both enzymes are active and stable at this pH. The change to do both incubations at pH 5 is convenient. However, in our hands, the same analytical values were obtained for a number of starch containing samples when sam both incubations were run at pH 5 as compared to running the alpha-amylase incubation at pH 7 (as per 996.11). It is known that a small amount of maltulose can be formed on hydrolysis of starch by alpha-amylase at pH 7 or above. However, this occurs in the industrial hydrolysis of starch which is performed at a starch concentration of approx. 30% w/v. Starch analyses are performed at a starch concentration of just 0.03% (1,000-fold lower concentration).

Page 4, line 79. “the use of mildly…excludes the use of alkali or DMSO and thus excludes resistant starch from inclusion in the dietary starch fraction”. This is exactly what is measured in AOAC Method 996.11, unless there is a requirement to also measure RS. So where is the difference? Also, how can the author be sure that RS is not hydrolysed in the gut of horses or chickens (pigs will be much the same as humans).

Page 4, line72. Dietary starch is defined and includes glycogen. Of course these methods also measure glycogen and maltodextrins, but glycogen is unlikely to be in an animal ration, and maltodextrins would be rare (perhaps some in distillers grains).

Page 6, lines 119-121. Pure corn starch gave a recovery of 99.3%, but in the interlab results, this averaged at just 89,4%. Why?
Page 7, point (6). In our laboratory, we have not experienced non-linear color formation with GOPOD reagent over t he range 0 – 1.2 absorbance units. Is this a problem with enzyme purity?

Page 8, point (8). Ease of use/efficiency. The advantages claimed are exactly the same advantages as described in AOAC Method 996.11. Where is the difference?

Page 9, lines 182-184. Method uses the same temperature for AMFG and glucose analysis. This is already done in 996.11.

Page 9. Lines 195-197. Enzyme purity.
Enzymes must be free of glucose, but it is essential that they are also free of other enzymes active on other glucose containing polymers e.g. beta-glucan. Industrial AMG preparations are highly contaminated with beta-glucanase and to a lesser extent beta-glucosidase. This requirement should be highlighted.

Page 10, lines 214-215. For the participants in the interlab, did you state purity requirements for glucose oxidase and peroxidase. It is essential that high purity enzymes are used. Glucose oxidase is commonly contaminated with catalase and this results in instability and fading of the color formed in this reaction.

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

	Page 16. Point (b) is missing. Page 16 – Purity and source of alpha-amylase and AMG. Detailed specifications on the source of alpha-amylase are given. However, it must be remembered that these enzymes are made for industrial use. There may be variation from batch to batch in contaminants important in an analytical procedure but of no consequence in the intended industrial application. Industrial AMG cannot be used in analytical procedures because it contains glucose, but more importantly, because they contain contaminating activities that interfere with starch determination in plant samples. As far as I am aware, the Megazyme purified AMG (E-AMGDF) is the only AMG pure enough to use in such assays other than pure AMG, which is too costly to use in such assays. Page 17, line 336. Change “amylase” to “amyloglucosidase” Page 17 (e). A statement should be made about the required purity of glucose oxidase and peroxidase. Page 17, line 378. Phenol is generally not used in glucose determination reagents because it is carcinogenic and also is not very stable. The chemical most commonly used in its place is *p*-hydroxybenzoic acid.
ER 9	For me the term "dietary starch" is new, especailly in connection with animal feed and pet food. Fromenergetic viewpoint, I can agree to include maltodextrins, glycogen fromanimal and microbial origin in the new term dietary starch. However I have problems what to do with the4 different types of resistant starhes, the RS1, RS2, RS3 and RS4. Starch incubation with alpha-amylase at 100 C will hydrolyse the RS1, RS2 and RS4 resistant starch but certainly not the RS3 resistant starch, the so-called retrograded starch. Different animals have different intestinal tracks, for example, pets, pigs, cows some can digest resistant starches, others not. So the content of dietary starch in a feed sample depends also on which kind of animal consumes the feed.The for digestion available "dietary starch" in one sample containing resistant starch categories RS1+RS2+RS3+RS4 is most likely different for pets (originally carnivores and less capable to digest native starches), pigs, cows.

	Method Clarity
ER 1	Positive feedback from collaborators
ER 2	Good with the exception how the limitation of the method in application to matrices containing hydrophilic antioxidant contents/activity exceeding 10-20 micromol as ascorbic acid) per 0.1 g of test dry matter.
ER 3	Easy to read. No issues.
ER 4	Well written and understandable

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

ER 5	Satisfactory
ER 6	Method is clearly written I didn't have problems following it, with the exception of the units used for the enzyme activities. It would be preferable for the authors to define the units of activity for each enzyme since definitions vary from manufacturer to manufacturer. This will be fundamental if the enzymes used need to be replaced with others.
ER 7	good
ER 8	Well thought through study and well written

