AOAC INTERNATIONAL

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Stakeholder Panel & Working Group Meeting

FOOD ALLERGENS (WHOLE EGG & OATS)

Tuesday, March 14, 2017

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AOAC INTERNATIONAL
BYLAWS

As Amended September 26, 2010

ARTICLE I
Name

The name by which this Association shall be known is "AOAC INTERNATIONAL" (hereinafter referred to as the "Association").[1]

ARTICLE II
Purpose

The primary purpose of the Association is to promote methods validation and quality measurements in the analytical sciences.

ARTICLE III
Membership

Section 1. Types of Membership

There shall be three (3) types of membership in the Association: Individual Members, Sustaining Member Organizations, and Organizational Affiliates.

A. Individual Members

There shall be four (4) categories of Individual Members in the Association: Members, Retired Members, Student Members, and Honorary Members.

B. Sustaining Member Organizations

There shall be one (1) category of Sustaining Member Organizations.

C. Organizational Affiliate

There shall be one (1) category of Organizational Affiliate.

Section 2. Qualifications for Membership

A. Individual Members

[1] Members

Qualifications for Members shall be a degree in science, or equivalent as approved by the Board of Directors, and interest in supporting and furthering the purpose and goals of the Association. Such scientists shall be eligible for membership provided they are engaged, or have been engaged, directly or indirectly, in a field relevant to the purpose of the Association.

[2] Retired Members

[1] AOAC INTERNATIONAL was incorporated in the District of Columbia on January 20, 1932, as the Association of Official Agricultural Chemists. On November 10, 1965, the name of the corporation was changed to the Association of Official Analytical Chemists, and on September 12, 1991, the current name was adopted.

A current Member who is no longer actively engaged, directly or indirectly, in a field relevant to the purpose of the Association but who has served the Association as a Member for at least ten (10) years shall be eligible for Retired Member status upon written request and payment of the annual Retired Member dues. Any special benefits accorded Retired Members shall be determined by the Executive Director.

[3] Student Members

Any full-time student working toward an undergraduate or graduate degree in the areas of chemistry, microbiology, food science or other related science shall be eligible for Student Membership in AOAC INTERNATIONAL.

[4] Honorary Members

Honorary Members shall be persons recognized for their substantial contribution toward the achievement of the objectives of the Association. They shall be nominated by the Board of Directors and may be elected by a two-thirds vote of the Individual Members voting.

B. Sustaining Member Organizations

A Sustaining Member Organization shall be any agency of a local, state, provincial, national, or international government; a university, college, or academic department; or any firm, business, or organization with an interest in supporting and furthering the purpose of the Association. Every Sustaining Member Organization must have a designated representative(s). All such Sustaining Member Organization representatives must meet the qualifications for Members and become Individual Members with all the rights and privileges thereof.

C. Organizational Affiliate

An Organizational Affiliate Organization shall be any agency of a local, state, provincial, national, or international government; a university, college, or academic department; or any firm, business, or organization with an interest in supporting and furthering the purpose of the Association. Every Organizational Affiliate must have a designated representative(s). All such Organizational Affiliate representatives must meet the qualifications for Members and become Individual Members with all the rights and privileges thereof.

Section 3. Application for Membership

Applications or requests for membership shall be submitted to the Association's headquarters office. Membership shall become effective upon approval of the application or request, payment of any required membership dues, entry on the membership rolls, and assignment of a member number.

Section 4. Expulsion

The Board of Directors, at any duly called meeting of the Board, by a two-thirds vote of those holding office, may terminate the membership of any member who in its judgment has violated the Bylaws or has been guilty of conduct detrimental to the best interests of the Association. Any member convicted of a felony is subject to immediate expulsion from the Association. Expulsion of a member by the Board of Directors shall be final and shall cancel all rights, interest, or privileges of such member in the services or resources of the Association. Any member, for whom expulsion is proposed, for reasons other than conviction of a felony, shall be entitled to not less than 60 days advance notice of the charges, the date upon which a hearing will be scheduled, and the right to present evidence in defense. The date and place of any such hearing, if held other than at the headquarters or annual meeting site of the Association, must be reasonable with respect to the location of any individual so charged.

Section 5. Dues, Membership Year, and Waivers

A. Annual dues for membership in the Association shall be fixed by the Board of Directors, subject to approval by the majority of the Individual Members voting by ballot by any of the following means (whichever is deemed appropriate by the Board at the time): mail, telephone call, telegram, cablegram, electronic mail or other means of electronic or telephonic transmission.

B. Honorary Members of the Association shall be exempt from payment of dues and annual meeting registration fees.

C. The membership year and the delinquency date shall be determined by the Board of Directors.

D. The authority to grant waivers of membership dues rests with Executive Director.

E. Student Member dues shall be one-third of regular Member dues, rounded up to the nearest $5.00 increment.

Section 6. Members in Good Standing; Rights and Privileges

All Individual Members who maintain their membership by payment of dues as required under these Bylaws and who otherwise qualify shall be considered in good standing and entitled to full privileges of membership.

ARTICLE IV
Officers

Section 1. Elected Officers

The elected officers of the Association shall be Individual Members and shall consist of a President, President-Elect, Secretary, Treasurer, and Immediate Past President.

A. President

The President shall be the principal elected officer of the Association, shall preside at meetings of the Association and of the Board of Directors and of the Executive Committee, and shall be a member ex-officio, with right to vote, of all committees except the Nominating Committee. He or she shall also, at the annual meeting of the Association and at such other times as he or she shall deem proper, communicate to the Association or the Board of Directors such matters and make such suggestions as may in his or her opinion tend to promote the welfare and further the purpose of the Association and shall perform such other

duties as are necessarily incident to the office of President or as may be prescribed by the Board of Directors.

B. President-Elect

In the absence of the President, or in the event of the President's inability or refusal to act, the President-Elect shall perform the duties of the President, and, when so acting, shall have all the powers of and be subject to all the restrictions upon the President. The President-Elect shall perform such other duties as from time to time may be assigned to him or her by the President or by the Board of Directors.

C. Secretary

The Secretary shall give notice of all meetings of the Association, keep a record of all proceedings, attest documents, and, in general, perform such other duties as are usual of the office of Secretary and such other duties as may be assigned by the President or by the Board of Directors.

D. Treasurer

The Treasurer shall be responsible for the funds and securities of the Association; serve as financial officer of the organization and as Chairperson of the Finance Committee; manage the Board of Director's review of and action related to the Board of Director's financial responsibilities; serve as the chief Board liaison in overseeing and reviewing the annual audit, and in general, perform such other duties as are usual of the office of Treasurer and such other duties as may be assigned by the President or by the Board of Directors.

E. Immediate Past President

The Immediate Past President shall serve as advisor to the President and Directors and perform such other duties as may be assigned from time to time by the President or by the Board of Directors.

Section 2. Appointed Officers

The appointed officers shall include the Executive Director and such other appointed officers as may be designated by the Board of Directors from time to time.

A. Executive Director

The day-to-day administration and management of the Association's offices shall be vested in a salaried manager employed or appointed by, and directly responsible to, the Board of Directors. This manager shall have the title of Executive Director with responsibility for the management and direction of all operations, programs, activities, and affairs of the Association, as approved or delegated by the Board of Directors. The Executive Director shall have direct responsibility for employment and termination of employment and the determination of compensation for staff members within the budgetary framework determined by the Board of Directors. The Executive Director functions as the chief operating officer of the Association within the guidelines established by the policies and procedures of the Board of Directors and, as necessary, with the concurrence of the President. The Executive Director shall have such other duties as may be prescribed by the Board.

B. Other Appointed Officers

Other appointed officers shall have such duties as may be prescribed by the Board.

ARTICLE V
Nominations, Elections, Terms, and Appointments to the Board of Directors

Section 1. Nominating Committee

The Nominating Committee shall annually recommend to the Board of Directors a slate of Individual Members as potential nominees for the elected positions where vacancies will occur. The Nominating Committee shall consist of five (5) members who shall be three (3) immediate Past Presidents, as available, and two (2) Individual Members-at-Large of the Association. If three Past Presidents are not available to serve, other Individual Members-at-Large shall be appointed by the President to the extent necessary to form the five (5)-member committee.

Section 2. Elections and Terms of Office

The President-Elect, the Secretary, Treasurer, and the Directors of the Board of Directors shall be elected by a majority of Individual Members voting, from a slate of nominees recommended annually by the Board of Directors.

Terms of office for all Officers and Directors shall begin with the adjournment of the annual meeting following their election and shall end with the adjournment of the annual meeting occurring nearest the expiration of their term. The six (6) Directors shall be elected to staggered three-year terms with two Directors elected to full three-year terms each year, but not to more than two (2), consecutive, three-year terms. Appointment or election to fill an unexpired term shall not affect the eligibility of a person to subsequently be elected to two (2) full terms. The Secretary shall be elected to a one-year term and may be re-elected to successive one-year terms. The Treasurer shall be elected for a one-year term and may be re-elected to successive one-year terms. The President-Elect shall be elected to a one-year term; whereupon the current President-Elect shall become President and the current President shall become the Immediate Past President, each serving a one-year term.

Section 3. Appointments

Directors-at-Large are appointed by the Board in accordance with Article VI, Section 2. Directors-at-Large are appointed for one (1) year terms, renewable at the discretion of the elected Board.

ARTICLE VI
Board of Directors

Section 1. Composition

The Board of Directors shall consist of eleven (11) elected members to include the President, President-Elect, Secretary, Treasurer, Immediate Past President, six (6) Directors, and up to three (3) appointed Directors-at-Large, all of whom shall be Individual Members of the Association. The elected Board shall reflect the makeup of the Association membership and shall not be dominated by any single interest.

Section 2. Powers and Duties

The Board of Directors shall provide supervision, control, and direction of the affairs of the Association, shall determine the Association's policies or changes therein within the limits of the Bylaws, shall actively prosecute

its purpose, and shall have discretion in the disbursement of its funds. It may adopt such rules and procedures for the conduct of its business as shall be deemed advisable, and may, in the execution of the powers granted, appoint such agents as it may consider necessary. The Board of Directors may appoint up to three (3) Directors-at-Large, if, in their opinion, such appointments advance the purpose of the Association. Directors-at-Large shall be accorded the same voting privileges as elected Directors.

Section 3. Meetings

Except that the Board shall have a regular meeting at the time and place of the annual meeting, the Board shall meet, in person or via telephone conference call, upon call of the President at such times and places as he or she may designate within the policies adopted by the Board, and shall be called to meet upon demand of a majority of its members. Notice of all meetings of the Board of Directors shall be sent by any of the following means (whichever is deemed appropriate by the President at the time): mail, telephone call, telegram, cablegram, electronic mail or other means of electronic or telephonic transmission to each member of the Board at his or her last recorded address or number at least fourteen (14) days in advance of in-person meetings or forty-eight (48) hours in advance of conference call meetings.

Section 4. Quorum

A quorum for any meeting of the Board is six (6) Board members elected in accordance with Article V (1). Any less number may: (1) set a time to adjourn, (2) adjourn, (3) recess, or (4) take measures to obtain a quorum.

Section 5. Absence

Any member of the Board of Directors unable to attend a meeting of the Board shall notify the President and state the reason for his or her absence. If a member of the Board is absent from two (2) consecutive meetings, he or she may be removed by a two-thirds vote of the Board Members then in office.

Section 6. Compensation

Members of the Board of Directors, as such, shall not receive any compensation for their services as Board members, but the Board may, by resolution under policies it may adopt, authorize reimbursement of expenses incurred in the performance of members' duties. Such authorization may prescribe conditions and procedures for approval and payment of such expenses. Nothing herein shall preclude a Board member from serving the Association in any other capacity and receiving compensation for such services, if compensation is customarily paid for such services.

Section 7. Resignation or Removal

Any member of the Board may resign at any time by giving written notice to the President, Secretary, Treasurer, or to the Board of Directors. Such resignation shall take effect at the time specified therein, or, if no time is specified, at the time of acceptance thereof as determined by the President or the Board.

Any member of the Board may be removed by a three-fourths vote of the Board members then in office and present at any regular or special meeting of the Board.

Section 8. Vacancies: Members of the Board

If a vacancy should occur in the membership of the elected Board of Directors, any Past President may be appointed by action of the remaining members of the Board to temporarily fill such vacancy until the next

regularly scheduled election. At the next regularly scheduled election nominations will be presented to fill the vacancy for the unexpired portion of the term remaining.

Section 9. Vacancies: President and Other Officers

If the office of the President shall become vacant, the President-Elect shall thereupon become President of the Association for the unexpired term, followed by his or her duly elected term. In the event the office of President becomes vacant at a time when the office of President-Elect is also vacant, the Presidency shall be filled for the remainder of the term by the action of the Board of Directors. If any other officer position shall become vacant, the office may be filled for the remainder of the term by action of the Board.

ARTICLE VII
Committees

Section 1. Committee Formation

The Board of Directors shall form and adopt terms of reference for such standing or special boards, committees, subcommittees, task forces, or task groups as may be required by these Bylaws or as the Board may determine necessary to carry out the affairs of the Association.

Section 2. Committee Appointments

Subject to the requirements of these Bylaws and the specific terms of reference adopted by the Board, the President shall make the appointments to fill the vacancies occurring in the Association's standing or special boards, committees, subcommittees, task forces, or task groups.

ARTICLE VIII
Official Methods of Analysis

The Board of Directors (BoD) is empowered to develop written policies and procedures for the study, adoption, and change in status of the Official Methods of Analysis of AOAC INTERNATIONAL. Implementation of the policies and procedures shall be delegated to an Official Methods Board (OMB).

Section 1. Composition of the Official Methods Board

The Official Methods Board shall consist of a chair and a vice chair, and members who are recommended by the chair. The chair, vice chair and members are appointed by the President of AOAC INTERNATIONAL. The OMB shall be composed of members representing a balance of government, industry, and academia as appropriate to the scope of the group and shall not be dominated by any single interest.

Section 2. Purpose of the Official Methods Board

The OMB shall serve the Association in a scientific and advisory capacity on methods and the process of their adoption. The OMB shall be responsible for implementation of procedures adopted by the BoD, according to the principles in section 3 below.

Section 3. Principles of the Official Methods Program

A. Adequate records of technical data, discussions, and decisions on the study, adoption, and change of status of Official Methods of Analysis shall be maintained for a reasonable time.

B. Timely notice of proposed method studies, adoption, or change in status shall be published in an Association publication that is circulated to the members.

C. Opportunity shall be provided for materially interested parties to submit input during method study and adoption procedures and to submit comments on the adoption, use of, or change in status of specific methods.

D. Methods submitted to the OMB for inclusion in the OMA shall be thoroughly studied, scientifically reviewed, and available in published form prior to adoption as Final Action by the OMB.

E. The OMB shall adopt methods as Final Action.

ARTICLE IX
Meetings

Section 1. Annual Meeting

The annual business meeting of the Association shall be held at the time and place decided by the Board of Directors. A special meeting of the entire Association may be called by the Board of Directors; announcement thereof shall be made at least thirty (30) days prior to the time of said meeting.

Section 2. Quorum

One hundred Individual Members who are present in person or by proxy and entitled to vote shall constitute a quorum at any meeting of the Association which is duly called pursuant to the provisions of these Bylaws.

ARTICLE X
Voting

Section 1. Voting by Ballot

By direction of the Board of Directors, unless otherwise required by these Bylaws or conducted under alternative procedures established under these Bylaws, voting on any matter, including the election of officers and directors, the election of Honorary Members, amendment of the Bylaws, and the approval of dues, may be conducted by ballot of the voting membership by any of the following means (whichever is deemed appropriate at the time): mail, telephone call, telegram, cablegram, electronic mail or other means of electronic or telephonic transmission, and the question(s) thus presented shall be determined according to the votes received, provided in each case votes of at least five (5) percent of the voting membership shall be received. Any and all action taken in pursuance of a vote by any of the means indicated above (whichever the Board deemed appropriate at the time)

in each case shall be binding upon the Association in the same manner as would be action taken at a duly called meeting and shall become effective, unless otherwise provided for in these Bylaws or otherwise stated in the ballot, on the day following certification of the vote.

Section 2. Voting by Proxy

At any duly called meeting of Individual Members, a member-of-record, as determined thirty (30) days prior to any meeting and who is entitled to vote, may vote by proxy executed in writing by the Individual Member or his or her duly authorized attorney-in-fact. No proxy shall be valid for more than eleven (11) months after the date of its execution unless otherwise provided in the proxy.

ARTICLE XI
Earnings and Assets

Section 1. Non-Profit Status

A. Regardless of any provision of the Bylaws which may be construed otherwise:

[1] No part of the net earnings of the Association shall under any circumstances inure to the benefit of any member or individual.

[2] The Association shall not be operated for a private profit.

B. On lawful dissolution of the Association and after settlement of all just obligations of the Association, the Board of Directors shall distribute all remaining assets of the Association to one (1) or more organizations selected by the Board of Directors which have been held exempt from Federal Income Tax as organizations described in section 501(c)(3) of the Internal Revenue Code of 1954.

Section 2. Political Activities

A. No substantial part of the Association's activities shall consist of carrying on propaganda or otherwise attempting to influence local, state, or national legislation. All activities of the Association shall be determined by the Board of Directors.

B. The Association shall not participate or intervene in any manner in any campaign on behalf of any candidate for a political office.

ARTICLE XII
Sections

Section 1. Sections

The Board of Directors shall set geographic limits and grant authority to groups of Individual Members of the Association residing or working in the same geographical areas for the establishment of Sections.

Section 2. Purpose of Sections

The purpose of Sections shall be to promote and further the purpose of the Association.

Section 3. Membership in Sections

Individuals interested in the purpose of the Section shall be eligible for Section membership. Only Individual Members of the Association shall be eligible for election to the Executive Committee of the Section.

Section 4. Bylaws of Sections

Subject to approval of the Board of Directors, each Section shall adopt, for its own governance, bylaws not inconsistent with these Bylaws.

Section 5. Dissolution of Sections

When any Section shall cease to function as a Section for a period of more than one year, or if its membership shall be less than ten (10) Individual Members of the Association for a period of one (1) year, the Board of Directors may terminate the existence of such Section.

Section 6. Actions of Sections

No act of a Section or its members shall be considered an act of the Association unless expressly authorized, ratified, or affirmed by the Board of Directors.

ARTICLE XIII
Technical Divisions

Section 1. Purpose

Technical Divisions shall represent communities of interest within the Association which have the purpose of furthering the purpose of the Association through the development of the analytical sciences either in a commodity-based or scientific discipline-based field. Their activities shall not duplicate the organizational structure nor conflict with the policies or procedures for the adoption of official methods of analysis by the Association.

Section 2. Creation, Combination, Discontinuance, or Change

Technical Divisions may be created, existing Technical Divisions may be combined or discontinued, or the name of a Technical Division may be changed under policies and procedures adopted by the Board of Directors. Each Technical Division shall adopt bylaws not inconsistent with these Bylaws. The jurisdiction of each Technical Division shall be described in its bylaws. No act of any Technical Division or its members shall be considered an act of the Association unless expressly authorized, ratified, or affirmed by the Board of Directors.

ARTICLE XIV
Indemnification

The Association shall have the power to pay, by indemnity, reimbursement, or otherwise, to or for the use of any person designated by resolution of the Board of Directors who was or is a party or is threatened to be made a party to any threatened, pending, or completed action, suit, or proceeding, whether civil, criminal, administrative, or investigative (other than an action by or on behalf of the Association), by reason of the fact he or she is or was a director, officer, committee member, employee or agent of the Association, or was serving as such for another at the request of the Association, against expenses (including legal, accounting, witness and other), judgments, fines, and amounts paid in settlement so long as such person was not found by a court of competent jurisdiction to have been willfully negligent of the interests of the Association or such person had reasonable cause to believe that his or her conduct was lawful.

ARTICLE XV
Parliamentary Authority

The rules contained in the current edition of *Robert's Rules of Order Newly Revised* shall govern the Association in all cases in which they are applicable and in which they are not inconsistent with these Bylaws or any special rules of order the Association may adopt.

ARTICLE XVI
Amendments to the Bylaws

These Bylaws may be amended, repealed, or altered, in whole or in part, by a three-fourths vote: (a) of the Individual Members at any annual business or duly called special meeting of the Association, provided notice of any amendment proposed for consideration shall be sent by any of the following means (whichever may be deemed appropriate at the time): mail, telephone call, telegram, cablegram, electronic mail or other means of electronic or telephonic transmission to the last recorded address or number of each Individual Member at least thirty (30) days prior to the date of the meeting; or (b) by approval of the Individual Members through ballot sent by any means indicated above in accordance with the provisions of Article X, Voting.

All proposed amendments of these Bylaws shall be presented in writing to the Board of Directors. The Board shall present the proposals to the Association membership, with recommendations. All amendments to the Bylaws, unless otherwise stated, will become effective at the adjournment of the meeting where action is taken or on the day following the certification of a vote by mail ballot.

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.

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.

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.

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

AOAC INTERNATIONAL

International Stakeholder Panel on Alternative Methods (ISPAM)

Meeting at the Gaithersburg Marriott Washingtonian Center

9751 Washingtonian Boulevard, Gaithersburg MD 20878, USA

STAKEHOLDER PANEL DRAFT MEETING AGENDA

Tuesday, March 14, 2017

Meeting Start Time: 8:30AM (Eastern US)

ISPAM Chair: Erin Crowley

(Q Laboratories, Inc.)

Location: Salon C/D/E

(Registration Opens at 7:30AM)

I. WELCOME & INTRODUCTIONS (Goodwin/Crowley – 8:30AM-8:45AM)

Jonathan Goodwin (AOAC) will open the meeting by welcoming attendees, leading introductions, and introducing ISPAM Chair, Erin Crowley (Q Laboratories, Inc.). Crowley will call the meeting to order.

II. AOAC ISPAM GOALS/OVERVIEW/UPDATE (Crowley – 8:45AM-9:15AM)

Crowley will review the AOAC ISPAM meeting agenda and goals. She will also provide an overview of ISPAM and its working group activities, including an update on development of standard method performance requirements (SMPR®) for egg and the new working group for gluten. She will also provide an overview of expected ISPAM activities between March 2017 and the next ISPAM meeting in September 2017.

III. AOAC STANDARDS DEVELOPMENT POLICIES & PROCEDURE OVERVIEW (McKenzie – 9:15AM-9:45AM)

Deborah McKenzie (AOAC) will provide information on the AOAC Standards Development process.