	Pros/Strengths
ER 1	Single vessel
ER 2	Relatively more efficient method. Very well studied and validated in SLV. 15 labs. collaboratively studied the method and analyzed 10 homogenous test materials (animal feeds and pet foods) using the described method for dietary starch (ranging starch contents of 1-70%). The average within lab. Repeatability as sr for % Dietary starch was 0.49 with a range of 0.03 to 1.56, and among –laboratory repeatability of standard deviation sR averaged 0.96 with a range of 0.09 to 2.69. HORRAT averaged 2.0 for all test samples and 1.9 for samples containing dietary starch more than 2%.
ER 3	Measurement of carbohydrates by enzyme-digestion and analysis of the liberated mono-saccharides is an established approach which has worked well for a range of carbohydrates. The collaborative data from this study demonstrates this approach works well for dietary starches due to properly accounting for sucrose & inherent glucose interferences, and in deterring formation of maltulose. Dietary starch is digested to glucose and the increase in glucose level is used to calculate %dietary starch. Potential interferences are either accounted for (inherent glucose) or excluded (deter inherent sucrose digestion and deter maltulose formation).
ER 4	Traditional chemistry that has been well studied. Can be carried out in modestly equipped laboratories by technical personnel with modest training.
ER 5	Relatively straightforward procedures Satisfactory recovery on glucose and corn starch. Low interference from sucrose , β-glucan and cellulose. Good repeatability and reproducibility.
ER 6	- A simple method that does not need specialized equipment. - option to use alternative methods for glucose analysis is mentioned if a lab does not wish to use the GOPOD assay
ER 7	no comment
ER 8	The specific advantages of this method over AOAC Method 996.11 are not clear. With both methods, good reproducibility and recovery of starch was obtained over a wide range of samples. This method is no easier to perform than 996.11.

	Cons/Weaknesses
ER 1	None
ER 2	The method underestimates dietary starch in feeds and foods whose antioxidant content is known to exceed 10-20 micromol of hydrophilic antioxidant (as ascorbic acid) per 0.1 g of test dry matter. The method in the current format may not be easily applicable to foods/feeds high in phenolic compounds (e.g. beets, red sorghum grain).

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

ER 3	Spectrophotometric measurement does work well and is easy, quick, and reliable. Quantitative of sugars by HPLC is also simple (a bit more expensive though) but will allow tracking of sucrose to assure its digestion did not occur. The method's steps do prevent sucrose digestion by relying on high-purity enzymes. Since these enzymes are more expensive, laboratories using lower cost enzymes would be at risk of reporting less accurate, higher values.
ER 4	Lack of sophisticated instrumentation will be unappealing to those inclined to high level tech methods.
ER 5	Quadratic fit may be difficult for some users to use, and automation of the whole quantitation process is somewhat difficult to achieve. Scaling up is somewhat limited because of the need to measure absorbency within 30 min of GOPOD reaction. High content of anti-oxidant will prevent accurate determination of glucose, forcing other glucose detection methods into consideration.
ER 6	- potential interference of substances with anti-oxidant activity (if this is unknown it needs to be assessed somehow, or an alternative glucose assay should be used) - although it is mentioned that glucose assays other than GOPOD can be used, it does not appear to have been tested or validated. - it is mentioned that leaving the sample (taking a break) after dilution of fully digested samples has an impact on recovery - but why should that be the case?
ER 7	none
ER 8	This is a good method, but would appear not to be an improvement over AOAC

	Supporting Data Comments
ER 1	Impressive data package
ER 2	15 labs. collaboratively studied the method and analyzed 10 homogenous test materials (animal feeds and pet foods) using the described method for dietary starch (ranging starch contents of 1-70%). The average within lab. Repeatability as sr for % Dietary starch was 0.49 with a range of 0.03 to 1.56, and among –laboratory repeatability of standard deviation sR averaged 0.96 with a range of 0.09 to 2.69. HORRAT averaged 2.0 for all test samples and 1.9 for samples containing dietary starch more than 2%.
ER 3	Excellent study
ER 4	Excellent data package. Well done study.
ER 5	Well-organized summary tables about statistics of all matrix results Good study on the glucose standard responses across different batches
ER 6	This looks to be a straight forward assay which did not appear to be problematic for most of the labs involved in the MLT. The authors have mentioned that alternative assays for glucose could be used instead of GOPOD (and may be essential for samples with high anti-oxidant contents). It would be interesting to know if this has been tested in any of the labs because although it is mentioned it does not appear to have been verified.
ER 7	good
ER 8	Method should be accepted with some changes to text

	Method Optimization
ER 1	Done
ER 2	The method has been optimized for its efficiency and better recovery of starch.
ER 3	Keep as written (see comment in Cons/Weaknesses for optional digestion)
ER 4	No further work needed.

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

ER 5	Same temperature for both enzymatic procedures, allowing better efficiency. Changed to quadratic curve due to the slight non-linearity of the standards.
ER 6	It would be interesting to understand why leaving the sample (taking a break) after dilution of fully digested samples has an impact on recovery (line 699, p31)
ER 7	good
ER 8	n/a

	Analytical Range
ER 1	1-100%
ER 2	0-100 mg starch in the assay
ER 3	Range studied was 1.00% - 69.6%. Corn starch was used as a spiking agent which suggests this material can be tested directly on this material (89% dietary starch) as long as enzymes are keep in sufficient excess/
ER 4	See method collaborative study report.
ER 5	~1% to 100%
ER 6	about 1 (lowest amount in samples tested in MLT) - 100% starch (considering corn starch used as control)
ER 7	good
ER 8	Acceptable

	LOQ
ER 1	Approx. 0.3% (probably a little larger)- definitely less than 1%
ER 2	0.9% of starch sample weight basis
ER 3	0.3%. Acceptable limit.
ER 4	See method collaborative study report.
ER 5	0.3%
ER 6	This has been estimated as 0.2% dietary starch by using reagent blanks. The approach seems reasonable, although one may expect the practical LoQ to be higher when applied to samples (and is probably not independent of the free glucose content of a sample)
ER 7	good
ER 8	Acceptable