IV. UPDATE ON ISPAM WORKING GROUP ON FOOD ALLERGEN ASSAYS (Working Group Co-Chairs – 9:45AM-10:30AM)

Samuel Godefroy (Université Laval) & Jupiter Yeung (Nestlé) will present an update on the activities of the Food Allergens Assays Working Group and the progress on development of standard method performance requirements (SMPRs®) for egg.

V. WORKING GROUP CHAIR PRESENTATION ON GLUTEN ASSAYS INCLUDING FITNESS-FOR-PURPOSE (10:45AM-12:00PM)

Information will be presented on the background, analytical challenges, regulatory requirements, community efforts, and fitness-for-purpose for the determination of gluten in oats.

1. **Overview** (Joe Boison, CFIA (Working Group Chair))
2. **Background/History** (Paul Wehling, General Mills)
3. **Analytical Challenges** (Tracy Mui, PepsiCo)
4. **Regulatory & Fitness-for-Purpose*** (Terry Koerner, Health Canada/Joe Boison, CFIA)

AOAC INTERNATIONAL

International Stakeholder Panel on Alternative Methods (ISPAM)

Meeting at the Gaithersburg Marriott Washingtonian Center

9751 Washingtonian Boulevard, Gaithersburg MD 20878, USA

WORKING GROUP DRAFT MEETING AGENDA

Tuesday, March 14, 2017

Meeting Start Time: 1:00PM (Eastern US)

ISPAM Chair: Erin Crowley

(Q Laboratories, Inc.)

Location: Salon C/D/E

I. WORKING GROUP ON FOOD ALLERGEN ASSAYS (Godefroy/Yeung/Coates)

1. Standard Method Performance Requirement (SMPR®)

II. WORKING GROUP ON GLUTEN ASSAY (Boison/Wehling/Mui/ Coates)

1. Standard Method Performance Requirement (SMPR®) Orientation (Coates)
2. Discussion on Analytical Challenges & Regulatory Issues (Boison/Wehling/Mui)
3. Review of Endorsed Fitness-for-Purpose (Boison)
4. Standard Method Performance Requirement (SMPR®) Development (Boison/Coates)

III. NEXT STEPS (Crowley/McIver)

Erin Crowley (Q Laboratories) and Krystyna McIver (AOAC) will discuss next steps for the working group activities, wrap up all discussions and answer any additional questions.

MEETING ITINERARY:

Registration	**(7:00AM)**
Meeting Start Time	**(8:30AM)**
Morning Break	**(10:30AM)**
Lunch	**(12:00PM)**
Afternoon Break	**(2:45PM)**

***Requires a vote**
Draft meeting agenda is subject to change w/out notice

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Erin Crowley, Ph.D.
Q Laboratories, Inc. & ISPAM Chair

Erin Crowley was recently appointed as Chief Scientific Officer at Q Laboratories, Inc in Cincinnati, Ohio. Prior to this, she was the Microbiology Research and Development Supervisor at Q Laboratories, Inc. in Cincinnati, Ohio since 2006. For the past 10 years, Erin and her R&D team have served as an independent third-party laboratory with a primary focus on providing high quality method validation for microbiological rapid detection methods. These validations include Independent laboratory evaluations for pathogen detection, qualitative methods and confirmatory biochemical assays for AOAC Official Methods of Analysis, AOAC Research Institute Performance Tested Methods Program and MicroVal Certification Program. In addition to being an active member of the International Association of Food Protection (IAFP) and AOAC, Erin currently serves as Chair of the AOAC Official Methods Board, Chair of the International Stakeholder Panel on Alternative Methods (ISPAM) and a member of the MicroVal Technical Committee (MVTC). Erin earned a B.S. from the University of Cincinnati in Cincinnati, Ohio and an M.A. from Tufts University in Medford, MA.

Overview of International Stakeholder Panel on Alternative Methods Activities (ISPAM)

March 14, 2017

Erin Crowley

Chair, ISPAM

Chief Scientific Officer, Q Laboratories, Inc.

Agenda

International Stakeholder Panel on Alternative Methodology (ISPAM)

Tuesday, March 14, 2017
Meeting Start Time: 8:30AM (Eastern US)

I. WELCOMES & INTRODUCTIONS (Goodwin/Crowley – 8:30AM-8:45AM)

Jonathan Goodwin (AOAC) will open the meeting by welcoming attendees, leading introductions, and introducing ISPAM Chair, Erin Crowley (Q Laboratories, Inc.). Crowley will call the meeting to order.

II. AOAC ISPAM GOALS/OVERVIEW/UPDATE (Crowley – 8:45AM-9:15AM)

Crowley will review the AOAC ISPAM meeting agenda and goals. She will also provide an overview of ISPAM and its working group activities, including an update on development of standard method performance requirements (SMPR®) for egg and the new working group for gluten. She will also provide an overview of expected ISPAM activities between March 2017 and the next ISPAM meeting in September 2017.

III. AOAC STANDARDS DEVELOPMENT POLICIES & PROCEDURE OVERVIEW (McKenzie – 9:15AM-9:45AM)

Deborah McKenzie (AOAC) will provide information on the AOAC Standards Development process.

Agenda

IV. UPDATE ON ISPAM WORKING GROUP ON FOOD ALLERGEN ASSAYS (Working Group Co-Chairs – 9:45AM-10:30AM)

- Samuel Godefroy (Université Laval) & Jupiter Yeung (Nestlé) will present an update on the activities of the Food Allergens Assays Working Group and the progress on development of standard method performance requirements (SMPRs®) for egg.

V. WORKING GROUP CHAIR PRESENTATION ON GLUTEN ASSAYS INCLUDING FITNESS-FOR-PURPOSE

(10:45AM-12:00PM)

Information will be presented on the background, analytical challenges, regulatory requirements, community efforts, and fitness-for-purpose for the determination of gluten in oats.

- 1. **Overview** (Joe Boison, CFIA-Working Group Chair)
- 2. **Background/History** (Paul Wehling, General Mills)
- 3. **Analytical Challenges** (Tracy Mui, PepsiCo)
- 4. **Regulatory & Fitness-for-Purpose*** (Joe Boison, CFIA)

- **Lunch on your own 12-1 pm**

Agenda

WORKING GROUP DRAFT MEETING AGENDA

Tuesday, March 14, 2017

Meeting Start Time: 1:00PM (Eastern US)

I. WORKING GROUP ON FOOD ALLERGEN ASSAYS (Godefroy/Yeung/Coates)

1. Standard Method Performance Requirement (SMPR®)

II. WORKING GROUP ON GLUTEN ASSAY (Boison/Wehling/Mui/ Coates)

- 1. Standard Method Performance Requirement (SMPR®) Orientation (Coates)
- 2. Discussion on Analytical Challenges & Regulatory Issues (Boison/Wehling/Mui)
- 3. Review of Endorsed Fitness-for-Purpose (Boison)
- 4. Standard Method Performance Requirement (SMPR®) Development (Boison/Coates)

III. NEXT STEPS (Crowley/McIver)

- Erin Crowley (Q Laboratories) and Krystyna McIver (AOAC) will discuss next steps for the working group activities, wrap up all discussions and answer any additional questions.

International Stakeholder Panel on Alternative Methods (ISPAM)

- Driven and supported by AOAC Organizational Affiliates and contributing members who participate in the AOAC Research Institute Program
- ISPAM was formed initially to develop harmonized, internationally accepted standard validation guidelines for alternative (rapid) chemical and microbiological methods by leveraging global networks of experts to reach consensus on an analytical validation protocol.
  - The goal is to achieve optimal efficiency and avoid duplication of efforts in order to meet regulatory and product safety testing requirements.
- The ISPAM as a whole is made up of 60+ member stakeholders who, at open stakeholder meetings, discuss and deliberate recommendations of smaller working groups.
- Only a vetted balanced group and representative stakeholders from industry, government, academia and international organizations, demonstrate stakeholder consensus to approve or not the recommendations of the working groups.

Vetting of Stakeholder Panel Voting Members

- All stakeholders may share and contribute to the discussions
- However, consensus of the stakeholders is demonstrated via voting conducted with a vetted, balanced representative group of stakeholders
- All identified and perspectives are represented.
- Stakeholder panels deliberate and reach consensus on standards.

ISPAM Voting Panel – Mid-Year Meeting 2016

Table 2: Representative Voting Members (29 out of 58 registrants)

Broad Perspective	Specific Perspective	Region	Organization(s)
Academia	Research	US	FARRP-Univ. of Nebraska
Government	Regulatory	Canada	Health Canada / CFIA
Government	Regulatory	Canada	Canadian Grain Commission
Government	Regulatory	US	US FDA
Government	Regulatory	Belgium	European Commission
Government	Regulatory	Austria	AGES
NGO	Product Certification	US/France	GFCO-GIG / AFNOR
NGO	Research	Austria	MoniQA
NGO	Independent	Germany	German Center for Food Chemistry
NGO	Food	France	AOAC Food Allergen Community
Industry	Food	US	General Mills
Industry	Food	US	PepsiCo/Quaker Oats
Industry	Food	Japan	Nippon Ham
Industry	Food	US	Nestle

ISPAM Voting Panel – Mid-Year Meeting 2016

Broad Perspective	Specific Perspective	Region	Organization(s)
Industry	Food	US	Grain Millers
Industry	Food	US	Abbott/Hershey
Industry	Method Developer	US	Elution/3M
Industry	Method Developer	Germany	R-Biopharm/ Roka Biosciences
Industry	Method Developer	US	Neogen / Rheonix
Industry	Method Developer	US	Romer Labs/Morinaga
Industry	CRO	US/New Zealand	Eurofins / AsureQuality
Industry	CRO	US	Merieux NutriSciences/
Industry	CRO	US	Microbac Laboratories
Industry	CRO	France	Food Consulting Services
Industry	Product Certification	Canada	Allergen Control Group
Industry	Consulting	Netherlands	FoodPhysica (Clyde Don Consulting)
Industry	Biotechnology	France	BioAdvantage
Industry	Consulting	Australia	DTS Facta
Industry	Technology Provider	US	Agilent / SCIEX / Waters

ISPAM: The Year in Review
Mid-Year Meeting 2016

- Panel discussion on Global Food Safety Needs
  - Speakers
    - GMA
    - FDA-ORA
    - USDA-FSIS
    - Chinese Institute of Food Science and Technology
    - University of Buenos Aires
    - Labororatorio Technologico del Uruguay
  - Most critical needs
    - Allergen detection methods
    - Enrichment issues with Pathogen Detection
    - Environmental Sampling Plans and Testing- Data Acceptance
    - Whole Genome Sequencing- Standards

ISPAM Mid-Year Meeting 2016

- AOAC-RI Board of Directors agreed to form a working group on food allergens
  - Focus on rapid method technology
  - Molecular, Immunoassay, new and emerging technologies
  - Complement to SPSFAM current WG for select allergen detection using mass spectroscopy.

Background and positioning of Working Group

- Working Group reporting to the AOAC International Stakeholder Panel on Alternative Methodologies (ISPAM) to improve food allergen methods.

- WG tasked specifically to develop recommendations for the establishment of Standard Method Performance Requirements (SMPR) for food allergen methodologies

- WG creation follows a priority setting exercise for work on Food Allergens SMPR – ***Thought Leader Advisory Meeting*** on June 15th at AOAC HQ.