	Accuracy/Recovery
ER 1	99.3 pure corn starch, 90@ control corn starch.
ER 2	89.9% +/- 3.7%
ER 3	993.8% wi+/- 0.8% is excellent
ER 4	See method collaborative study report.
ER 5	Pure corn starch: 99.3% ± 0.8% (Theoretical = 100%) Corn Starch: 89.9% ± 3.7% (Estimated = 89.4)
ER 6	This does not appear to have been extensively tested. Pure starch products have been assayed and the recoveries are greater than 95%, Dextrins appear to be more problematic, but this does not seem to have been discussed.
ER 7	good

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

ER 8	Good

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

	Precision
ER 1	Average RSDr 3.5%
ER 2	RSDr % = 1.23 - 8.61%
ER 3	Acceptable. Soybean meal was the highest but this was likely due to a possible lower degree of sample homogeneity.
ER 4	See method collaborative study report.
ER 5	2-3% most samples; >8% Alfalfa pellets (low level @ ~1%); ~6% Dry Dog Kibble; 5% Soybean Meal (low level @~1%)
ER 6	RSD(r) varies from 1.2 - 8.6 %, and is generally below 5% which I would generally regard as acceptable.
ER 7	good
ER 8	Good

	Reproducibility
ER 1	Average RSDR 6.1%
ER 2	RSDR% = 3.87 - 11.16%
ER 3	Acceptable. Soybean meal was the highest but this was likely due to a possible lower degree of sample homogeneity.
ER 4	See method collaborative study report.
ER 5	4-6% most samples; ~10% for Alfalfa pellets and Soybean Meal
ER 6	RSD(R) varies from 3.9- 11.2 %, and is generally below 6% which I would also consider acceptable.
ER 7	good
ER 8	Good

	System Suitability
ER 1	Good systems suggested (Starch, sucrose, glucose)
ER 2	The use of corn starch as control sample to evaluative quantitative recovery in the assay.
ER 3	see above
ER 4	Definitely suitable for purpose
ER 5	N/A
ER 6	The use of enzymatic hydrolysis to convert starch to glucose, and the GOPOD assay to specifically assay the starch means the method is very selective. Potential interferences have been identified and suitable controls are mentioned.
ER 7	good
ER 8	Acceptable

OMAMAN-13: Determination Of Dietary Starch In Animal Feeds And Pet Food By An Enzymatic-Colorimetric Method Collaborative Study*

Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

	First Action Recommendation
ER 1	Yes
ER 2	Yes, I do recommend the method to be adopted as First Action Method by ERP after authors have explained the following two limitation of the method in application. 1. The method in the current format is not be easily applicable to foods/feeds high in phenolic compounds (e.g. beets, red sorghum grain). 2. Oats beta-glucan interfere in the assay and provide values above LOD = 0.31 +/- 0.09%.
ER 3	Yes. Recommend consideration of allowing HPLC as an option to measure liberated glucose to calculate %dietary starch. This approach would also measure free, inherent glucose and track if sucrose-digestion has occurred.
ER 4	Yes
ER 5	Yes.
ER 6	Yes
ER 7	yes
ER 8	Yes

	After First Action Recommendation
ER 1	Use feedback
ER 2	NA
ER 3	Recommend consideration of allowing HPLC as an option to measure liberated glucose to calculate %dietary starch. This approach would also measure free, inherent glucose and track if sucrose-digestion has occurred. Decision needed if single or multiple lab work is needed to verify.
ER 4	Just the normal 2 year feedback period. Collaborative is completed and complete.
ER 5	N/A
ER 6	It would be good to test the performance of the method when an alternative glucose assay is used. Clarify the reason why dextrin recovery is low.
ER 7	no
ER 8	That included in the text above. (Please clarify)

AOAC OMA 2014.08	
POLYCYLIC AROMATIC HYDROCARBONS IN SEAFOOD GAS CHROMOTOGRAPHY MASS SPECTROMETRY	
Was the method's applicability considered? **Please state the method's applicability statement:**	
Yes	Applicable for the determination of 32 PAH's in mussel, oyster, and shrimp.
Yes	Applicable for the determination of the following PAHs in mussel, oyster, and shrimp: 1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[g, b,h,i]perylene, benzo[k] fluoranthene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorine, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene. These were representative PAH analytes selected for the collaborative study. The method has been single laboratory validated for 32 PAHs in fish and shrimp and therefore, is expected to be applicable to other GC-amenable PAHs and seafood matrices.
Yes	This method was developed in response to the most recent oil spill in the Gulf coast.
Yes	Applicable for the determination of the following PAHs in mussel, oyster, and shrimp: 1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[g,h,i]perylene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene. These were representative PAH analytes selected for the collaborative study. The method has been singlelaboratory validated for 32 PAHs in fish and shrimp (1), and, therefore, is expected to be applicable to other GC-amenable PAHs and seafood matrices. The concentration ranges evaluated within the collaborative study are given in Table 2014.08A.]
Yes	Applicable for the determination of the following PAHs in mussel, oyster, and shrimp: 1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[g,h,i]perylene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorine, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene. These were representative PAH analytes selected for the collaborative study. The method has been single-laboratory validated for 32 PAHs in fish and shrimp, and, therefore, is expected to be applicable to other GC-amenable PAHs and seafood matrices.
Yes	[Applicable for the determination of the following PAHs in mussel, oyster and shrimp: 1,7-dimethylphenanthene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[g,h,i]perylene, [k]fluoranthene, chrysene, dibenz[a,h]anthraccene, fluoranthene, Fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene. These were representative PAH analytes selected for the collaborative study. The method has been single-laboratory validated for 32 PAHs in fish and shrimp and, therefore, is expected to be applicable to other GC-amenable PAHs and seafood matrices.]

Yes	The method fit for its purpose when it was used in Calgary Laboratory, CFIA. We found the method was very robust.

Were the method's safety concerns addressed prior to the approval for AOAC First Action Official Methods status?	
Yes	
Yes	
Yes	
Yes	
Yes	
Yes	
Yes	

Is the documentation for the safety evaluation available and completed?	
Yes	
Yes	
Yes	
Yes	
Yes	
Yes	
Yes	

Are there any reference materials considered or addressed?	
Yes	
Yes	
Yes	
Yes	
Yes	
Yes	
No	

Is there documentation regarding reference materials available and noted? If so, where is it located?
Yes, Page 63-71 of the ERP book
Yes, the reference material is available through NIST SRM 2977 and SRM 1974c) and is included in the submission package
i believe reference materials were used in the original journal publication Simplified and rapid determination of polychlorinated biphenyls, polybrominated diphenyl ethers, and polycyclic

aromatic hydrocarbons in fish and shrimps integrated into a single methodOriginal Research Article
Pages 84-91
Kamila Kalachova, Jana Pulkrabova, Lucie Drabova, Tomas Cajka, Vladimir Kocourek, Jana Hajslova
analytica chimica acta

D. Reference Standards
NIST standard reference, Standard Reference Material (SRM) 2977 should be mentioned in the method.

The study, as reported in Mastovska et al.: Journal of AOAC International Vol. 98, No. 2, 2015 used Reference Material 1974b, which is a mussel matrix with
certified concentrations of incurred PAHs and other organic
contaminants

NIST SRM 1974b was used for method qualification, but no data was presented.

Labeled PAHs: http://shop.isotope.com/category.aspx?id=10032756

Unlabeled PAHs: http://shop.isotope.com/category.aspx?id=10032758

PAH mixes: http://shop.isotope.com/category.aspx?id=10032698

There were certificates of Standard Reference Material (SRM) 2977 and Standard Reference Material (SRM)1974, but no data were found from those reference materials to further demonstrate the method performance.

What type of single laboratory validations were used for this method [i.e, Harmonized PTM, Precollaborative, Independent, Collaborative, etc.] and where is it referenced in the method documentation?

Collaborative, Performance characteristics on page 72 of the ERP book

A Collaborative Study was done. This is stated in the title of the document as well as in the abstract, and in the document starting on page 479 of the publication

Collaborative

A full collaborative study was conducted and reported in Mastovska et al.: Journal of AOAC International Vol. 98, No. 2, 2015

Collaborative study was conducted, with 10 labs successfully completing the validation process.

Precollaborative Independent,
Reference 1 in Mastovska et al.:J. AOAC Int., 98(2)2015.

Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood Using Gas Chromatography-Mass Spectrometry: Collaborative Study.
It was a collaborative study, which was published at J AOAC Int, to demonstrate the method performance.

Are the approved validation protocols available and documented?

Yes

Yes

Yes

Yes

Yes

Yes

Yes
Is the method documentation available that demonstrates Reproducibility/Uncertainty and Probability of Detection? If so, please cite the reference.

STATISTICAL REVIEW: Is the documentation for the statistical review prior to AOAC First Action Official Methods status available and completed?
yes
Yes
yes
complete statistical review and comparison to HorRat was presented in Mastovska et al.: Journal of AOAC International Vol. 98, No. 2, 2015. The data from the study was available before the method was recommended for 1st Action but it was not yet published.
Yes.
Yes, in the section from pp 109-118 in the ERP book for the PAH method. Also extensively covered in Mastovska et al.:J. AOAC Int., 98(2)2015.
The method demonstrated good reproducibility as reported its collaborative study published at J AOAC Int. I could not locate the documentation on measurement uncertainty, except in the Certificate of Analysis for Reference Materials.
Please cite below the OMA Appendicies, Community Guidance, or SMPR by which this method was reviewed against.
Community guidance is on page 22 of the ERP Book 1. Does this method sufficiently follow the collaborative study protocol 2. Is the method Scientifically sound and can be followed 3.What are the strengths and weaknesses of the method? 4.How do the weaknesses weigh in your recommendation of the method? 5.Will the method serve the community that will use the method? 6.What additional information be needed to further support the method?
Method performance criteria are set instead of prescribing specific products/instruments needed to successfully complete analysis.
In response to Gulf oil spill. I believe there was stakeholder panel meetings and a working group regarding

method requirements and scope.

SMPRs were not prepared for this method. This method was not developed through the stakeholder process. It was a method chosen in response to an emergency. The method performance was compared to the NIST method for PAHs.

The 19 PAHs studied were those chosen by NOAA as representative and were used by all labs responding to the Gulf Oil Spill. ERP members found this scope and performance appropriate at October 2014 ERP meeting.
Method Scope and Applicability ERP3 statement was most representative
ER 3 Scope of the method includes 19 specific PAHs in seafood. The matrices tested, shrimp, oyster and mussel, are typically 5%
lipid content and below (USDA Nutrient database). Lipid content of commodities amendable for this method is an important consideration and should be addressed in the text of the method. Higher fat samples are addressed briefly in the
method, indicating that a reduction of volume of extract should be applied to the silica SPE cartridge. This is a reasonable
modification to the method but has implications for overall detectability, especially for BaP. It is possible that to meet fat
removal criteria, modifications for calibration curves and/or sample preparation will need to be made. Modifications may be
significant and therefore some comment on an upper limit of the lipid content applicable for the method as written would
be useful.

No available SMPR. This method was created in an effort to address an emergency response to the gulf oil spill.