Background on Food Allergens Methods

- Most Methods are Antibody based methods:
  - ELISA tend to dominate existing methodologies being applied for food control purposes
  - Other detection approaches are possible e.g. SPR-based biosensors

- Some methods are DNA/RNA based methods – used generally as complementary methods (particularly in jurisdictions where the regulations are based on protein requirements)

- Methods based on Mass-Spectrometry detection (with the associated separation technique) are confirmatory approaches.

Outputs of the AOAC Priority Setting process – Meeting June 15th 2016

- Recommendations were made to consider the following priorities:
  - Egg
  - Milk
  - Tree nuts (priority to be determined)
  - Peanut
  - In a parallel track to examine Gluten based methodologies

- Recommendation to consider ELISA-based methodologies as a priority, both plate-based methods (Quantitative approaches) and Lateral flow device based techniques (semi-quantitative methods generally).

- Leverage work conducted previously under the auspices of AOAC's Food Allergen Community between 2006-2015 and SPSFAM in 2015-2016

- **WG co-chaired by Samuel Godefroy (University of Laval) and Jupiter Yeung (Nestle)**

PURPOSE

To develop recommendations for SMPRs for ELISA-based Food Allergen Methods using the priority sequence established by the Thought Leader Advisory group

Outcomes of AOAC Annual Meeting 2016

The determined the following priority allergens in food (food will be defined in the SMPR®):

1. **Eggs**
2. **Milk**
3. **Peanut**
4. **Tree nut (hazelnut, almond)**
5. **Celery**
6. **Mustard**
7. **Gluten**

Fitness for Purpose Statements – Quantitative and Qualitative

- **Endorsed by ISPAM**
- **Working Group Formed**
- **Core Group formed to begin writing the draft**

Outcomes of AOAC Annual Meeting 2016

Fitness for Purpose Statement (Quantitative):

- Quantitative analysis of sources of food allergens according to the priority sequence in environmental samples and foods by ELISA-based technologies with consideration of alternative technologies

Fitness for Purpose Statement (Qualitative):

- Qualitative analysis of sources of food allergens according to the priority sequence in environmental samples and foods e.g. by Lateral Flow device technologies

Report on Progress Since Annual Meeting

- Teleconferences with Core Drafting Group and Working Group
- Draft SMPR for Egg distributed to the WG and 90 comments received after first pass
- Core Drafting Group met in-person on February 3, 2017 at AOAC Headquarters
  - Review all comments in person
  - Revised based on comments
  - Distributed to WG for March 13th meeting prior to presenting to ISPAM Stakeholders today
  - DRAFT AOAC Allergen SMPR "Quantitation of Chicken Egg by ELISA-based* Methods"

Report on Progress Since Annual Meeting

- Official Launch of Working Group (WG) on Gluten Assays
  - First ISPAM WG formed through the WG initiative and fully funded by ISPAM Stakeholders
  - Will start development of a Standard Method Performance Requirement (SMPR®) for the detection of gluten in oats
  - Chaired by Dr. Joe Boison, CFIA

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Deborah McKenzie
AOAC INTERNATIONAL

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INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS(ISPAM)

AOAC Standards Development Process
and
Launch of AOAC Stakeholder Panel Working Groups

Deborah McKenzie
Gaithersburg, MD
March 14, 2017

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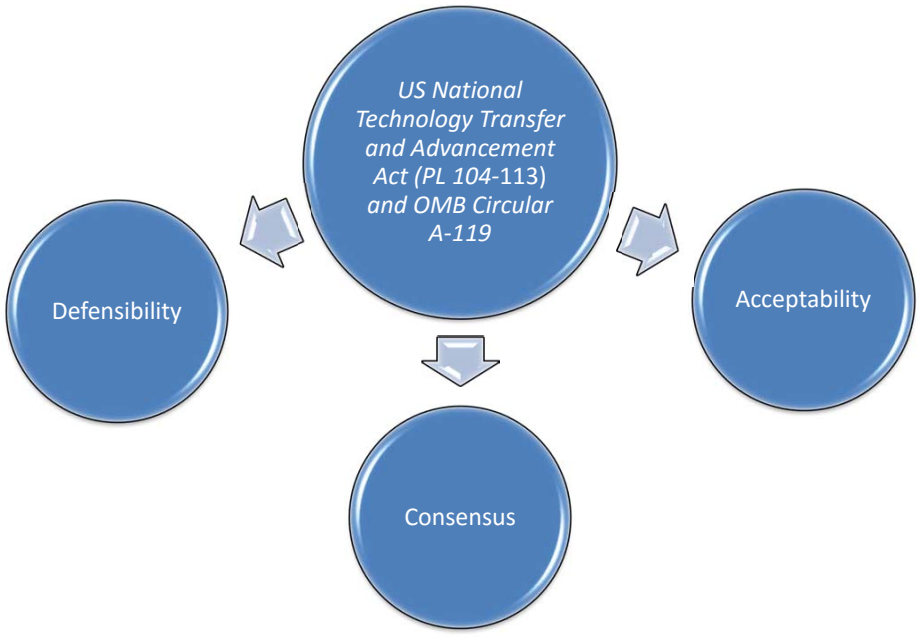
AOAC Standard Development Process



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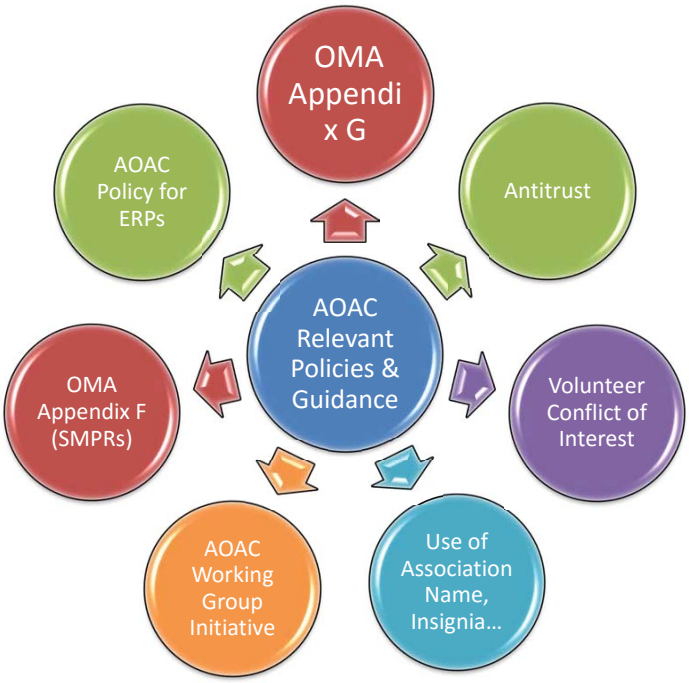
AOAC Standards Development



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AOAC Standards Development

- AOAC develops voluntary consensus standards using the following principles:

Transparency

Openness

Balance

Due Process

Consensus

Appeals

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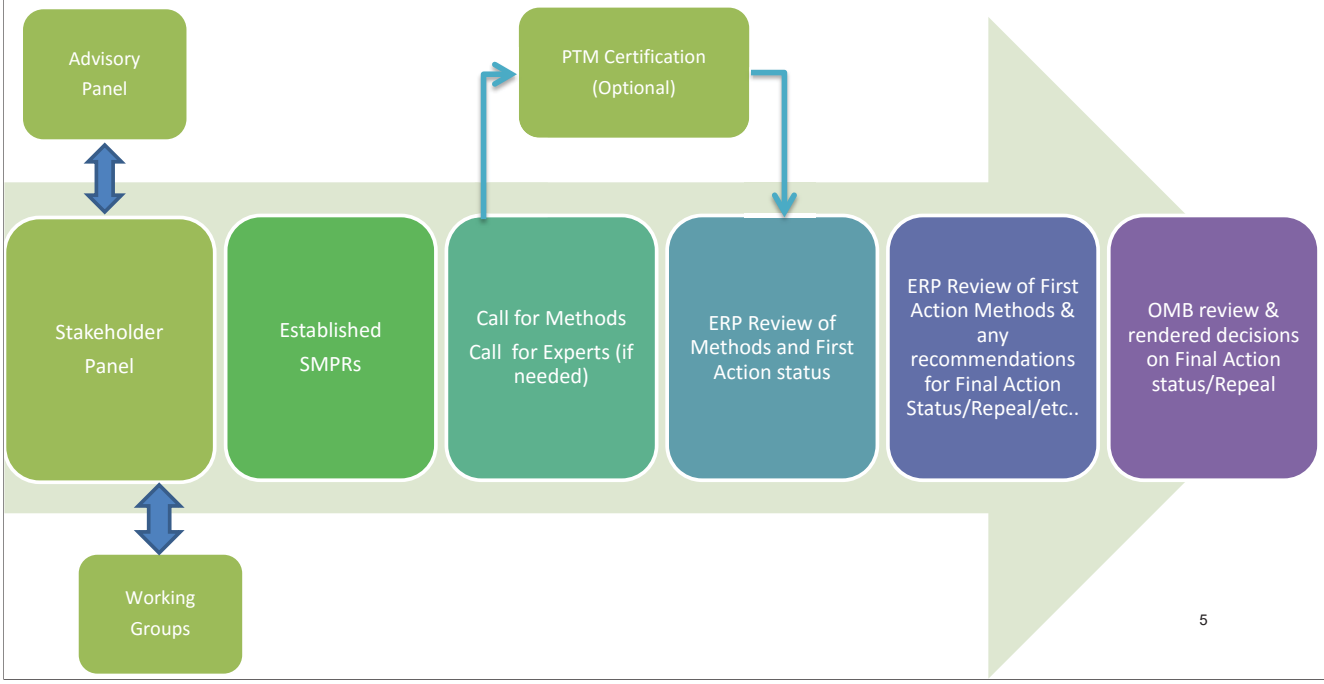
AOAC Standards Development

Initiating SMPRs to Final Action Consideration of Methods



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Stakeholder Panel Working Groups

- Present background and history on priority allergens for stakeholder panel
- Develop draft SMPR
- Will present motions to the stakeholder panel on components of the standard method performance requirements
- Can participate in ISPAM related in-person meetings

ISPAM Working Groups

ISPAM Working Groups	Status
Working Group on Microbiology Validation Harmonization	Active
Working Group on Qualitative Chemistry Guidelines	Inactive
Working Group on Statistics	Inactive
Working Group on Produce Sampling Standard	Inactive
Working Group on Produce – Salmonella in Leafy Greens	Inactive
Working Group on Food Allergen Assays	Active
Working Group on Gluten Assays	Newly Active

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AOAC Stakeholder Panel Role and Responsibilities

- To harmonize method validation guidance
- To form working groups to draft SMPR(s) based on specific priorities as specified
- To provide comments on draft standard method performance requirements
- To respond to calls for methods and calls for experts as applicable or appropriate
- Most importantly, share your perspective.
  - To attend stakeholder panel meetings and deliberate on and adopt voluntary consensus standards