Expert Review Panel Chair Report

Only negative comment was there was no data from certified reference material to demonstrate the method performance. However, the discussion of the Expert Review Panel concluded that the use of
a certified reference material was not required and did not delineate scientific reasoning to not move the method forward.

Based upon the feedback submitted, are there any recommended changes to the AOAC First Action method as written?

No

No

No

Yes,
The need to include the NIST reference material was noted and should be mentioned in the method.
Negative Vote Discussion: One member of the expert review panel voted against the motion. Due to the reviewer's comments, he inquired about the method not using a certified reference material for PAHs in seafood. Standard Reference Material (SRM) 1974b Mussel Tissue is mentioned as part of the qualification of the labs as a practice sample, but no data was reported using SRM 1974b for validation of the proposed method. The availability of SRM 1974c (which has replaced SRM 1974b) provided an excellent opportunity to use a CRM to validate an AOAC method. The discussion of the Expert Review Panel concluded that the use of a certified reference material was not required and did not delineate scientific reasoning to not move the method forward. This method was created in an effort to address an emergency response to the gulf oil spill. The information provided in reference to the selection of the 19 target PAH compounds and the matrices

selected were noted in the Fitness for Purpose statement established by the Stakeholder Panel on Petroleum Contaminants in Seafood in 2010. The ERP captured a revote.
1.
It was not advisable or possible to use this material in the Collaborative Study due to the cost of the material. It also has analytes which are so low in concentration that NOAA labs had trouble getting repeatable quantitations. The criteria for acceptance allowed for more that one analyte to be outside the acceptable range. It is hoped that this new method may provide more sensitivity and precision but reproducibility data for the NIST reference material using this new method is not yet available.

These comments were addressed in the writing of the 1st Action Method
Method is well-written but needs more specificity in select sections. For example, on page 6, under (5), it is stated all
analytes of reagent blanks must be below the concentrations in the lowest calibration standard. Needs more clarification....how far below? Also, since stability of some PAHs was questionable, a Stability Study needs to be carried out
with PAH standards stored at varying temperatures and times. The Safety Section must be in the front of the method since
safety is more important than any other part of the protocol.

This comment has not yet been addresses but could be a simple edit of the method if the ERP members agree. The authors should use
correct nomenclature for the PAHs, i.e., Benzo[ghi]perylene not benzo[g,h,i]perylene

ERP may want to consider updates to the method as recommended by ERP3
Weaknesses include: 1. Method scope of 1 ug/kg LOQ of BaP was not tested as a fortification level. As I read the method,
the lowest fortification level for BaP was 2 ug/kg. 2. Polypropylene tubes used for extraction will likely cause users of the
method issues with PAH contamination. Discussion of alternatives would be helpful. 3. PAH GC-MS analysis has significant
differences than typical analysis of most other types of compounds. Guidance for GC-MS parameters would likely be helpful
for users of the method. These include parameters like inlet temperature, transfer line temperature, ion source temperature,
column loadability and efficient flow conditions. 4. There is no recommendation on how to report data on chrysene and
triphenylene if the recommended, but not required, 50% valley separation is not met. Can chrysene and triphenylene be
reported together? 5. Ion ratios are mentioned as a requirement for identification but there is no indication as to the RSD
value that is acceptable or some other qualification. 6. When a linear calibration curve is not possible, allowance for a "wellcharacterized"
quadratic formula is made but with no discussion of what "well-characterized" means. Some guidance would be useful because some user will not be accustomed using quadratic calibration curves.

This comment might be addressed in the method if agreed by the ERP

18.2 megaohm water should be used for any GC/MS method (page 9, Section C).

No
Yes, My own feedback: The source temperature recomended (>or = 280 C) is too low. In general, the highest possible source temperature should be used. At least 300 C and higher if the instrument has the capability.
No
Has the method author addressed any specific AOAC Final Action requirements as noted by the ERP, if any?
yes
yes
I know that the author has prepared responses but they are not in the ERP book or I can't find them
N/A. No final action requirements were proposed.
Has this method received any additional recognitions?
no
na
na
Method of the Year Collab Study of the Year
N/A
Collaborative Study of the Year award.
Method of the Year, 2015.
Please cite the OMA Method, Number, etc.
no
AOAC OFFICIAL METHOD 2014.08: POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) IN SEAFOOD GAS CHROMATOGRAPHY-MASS SPECTROMETRY
AOAC Official Method 2014.08 and Mastovska et al, Journal AOAC, Vol 98, No 2, 2015
AOAC Official Method 2014.08 Polycyclic Aromatic Hydrocarbons (PAHs)in SeafoodGas Chromatography-Mass Spectrometry First Action 2014
OMAMAN-15: Determination of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry
AOAC Official Method 2014.08
AOAC Official Method 2014.08

If there are methods published in the JAOAC for this method, please cite the references:
no
J AOAC Int. 2015 Mar-Apr;98(2):477-505. doi: 10.5740/jaoacint.15-032.
Mastovska et al, Journal AOAC, Vol 98, No 2, 2015
Mastovska et al.: Journal of AOAC International Vol. 98, No. 2, 2015 477-505
JOURNAL OF AOAC INTERNATIONAL VOL. 98, NO. 2, 2015, pg 477-505.
Mastovska et al.:J. AOAC Int., 98(2)2015.
Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood Using Gas Chromatography-Mass Spectrometry: Collaborative Study. Vol 98, No 2, 2015, 447
AOAC Method Recommendation:
Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
REVIEWERS:
Steven C. Moser
Lowri DeJager
Julie Kowalski
Jo Marie Cook
Kai Liu
Philip L. Wylie
Jian Wang