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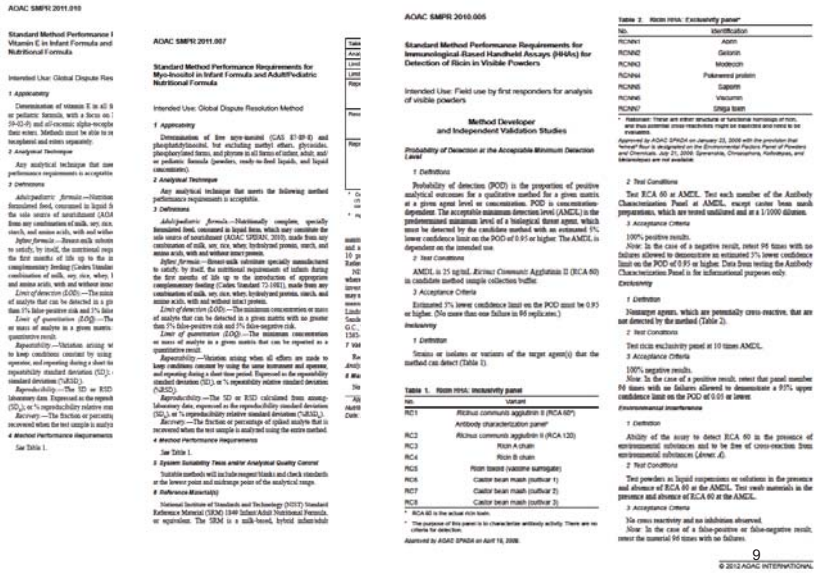
Standard Method Performance Requirements

- ***Commonly referred to as - - SMPRs***



AOAC SMPR 2011.010

Standard Method Performance Requirements for Vitamin E in Infant Formula and Nutritional Formula

AOAC SMPR 2011.007

Standard Method Performance Requirements for Myo-Inositol in Infant Formula and Adult/Pediatric Nutritional Formula

AOAC SMPR 2010.005

Standard Method Performance Requirements for Immunological Based Handheld Assays (HHAs) for Detection of Ricin in Visible Powders

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SMPRs

- Documents a community's analytical method needs
- Very detailed description of the analytical requirements
- Includes method acceptance requirements
- Used to qualify methods for AOAC approval in the *Official Methods*^{SM} program
- Published as a standard

Performance requirements parameters for quantitative methods

- Analytical Range
- Limit of Detection
- Limit of Quantitation
- Repeatability
- Recovery
- Reproducibility

4. Method Performance Requirements

Analytical range	0.01–5.0[a]	
Limit of detection (LOD)	≤0.004[a]	
Limit of quantitation (LOQ)	≤0.01[a]	
Repeatability (RSD_r)	0.01[a]	≤15%
	0.2[a]	≤7%
	0.5[a]	
	5.0[a]	
Recovery	0.01[a]	90–110%
	0.2[a]	
	0.5[a]	
	5.0[a]	
Reproducibility (RSD_R)	0.3	≤11%
	0.6	
	1.0	
	2.5	
	5.0	
Concentrations apply to (*1*) "ready-to-feed" liquids "as is"; (*2*) reconstituted powders (25 g into 200 g water); and (*3*) liquid concentrates diluted 1:1 by weight.		
[a] µg/100 g expressed as cyanocobalamin in reconstituted final product.		

11

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Stakeholder Panel Activity Since September 2016

- Working Group on Food Allergen Assays met in November and December to work on draft of quantitative SMPR for egg
- Comments were received from working group members and sent out for working group meeting.
- Working group co-chairs held an in-person meeting at AOAC on February 3rd to draft responses to comments
- Drafting subgroup of the working group met at AOAC on March 13th to work on draft of SMPR
- Working group will meet following ISPAM per agenda to continue working on the SMPR.

AOAC Standard Method Performance Requirements (SMPRs)

– Published in *Official Methods of Analysis of AOAC INTERNATIONAL*

– Manuscript published in *Journal of AOAC INTERNATIONAL*

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Stakeholder Panel Activity Since September 2016 (cont.)

- New working group formed via AOAC's Working Group Initiative for Gluten in Oats
- Advisory Panel formed from sponsors: General Mills, PepsiCo, Romer Labs, Neogen, R-Biopharm, Elution Technologies, and Grain Millers
- Working group launch presentations developed for presentation at this meeting.
- Stakeholders will be asked to deliberate on and endorse a final version of a fitness for purpose statement for gluten in oats
- Working group to meet after the ISPAM meeting per agenda to begin work on drafting the SMPR

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Stakeholder Panel Composition

- Product Manufacturers
- Analyte/Method Subject Matter Experts
- Technology Providers
- Method Developers
- Government and Regulators
- Contract Research Organizations
- Reference Materials Developers
- Ingredient Manufacturers
- Method End Users
- Academia & Research
- Non Governmental Organizations
- Other as identified

Anyone with a material interest can participate
Balanced group of representative voting stakeholders
Chair and voting stakeholders vetted by AOAC Official Methods Board

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Organizational Meeting Registrants

3M	FDA	Office of Dietary Supplements, NIH
Abbott Nutrition	FL Dept. of Agriculture & Consumer Services	Pepsico/Quaker Oats
Agilent	FARRP, University of Nebraska-Lincoln	Q Laboratories
Allergen Control Group, Inc. (ACG)	Food Consulting Services - B. Popping	R-Biopharm
AOAC Food Allergen Community	General Mills	Rheonix
AsureQuality, New Zealand	GFCO/GIG	Roka Bioscience Inc.
Bia Diagnostics	Grain Millers Inc.	Romer Labs
BioAdvantage	Health Canada	Sample6,Inc
BioAnalyt GmbH	Maxxam Analytics	SCIEX
Canadian Food Inspection Agency	McCormick & Company, Inc.	Shimadzu Scientific Instruments
Canadian Grain Commission, Grain Research Laboratory	Mérieux NutriSciences	U.S. Treasury (retired)
Covance	Microbac Laboratories	USDA, ARS
DOTS Corp.	Morinaga Institute of Biological Science, Inc.	Waters Corporation
DuPont	Neogen	
Eurofins	Nestle	

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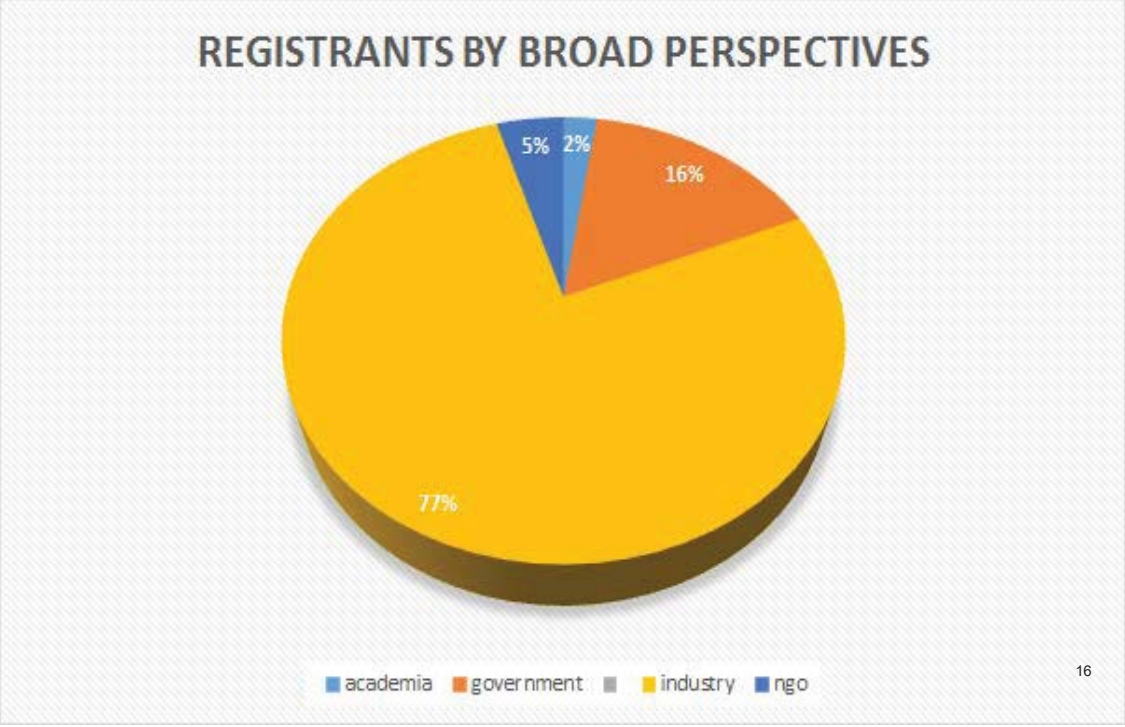
ISPAM Registrants by Broad Perspectives



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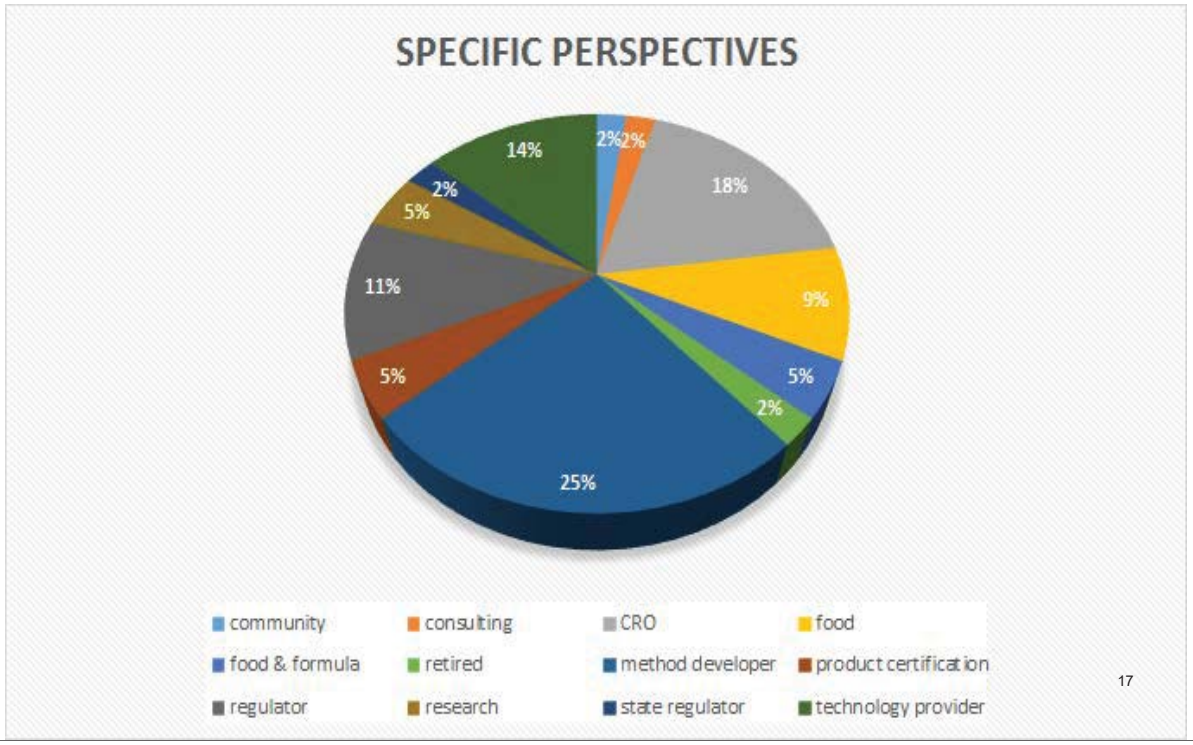
ISPAM Registrants by Specific Perspectives