AOAC OMA 2014.09	
Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea	
Was the method's applicability considered? **Please state the method's applicability statement:**	
Yes	A subset of the more than 600 compounds shown in the official method was chosen by an AOAC participating group.
Yes	Page 1429 under collaborative study protocol: scope/applicability published in JAOACI 98 (5) 1428-1454 (2015)
No	Did not see applicability information related to tea, although scope of analytes was well documented.
Yes	Method is applicable for the qualitative, quantitative and confirmatory analysis of 653 pesticides and chemical pollutant residues in tea
Yes	Applicability stated by the authors in the OMA 2014.09: "The method analyzes 653 Multiclass Pesticides and Chemical Pollutants in Tea by GC/MS, GC/MS/MS, and LC/MS/MS. The method could be applied for Green tea, Oolong tea, Black tea and Puer tea. LOQs for the 653 pesticides included in the SLV ranged from 0.03 to 1210 µg/kg. LODs of the GC/MS method ranged from 1.0 to 500 µg/kg, and the corresponding LOQs ranged from 2.0 to 1000 µg/kg. LODs of the GC/MS/MS method ranged from 1.0 to 900 µg/kg, and the corresponding LOQs ranged from 2.0 to 1800 µg/kg. LODs of the LC/MS/MS method ranged from 0.03 to 4820 µg/kg, and the corresponding LOQs ranged from 0.06 to 9640 µg/kg. A total of 482 of the 653 pesticides can be analyzed by GC/MS and GC/MS/MS, while 417 of the 653 pesticides can be analyzed by LC/MS/MS with LODs ≤100 µg/kg. There are 264 out of the 653 pesticides that can be analyzed by GC/MS, and 325 out of 653 by LC/MS/MS with LODs ≤10 µg/kg. There are 270 pesticides that can be analyzed by both GC/MS and LC/MS/MS. Of these, there are 264 pesticides that can be analyzed by GC/MS and 247 by LC/ MS/MS, with LODs ≤100 µg/kg for the GC/MS method. There are, however, 133 pesticides that can be analyzed by GC/MS and 200 by LC/MS/MS, with LODs ≤10 µg/kg" I still think this description is very confuse, and have some mistakes.
Yes	The method fit for purpose.
Yes	The method analyzes 653 Multiclass Pesticides and Chemical Pollutants in Tea by GC/MS, GC/MS/MS, and LC/MS/MS. The method could be applied for Green tea, Oolong tea, Black tea and Puer tea. LOQs for the 653 pesticides included in the SLV ranged from 0.03 to 1210 µg/kg. LODs of the GC/MS method ranged from 1.0 to 500 µg/kg, and the corresponding LOQs ranged from 2.0 to 1000 µg/kg. LODs of the GC/MS/MS method ranged from 1.0 to 900 µg/kg, and the corresponding LOQs ranged from 2.0 to 1800 µg/kg. LODs of the LC/MS/MS method ranged from 0.03 to 4820 µg/kg, and the corresponding LOQs ranged from 0.06 to 9640 µg/kg. A total of 482 of the 653 pesticides can be analyzed by GC/MS and GC/MS/MS, while 417 of the 653 pesticides can be analyzed by LC/MS/MS with LODs ≤100 µg/kg. There are 264 out of the 653 pesticides that can be analyzed by GC/MS, and 325 out of 653 by LC/MS/MS with LODs ≤10 µg/kg. There are 270 pesticides that can be analyzed by both GC/MS and LC/MS/MS. Of these, there are 264 pesticides that can be analyzed by GC/MS and 247 by LC/ MS/MS, with LODs ≤100 µg/kg for the

	GC/MS method. There are, however, 133 pesticides that can be analyzed by GC/MS and 200 by LC/MS/MS, with LODs ≤10 µg/kg.

Were the method's safety concerns addressed prior to the approval for AOAC First Action Official Methods status?	
Yes	
Yes	
Yes	
Yes	
Yes	
Yes	
Yes	

Is the documentation for the safety evaluation available and completed?	
No	
Yes	
No	
No	
Yes	
Yes	
Yes	

Are there any reference materials considered or addressed?	
Yes	
No	
No	
Yes	
No	
No	
No	

Is there documentation regarding reference materials available and noted? If so, where is it located?
Chemical and standards are addressed in the method in the appropriate Materials section.
There are no standard reference materials for these pesticide residues.
only documentation regarding preparation of working standards was noted. No information regarding sourcing of reference materials was made. Also, no information in the study regarding use or results of interlaboratory PT

reference materials available.(e.g. FAPAS)
I found reference materials (proficiency tests) available from FAPAS. Several of the method users stated there were reference materials available.
Looking in different databases I could not find information about pesticides in tea reference materials
No data from certified reference materials were presented.
Looking in databases, I could not find information about pesticides in tea reference materials
What type of single laboratory validations were used for this method [i.e, Harmonized PTM, Precollaborative, Independent, Collaborative, etc.] and where is it referenced in the method documentation?
Independent. See GF Pang's Journal of AOAC publications. One of the publications directly related to the method is listed as a reference at the end of the official method document.
Pre-collaborative SLV mentioned in the Introduction of the Collaborative Study report/publication
Noted multiple laboratory validations in the Method annex
There was a collaborative study done prior to first action. OMAMAN-14 details the study
The SLV was performed pre-collaborative. Reproducibility was tested by authors using a collaborative study (MLT). The information is included in the Method Manuscript.
The method performance was demonstrated in its collaborative study, which was published at J AOAC Int.
Pre-collaborative
Are the approved validation protocols available and documented?
Yes
Yes
No
Yes
Yes
Yes
Yes
Is the method documentation available that demonstrates Reproducibility/Uncertainty and Probability of Detection? If so, please cite the reference.

STATISTICAL REVIEW: Is the documentation for the statistical review prior to AOAC First Action Official Methods status available and completed?
i believe so
This was very thoroughly done
Unknown, not presented here.
As a ERP member that approved it to first action, the method was reviewed and met the guidelines of Appendix D: Collaborative study procedures to validate Characteristics of a Method of Analysis.
Yes
The reproducibility was presented in the paper but not measurement uncertainty.
Yes
Please cite below the OMA Appendicies, Community Guidance, or SMPR by which this method was reviewed against.
none
This is not applicable. Study was began long before SMPR development started at the AOAC.
1) Community Guidance as published in the provided review documents 2) Method author feedback
NA there was no SMPR to my knowledge
No SMPR exists for this method. AOAC validation guides used for SLV Appendix D of OMA was used as criteria for MLT. Representative pesticides were selected for the MLT based on guidance from AOAC Method Centric Committee on Pesticides Residues.
The main concern for this method was sample hydration that is samples need to be hydrated prior to organic solvent extraction. However, there were no concerns reported by end-users from method feedback.
No SMPR exists for this method. AOAC validation guides used for SLV Appendix D of OMA was used as criteria for MLT
Based upon the feedback submitted, are there any recommended changes to the AOAC First Action method as written?
No
No, Of the 15 feedback responses obtained all were highly complimentary, confirming that the method is reproducible in their hands
Yes, 1) There were multiple safety concerns although these were not documented. 2) more information on source of reference materials 3) More information on availability of Cleanert 4) More information on results of Harmonized PT results of the method

in real tea samples
Yes, There was one comment that the sample preparation is time consuming. i.e, rotatory evaporation. That is what the method uses, so to my knowledge that has not been addressed.
No
No
No
Has the method author addressed any specific AOAC Final Action requirements as noted by the ERP, if any?
yes
The author had addressed the feedback responses of the SLV study and those following the collaborative study
Yes - specifically the concern over lack of a hydration step was addressed in the first ERP meeting
The author did all what the ERP asked for
Has this method received any additional recognitions?
na
Method was nominated and won an award at the 2016 Annual meeting
None noted
It was the AOAC 2015 Method of the Year
The method was granted “2015 Method of the Year”
No sure
The method was granted “2011 Method of the Year”
Please cite the OMA Method, Number, etc.
AOAC Official Method 2014.09 Determination and Confirmation of Residues of 653 pesticides and Chemical Pollutants in Tea
OMA method 2014.09 has several additional publications following the validation of the collaborative study method
2014.09 Pang, et. al
2014.09
OMA 2014.09

AOAC Official Method 2014.09

OMA 2014.09

If there are methods published in the JAOAC for this method, please cite the references:

High-Throughput Analytical Techniques for Multiresidue, Multiclass Determination of 653 Pesticides and Chemical Pollutants in Tea-Part IV: Evaluation of the Ruggedness of the Method, Error Analysis, and Key Control Points of the Method.
Fan CL, Li Y, Chang QY, Pang GF, Kang J, Cao J, Zhao YB, Li N, Li ZY.

References 4-29 in the same publication JAOACI 98 (5)1428-1454 (2015)

Pang, et. al, J AOAC Int. 2013 Jul-Aug;96(4):887-96. (original paper)

CHANG ET AL.: JOURNAL OFAOAC INTERNATIONALVOL. 99, NO. 4, 2016
(Degradation study)

Vol 94 No. 4, 2011

1- Pang, Guo-Fang; Fan, Chun-Lin; Zhang, Feng; Li, Yan; Chang, Qiao-Ying; Cao, Yan-Zhong; Liu, Yong-Ming; Li, Zeng-Yin; Wang, Qun-Jie; Hu, Xue-Yan; Liang, Ping "High-Throughput Analytical Techniques for Determination of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea"

Journal of AOAC International, Volume 94, Number 4, July-August 2011, pp. 1253-1296

2- Chun-Lin Fan, Qiao-Ying Chang, Guo-Fang Pang,1 Zeng-Yin Li, and Jian Kang ,Guo-Qing Pan and Shu-Zhan Zheng, Wen-Wen Wang , Cui-Cui Yao and Xin-Xin Ji "High-Throughput Analytical Techniques for Determination of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea, Part II: Comparative Study of Extraction Efficiencies of Three Sample Preparation Techniques"
Journal of AOAC International, Volume 96, Number 2, March/April 2013, pp. 432-440(9)
3- Guo-Fang Pang, Chun-Lin Fan, Qiao-Ying Chang, Yan Li, Jian Kang, Wen-Wen Wang, Jing Cao,Yan-Bing Zhao, Nan Li, and Zeng-Yin Li Zong-Mao Chen, Feng-Jian Luo, and Zheng-Yun Lou "High-Throughput Analytical Techniques for Multiresidue, Multiclass Determination of 653 Pesticides and Chemical Pollutants in Tea—Part III: Evaluation of the Cleanup Efficiency of an SPE Cartridge Newly Developed for Multiresidues in Tea"
Journal of AOAC International, Volume 96, Number 4, July-August 2013, pp. 887-896(10)
4- Fan Chun-Lin, Li Yan, Chang Qiao-Ying, Pang Guo-Fang, Kang Jian, Cao Jing, Zhao Yan-Bing, et al. 2015. "High-Throughput Analytical Techniques for Multiresidue, Multiclass Determination of 653 Pesticides and Chemical Pollutants in Tea-Part IV: Evaluation of the Ruggedness of the Method, Error Analysis, and Key Control Points of the Method."
Journal of AOAC International 98 (1): 130-48. doi:10.5740/jaoacint.12-478.
5- Xi Chen, Yan Li, Qiao-Ying Chang, Xue-Yan Hu, Guo-Fang Pang, and Chun-Lin Fan High-Throughput Analytical Techniques for Determination of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea—Part V: A Comparative Study of the Influence of Tea Hydration on the Efficiency of Pesticide Multiresidue Determination Using Three Sample Preparation Methods and GC/MS/MS
Journal of AOAC International, Volume 98, Number 1, January-February 2015, pp. 149-159(11)
6- Qiao-Ying; Pang, Guo-Fang; Fan, Chun-Lin; Chen, Hui; Wang, Zhi-Bin "High-Throughput Analytical Techniques for Determination of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea, Part VI: Study of the Degradation of 271 Pesticide Residues in Aged Oolong Tea by Gas Chromatography-Tandem Mass Spectrometry and Its Application in Predicting the Residue Concentrations of Target Pesticides"
Journal of AOAC International, Volume 99, Number 4, July-August 2016, pp. 1049-
7-.Guo-Fang Pang and Chun-Lin Fan, Yan-Zhong Cao, Fang Yan, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang "