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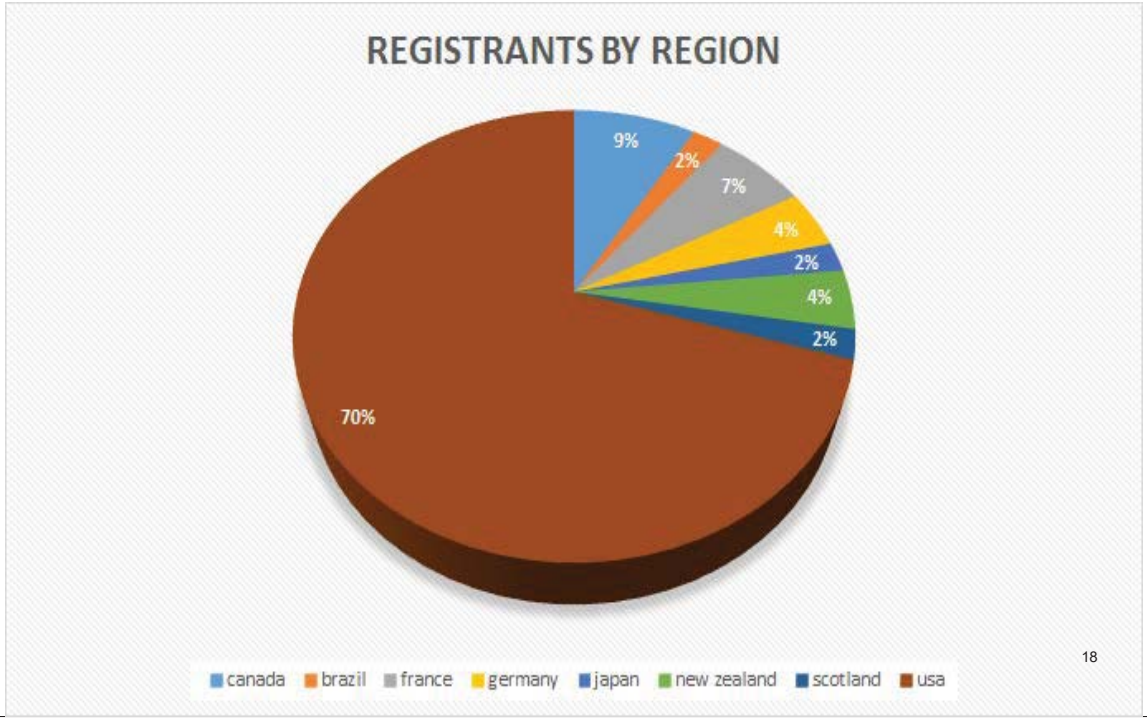
ISPAM Registrants by Regions



Proposed ISPAM Representative Voting Members

FARRP-Univ. of Nebraska	McCormick / Abbott Nutrition
Health Canada / CFIA	R-Biopharm
Canadian Grain Commission	Romer Labs/Roka Bioscience
US FDA	Neogen/3M
USDA	Morinaga
Florida Dept. of Agriculture and Consumer Services	BiaDiagnostics
GFCO-GIG	AsureQuality
Allergen Control Group	Merieux NutriSciences/ Eurofins
AOAC Food Allergen Community	Microbac Laboratories
General Mills	Food Consulting Services
PepsiCo/Quaker Oats	BioAdvantage
Nestle	SCIEX / Agilent
Grain Millers	Waters/Shimadzu

alternates

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Launching AOAC Stakeholder Panel Working Groups

- Working Group Chair or designee will present on the background, regulations, and analytical challenges of the priority. The WG chair will also propose a draft fitness for purpose statement that will serve as the basis for the working group's SMPR development
- ISPAM chair will entertain deliberation on the draft statement
- After due deliberation by ALL of the assembly, and potential tweaking, ISPAM chair will call for an endorsement of the fitness for purpose statement
- Information will be available for attendees to sign up to participate on the working group

Documentation and Communication

- AOAC carefully documents the actions of the Stakeholder Panel and the Working groups
- AOAC will prepare summaries of the meetings
  - Communicate summaries to the stakeholders
  - Publish summaries in the *Referee* section of AOAC's *Inside Laboratory Management*
- AOAC publishes its voluntary consensus standard
  - *Official Methods of Analysis of AOAC INTERNATIONAL*
  - *Journal of AOAC INTERNATIONAL*
- AOAC publishes the status of standards in the *Referee* section of AOAC's *Inside Laboratory Management*

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Roles and Responsibilities

- Stakeholder Panel
  - Establish working groups to develop standards
  - Comment, deliberate, and establish voluntary consensus standards
- Stakeholder Panel Working Groups
  - Develop draft standard method performance requirements
  - Reconcile comments
  - Present draft standard to stakeholders
- Official Method Board
  - Vet and approve stakeholder panel chair and representative voting stakeholders
  - Assign representative to serve as a resource to stakeholder panel
- AOAC Staff
  - Coordinate stakeholder panel, working groups, and facilitate their meetings
  - Document actions/decisions of working groups and stakeholder panel
  - Post SMPRs and collect comments for draft SMPRs

QUESTIONS?

THANK YOU

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Samuel Godefroy, Ph.D.
Université Laval
Working Group Co-Chair

Samuel Godefroy is a Full Professor of Food Risk Analysis and Regulatory Systems at the Department of Food Science, Faculty of Agriculture and Food Sciences, University Laval, Québec, Canada. He is also a professor at the Global Institute for Food Security (GIFS) at Queen's University of Belfast, in the UK. Samuel is currently leading the development of a Food Risk Analysis and Regulatory Excellence Platform (FRAREP) hosted by the Institute of Nutrition and Functional Foods (INAF) of Université Laval.

Before Joining Université Laval in the fall of 2015, Dr. Godefroy completed a secondment with the World Bank's Global Food Safety Partnership where he led the Strategic Development of this initiative. Under his leadership, this public-- private partnership developed and adopted its 2015--2020 strategic framework, to guide its actions in food safety capacity building globally.

Dr. Godefroy assumed senior food regulatory positions at the executive level with Health Canada for over 10 years, including the position of Director General of Health Canada's Food Directorate, the Federal food standard setting organization in Canada from 2009 to 2015.

Samuel served as Vice Chair of the FAO/WHO Codex Alimentarius Commission (CAC), the international food standard setting body from 2011 to 2014. During his tenure, Samuel led the development and facilitated the adoption by consensus of the organization's strategic plan for 2014-19.

Dr. Godefroy currently serves as a senior food science and regulatory expert on a number of advisory bodies and committees domestically and internationally, including expert advice to food safety projects led by the United Nations Industrial Development Organization (UNIDO) and serving on the International Advisory Committee of the China Centre for Food Safety Risk Assessment (CFSA). Samuel authored over 65 scientific publications and book chapters and serves on a number of international editorial boards of scientific journals related to food safety and nutrition.

Dr. Godefroy received his Ph.D. in Analytical Chemistry from the University of Pierre et Marie Curie (Paris VI). He holds degrees in Chemistry, Biochemistry and Chemical Engineering from the same University and from the École Nationale Supérieure de Chimie de Paris, France.

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Jupiter Yeung, Ph.D.
Nestlé
Working Group Co-Chair

Jupiter is a Principal Scientist for Global Food Safety for Nestle Nutrition. He joined Nestle in 2008. Jupiter has more than 20 years of food safety experience in physical, chemical and food allergen risk analyses and management to ensure safe and nutritious food supply to all stakeholders.

Prior to joining Nestle, Jupiter worked for GMA, academia and government. He holds a BSc in Pharmacy and PhD in Chemistry. He published over 120 manuscripts and book chapters on a wide range of subjects related to food safety, and health and wellness.

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Joe Boison, Ph.D.
Canadian Food Inspection Agency (CFIA)
Working Group Chair

Dr. Boison is a Senior Research Scientist with the Canadian Food Inspection Agency (CFIA), and holds 2 Adjunct Professor Faculty positions (one in the Chemistry Department and the other in the Department of Veterinary Biomedical Sciences) at the University of Saskatchewan. In 2003, he was appointed a Fellow of the World Innovation Foundation (FWIF), was awarded the CFIA President's National Award for Leadership Excellence in 2010 and in 2012, he was appointed a Fellow of the AOACI.

Dr. Boison has been a member of the Food Safety Research Network Team responsible for reviewing and evaluating research proposals submitted by scientists from Agriculture & Agri-Food Canada and the Canadian Food Inspection Agency for funding considerations. He is an executive member of the Spectroscopy Society of Canada, a member of the Standards and Measurement Committee for the American Society for Mass Spectrometry (ASMS), a member of the AOAC International, and a member of the Canadian Delegation to the Codex Committee on Residues of Veterinary Drugs in foods (CCRVDF).

Dr. Boison is regularly consulted within and outside Government (primary producers, program managers, test kit manufacturers, fellow scientists) with regards to residue testing methods for in-plant and on-farm use. In his current role as AOAC International's General Referee (GR) on Veterinary Drugs, Dr. Boison authors an annual review paper on the methods of analysis used in the regulatory analysis of veterinary drugs.

Dr. Boison's research and academic interests include development of chromatographic and mass spectrometric methods for the identification and confirmation of veterinary drug residues in biological fluids and tissues in support of regulatory enforcement and/or for pharmacokinetic and pharmacodynamic studies. 2. Development and adaptation of commercially available rapid tests for field and laboratory screening of drug residues in biological fluids and tissues. 3. Automation of laboratory methods for the analysis of veterinary and human drugs. 4. Teaching and development of graduate and undergraduate students to acquire expertise in bio-analytical mass spectrometry, metabolism and pharmacokinetic studies.

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INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS(ISPAM)

ISPAM SMPR Development For The Quantitative ELISA Analysis Of Gluten In Oats

Joe Boison, Ph.D. (Working Group Chair)
Gaithersburg, MD
March 14, 2017

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Stakeholder Engagement

- General Mills
- Quaker oats
- Grain Millers Inc.
- Neogen
- R-Biopharm
- Romer Labs
- Elution Technologies

Stakeholder Engagement (cont.)

- **Overview** (Joe Boison, CFIA-Working Group Chair)
- **Background/History** (Paul Wehling, General Mills)
- **Analytical Challenges** (Tracy Mui, PepsiCo)
- **Regulatory *** (Terry Koerner)
- **Fitness-for-Purpose*** (Joe Boison, CFIA)

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Overview

- The need to choose, characterize and validate a suitable method or methods for the quantitative analysis of gluten in oats.
- The current position and the difficulties and challenges to the development of accurate and precise methods for gluten in oats.
- A practical definition of gluten that can be used as the basis for method development.

Overview (cont'd)

- Paul Wehling, Medallion labs –
  - Definition of gluten
  - What is on the scope of the gluten method development project and what is not
  - Matrices to be covered and why
- Tracy Mui – PepsiCo
  - Current trends in assay results from gluten compliant and non-compliant gluten in oatmeal test results
- Terry Koerner – Health Canada
  - Health Canada Regulations
- Joe Boison – Canadian Food inspection Agency
  - Definition of the fit-for-purpose criterion for gluten method
  - Development of the Standard method Performance Requirements (SMPR)

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Electronic Working Group (eWG)

- Next Steps

Thank You!

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Paul Wehling, Ph.D.
General Mills/Medallion Labs

Paul Wehling has served as a volunteer for AOAC International since 2000, when he joined the Statistics Committee. He started his career at General Mills in 1983, and has been working in the analytical labs for most of that time. He is currently a Principal Scientist at General Mills, and is a Fellow of AOAC.

Medallion Labs

ISPAM GLUTEN IN OATS PROJECT

BACKGROUND AND HISTORY

Paul Wehling

WHAT IS GLUTEN?

- Codex Alimentarius Definition
  - Protein fraction from wheat, rye, barley, oats[1] or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5M NaCl.

  - Oats[1] - Oats can be tolerated by most but not all people who are intolerant to gluten. Therefore, the allowance of oats that are not contaminated with wheat, rye or barley in foods covered by this standard may be determined at the national level.

US AND CANADIAN REGULATIONS

- US FDA – August 2013
  - 21 CFR 101
  - Allows for GF Claims on oat products

- Health Canada – 2007 – "Celiac Disease and the Safety of Oats"
- Health Canada – July 2012 – Gluten-Free Claims Regulation (no oats)
- Health Canada – May 2015 – Marketing Authorization for Gluten-free Oats

PROJECT OBJECTIVE

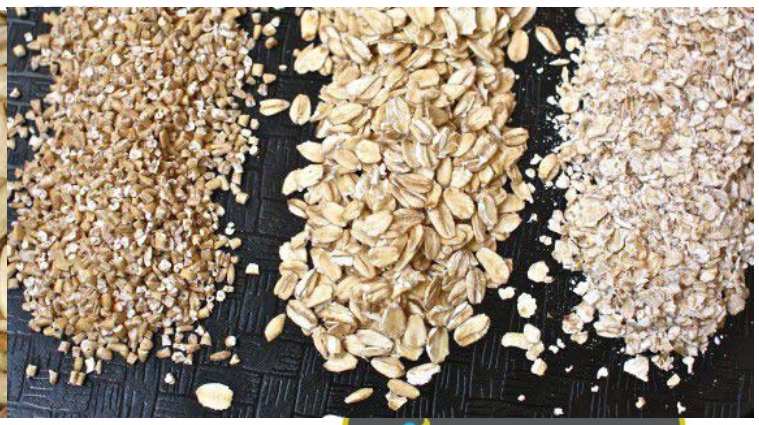
Validate methods which can precisely and accurately account for gluten contamination in oats from wheat, rye and barley.

CURRENT OMA METHODS

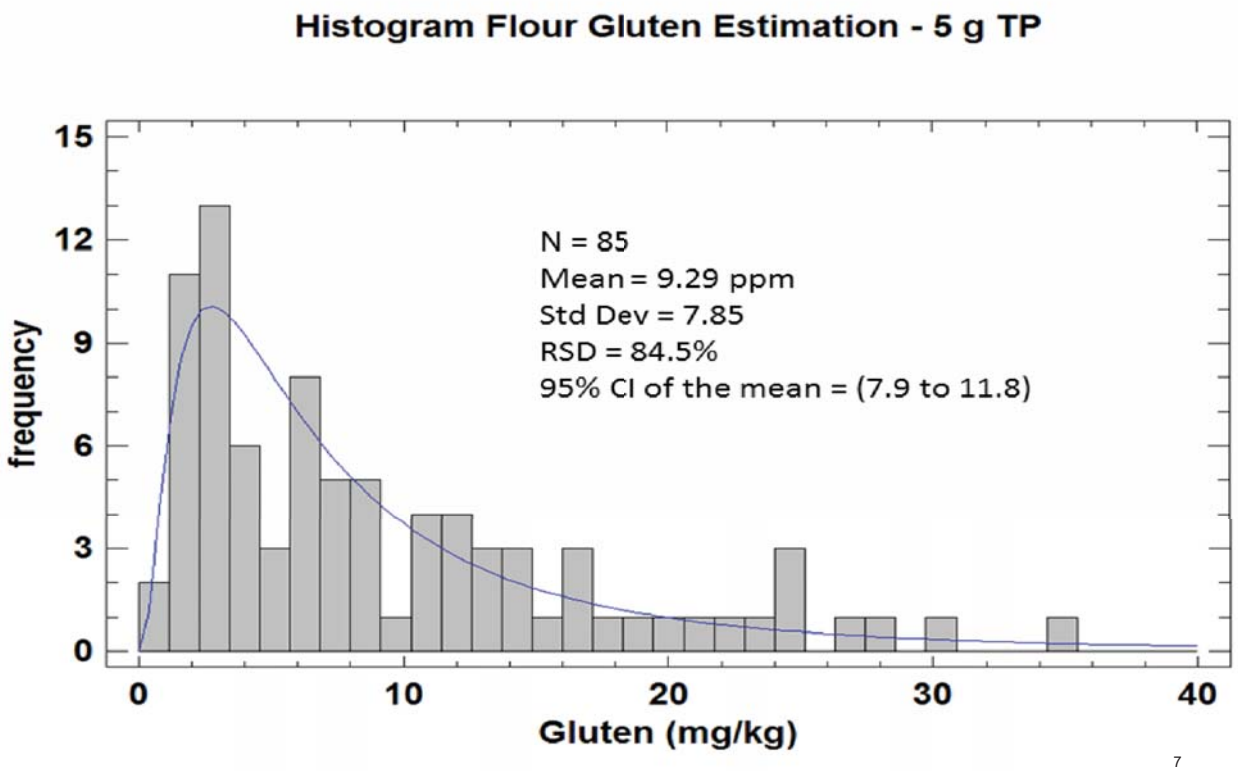
OMA No	Antibody	Action	Matrices	Comment
991.09	Skerritt	Final	“Foods”	Very low barley response
2012.01	R5	Final (2016)	Rice and Corn	Very high barley response
2014.03	G12	First (2014)	Rice Flour and Rice products	Some reported oat cross-reactivity
2015.05	R5	First (2015)	Fermented Cereals	Competitive Assay

SMPR CHALLENGES - PRECISION

Due to the inhomogeneous nature of the sample matrices, it may difficult to achieve RSDs as low as we have observed in other foods.



PRECISION CHALLENGES



SMPR CHALLENGES - PRECISION

- Method precision depends on sample homogeneity, particle size, test portion size, and extraction solvents/conditions.
- Pre-extraction preparation of samples (grinding/homogenizing) will be critical for managing variation.
- Test portion sizes may be larger than usual
- RSD criteria may be dependent on mean
- RSD Criteria for oats may be larger than usual, even for ELISA methods

SMPR CHALLENGES - RECOVERY

- Accuracy/ “Trueness” in gluten analyses has always been a difficult concept to formally define and experimentally estimate.
- In the past, barley has always been a minor consideration for choosing antibodies.
- How to develop criteria to evaluate relative responses of wheat, rye and barley?
- Criteria for kit response (extraction + antibody) should be evaluated individually for 3 grains.

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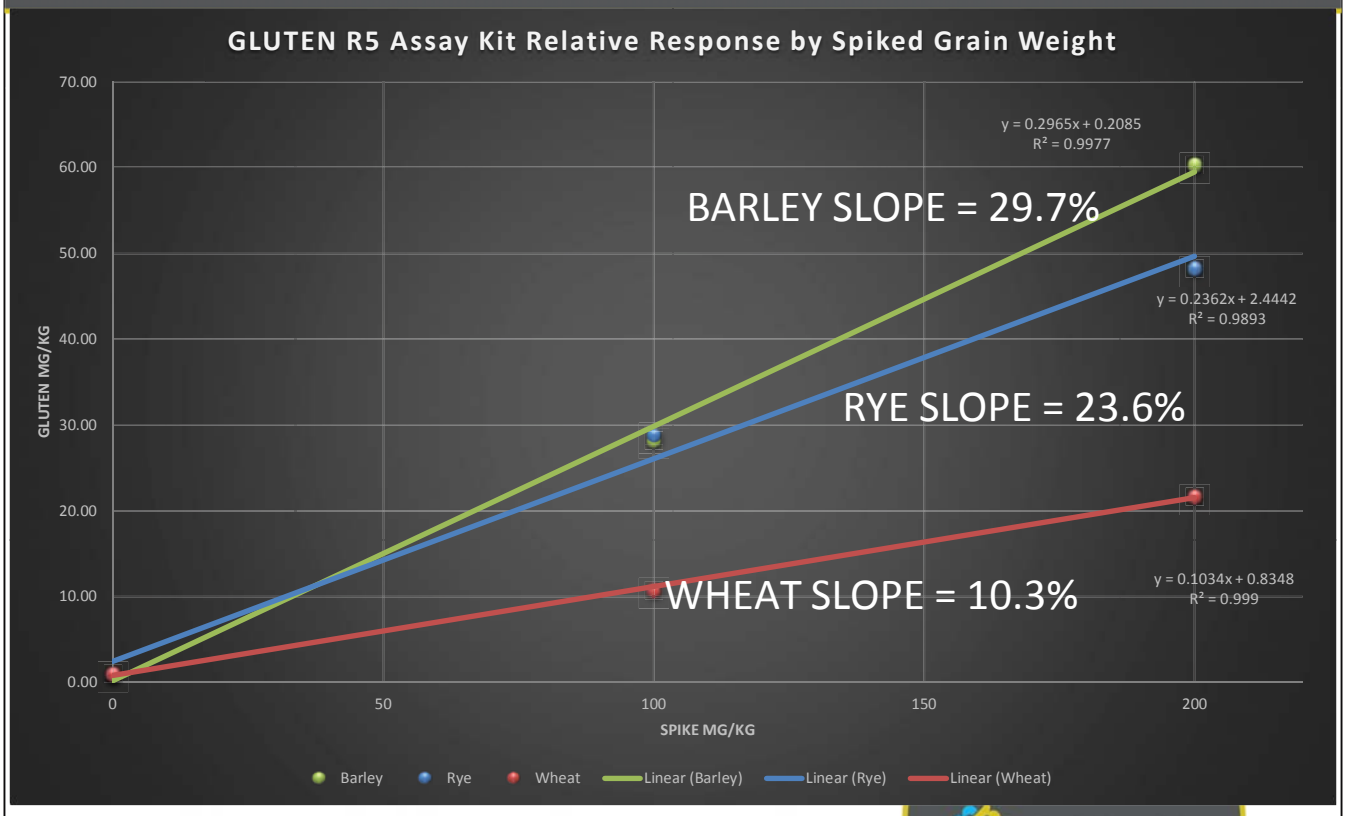


Medallion Labs
Trust The Minds of Medallion.

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Tracy Mui, Ph.D.
PepsiCo

Tracy Mui is a Manager in the Analytical Sciences group of PepsiCo where she manages the team which supports the Quaker, Tropicana, Naked Juice, and Gatorade brands. She also focuses on projects for food and beverage products and ingredients, including rapid method development and investigating new technologies that allow the lab to work more safely and efficiently. Tracy is also actively engaged with AOAC and holds a leadership role in TCJJP, where she keeps up-to-date with new concerns, methods and technologies in the industry.

PEPSICO

AOAC gluten working group meeting: PepsiCo's published data on gluten analysis in oats

Tracy Mui, R&D Analytical Sciences, Manager
Yumin Chen, R&D Analytical Sciences, Principal Scientist
Ron Fritz, R&D Statistics, Sr. Principal Scientist
Prabhakar Kasturi, R&D Analytical Sciences, Director

March 14, 2017

The views expressed in this presentation are those of the authors and do not necessarily represent position or policy of PepsiCo, Inc.