High Throughput Analytical Techniques for the Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea by GC/MS, GC/MS/MS, and LC/MS/MS: Collaborative Study, First Action 2014.09."
Journal of AOAC International 2015 Sep-Oct;98(5):1428-54

The method was published at J AOAC Int Vol 99, No 4, 2016, 1049.

1- Pang, Guo-Fang; Fan, Chun-Lin; Zhang, Feng; Li, Yan; Chang, Qiao-Ying; Cao, Yan-Zhong; Liu, Yong-Ming; Li, Zeng-Yin; Wang, Qun-Jie; Hu, Xue-Yan; Liang, Ping "High-Throughput Analytical Techniques for Determination of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea"

Journal of AOAC International, Volume 94, Number 4, July-August 2011, pp. 1253-1296

2- Chun-Lin Fan, Qiao-Ying Chang, Guo-Fang Pang,1 Zeng-Yin Li, and Jian Kang ,Guo-Qing Pan and Shu-Zhan Zheng, Wen-Wen Wang , Cui-Cui Yao and Xin-Xin Ji "High-Throughput Analytical Techniques for Determination of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea, Part II: Comparative Study of Extraction Efficiencies of Three Sample Preparation Techniques"
Journal of AOAC International, Volume 96, Number 2, March/April 2013, pp. 432-440(9)
3- Guo-Fang Pang, Chun-Lin Fan, Qiao-Ying Chang, Yan Li, Jian Kang, Wen-Wen Wang, Jing Cao,Yan-Bing Zhao, Nan Li, and Zeng-Yin Li Zong-Mao Chen, Feng-Jian Luo, and Zheng-Yun Lou "High-Throughput Analytical Techniques for Multiresidue, Multiclass Determination of 653 Pesticides and Chemical Pollutants in Tea—Part III: Evaluation of the Cleanup Efficiency of an SPE Cartridge Newly Developed for Multiresidues in Tea"
Journal of AOAC International, Volume 96, Number 4, July-August 2013, pp. 887-896(10)
4- Fan Chun-Lin, Li Yan, Chang Qiao-Ying, Pang Guo-Fang, Kang Jian, Cao Jing, Zhao Yan-Bing, et al. 2015. "High-Throughput Analytical Techniques for Multiresidue, Multiclass Determination of 653 Pesticides and Chemical Pollutants in Tea-Part IV: Evaluation of the Ruggedness of the Method, Error Analysis, and Key Control Points of the Method."
Journal of AOAC International 98 (1): 130-48. doi:10.5740/jaoacint.12-478.
5- Xi Chen, Yan Li, Qiao-Ying Chang, Xue-Yan Hu, Guo-Fang Pang, and Chun-Lin Fan High-Throughput Analytical Techniques for Determination of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea—Part V: A Comparative Study of the Influence of Tea Hydration on the Efficiency of Pesticide Multiresidue Determination Using Three Sample Preparation Methods and GC/MS/MS
Journal of AOAC International, Volume 98, Number 1, January-February 2015, pp. 149-159(11)
6- Qiao-Ying; Pang, Guo-Fang; Fan, Chun-Lin; Chen, Hui; Wang, Zhi-Bin "High-Throughput Analytical Techniques for Determination of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea, Part VI: Study of the Degradation of 271 Pesticide Residues in Aged Oolong Tea by Gas Chromatography-Tandem Mass Spectrometry and Its Application in Predicting the Residue Concentrations of Target Pesticides"
Journal of AOAC International, Volume 99, Number 4, July-August 2016, pp. 1049-
7-.Guo-Fang Pang and Chun-Lin Fan, Yan-Zhong Cao, Fang Yan, Yan Li, Jian Kang, Hui Chen, and Qiao-Ying Chang " High Throughput Analytical Techniques for the Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea by GC/MS, GC/MS/MS, and LC/MS/MS: Collaborative Study, First Action 2014.09."
Journal of AOAC International 2015 Sep-Oct;98(5):1428-54

AOAC Method Recommendation:

Recommend for AOAC Final Action Official Methods Status

Recommend for AOAC Final Action Official Methods Status

Recommend for AOAC First Action Continuance

Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
Recommend for AOAC Final Action Official Methods Status
REVIEWERS:
Julie Kowalski
Joe Boison
John Reuther
Amy Brown
Marina Torres
Jian Wang
Marina Torres