Non-compliance to FDA gluten regulation (< 20 ppm) is seen in oatmeal servings on the markets

	Product	# of Date codes	Qty. of servings tested		BLQ (<5 ppm)	5–20 ppm	20–80 ppm	>80 ppm	Positives (>5 ppm)	Observed non conforming (≥20 ppm)
7/14 Assessment (Internal Laboratory tested)	Gluten-free oatmeal from Producer #1	5	228	Count	216	7	4	1	12	5
				% of Total	94.74%	3.07%	1.75%	0.44%	5.26%	**2.19%**
	Gluten-free oatmeal from Producer #2	2	101	Count	95	4	2	0	6	2
				% of Total	94.06%	3.96%	1.98%	0.00%	5.94%	**1.98%**
	Gluten-free oatmeal from both	7	329	Count	311	11	6	1	18	7
				% of Total	94.53%	3.34%	1.82%	0.30%	5.47%	**2.13%**
12/14 Assessment (indep. Laboratory tested)	Gluten-free oatmeal from Producer #1	6	316	Count	302	6	5	3	14	8
				% of Total	95.57%	1.90%	1.58%	0.95%	4.43%	**2.53%**
	Gluten-free oatmeal from Producer #2	8	320	Count	304	14	0	2	16	2
				% of Total	95.00%	4.38%	0.00%	0.63%	5.00%	**0.63%**
	Gluten-free oatmeal from both	14	636	Count	606	20	5	5	30	10
				% of Total	95.28%	3.14%	0.79%	0.79%	4.72%	**1.57%**
Both studies	Internal and external studies combined	21	965	Count	917	31	11	6	48	17
				% of Total	95.03%	3.21%	1.14%	0.62%	4.97%	**1.76%**

Bold values indicate % servings noncompliant.

R. Fritz, Y. Chen. Kernel-based gluten contamination of gluten-free oatmeal complicates gluten assessment as it causes binary-like outcomes. Journal of Food Science and Technology, 2017, 52 (2), 359-365

Re-test of samples with compliant gluten reading reveal non-homogeneous distribution post grinding.

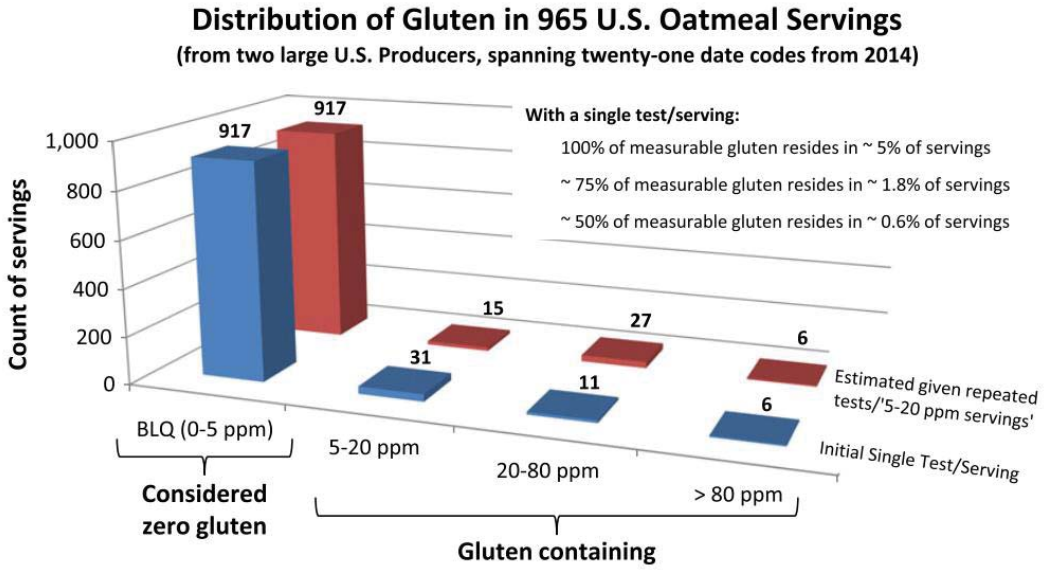
Re-test of samples with >5 ppm and <20 ppm gluten content reveal non-homogeneous distributions of gluten post grinding.

	Retests of in-market oatmeal finished goods (For samples found positive for gluten on 1st '0.25 g Test' but compliant, i.e., >5 and <20 ppm)												
	Original 1st 0.25 g Test Result	1st Retest Result	2nd Retest Result	3rd Retest Result	4th Retest Result	5th Retest Result	6th Retest Result	7th Retest Result	8th Retest Result	9th Retest Result	10th Retest Result	Resultant Avg. (n = 11)	Range of Outcomes
1	6.5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	2	7
2	6.6	14	10	13	7	8	8	39	BLQ	15	36	15	36
3	6.8	BLQ	6.5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	7.5	3	8
4	7.5	13	14	13	14	48	20	13	13	42	>160	>33	>153
5	7.7	34	16	14	6	20	8	BLQ	9	6.5	10	12	34
6	8.5	BLQ	BLQ	BLQ	7.5	BLQ	BLQ	BLQ	>160	13	BLQ	>18	>160
7	9.0	8.5	6.5	9.5	6	7	9	14	9	13	19	10	13
8	9.7	63	79	29	30	>160	31	30	70	65	>80	>59	>150
9	10.0	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	3	10
10	10.0	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	3	10
11	10.0	17	8	9.5	13	9.5	13	9	7	12	20	12	13
12	10.5	14	21	18	61	19	13	8.5	16	38	9.5	21	53
13	11.0	17	28	9.5	14	9.5	78	9.5	>80	24	12	>27	>71
14	12.5	15	8	BLQ	10	9	9	11	9	14	>160	>24	>160
15	13.0	BLQ	BLQ	BLQ	33	13	6	107	146	BLQ	87	38	146
16	13.4	31	25	18	26	14	32	75	27	20	52	30	62
17	15.0	24	17	18	21	15	13	9	8	17	10	15	16
18	15.5	36	40	18	16	14	7.5	76	42	14	6.5	26	70
19	16.0	BLQ	BLQ	6.5	BLQ	BLQ	>160	BLQ	BLQ	BLQ	BLQ	>19	>160
20	18.0	54	26	24	118	>160	24	28	28	95	39	>56	>142

%Averaging ⩾ 20 ppm 45–55%

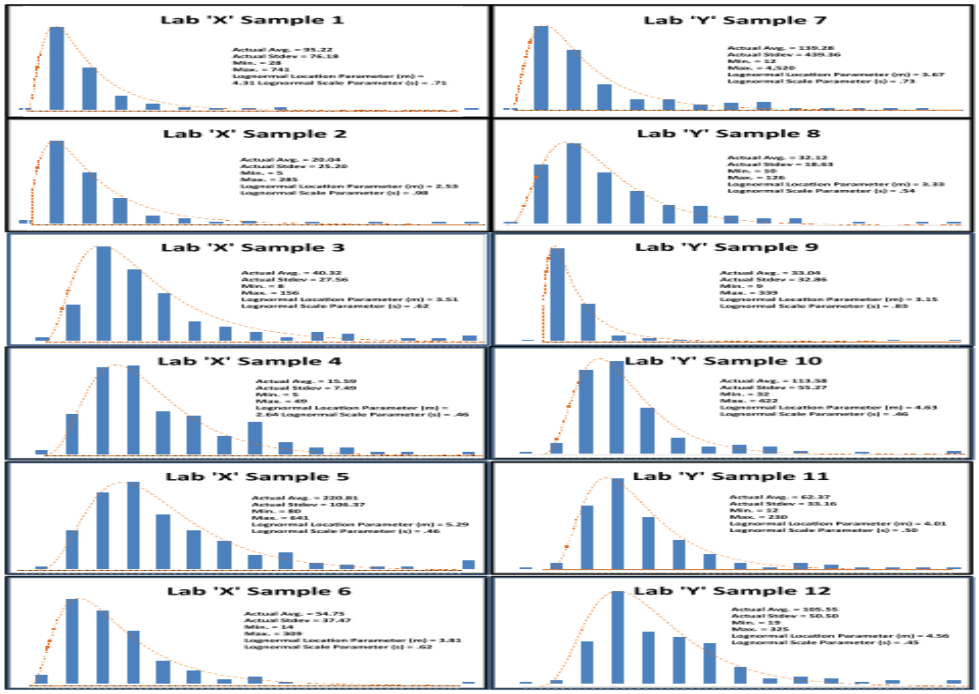
R. Fritz, Y. Chen, V. Contreras. Gluten-containing grains skew gluten assessment in oats due to sample grind non-homogeneity. Food Chem, 2017, Vol 216, 170-175

Distribution of gluten in 965 U.S. oatmeal – experimental data vs. adjustment after factoring in re-test results



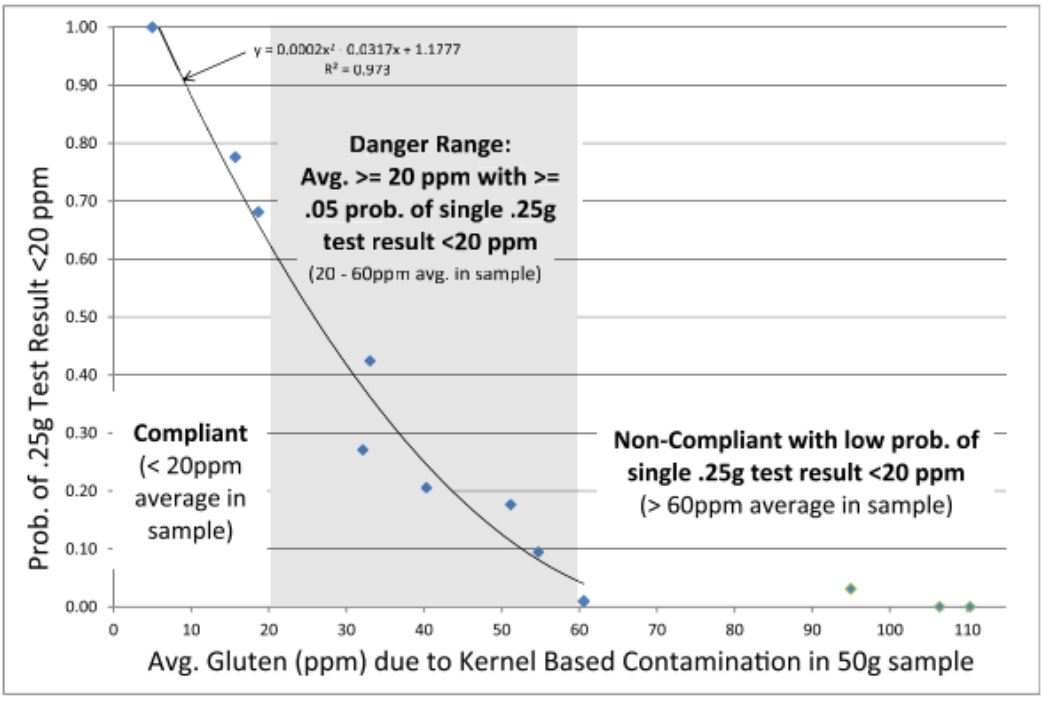
R. Fritz, Y. Chen. Kernel-based gluten contamination of gluten-free oatmeal complicates gluten assessment as it causes binary-like outcomes. Journal of Food Science and Technology, 2017, 52 (2), 359-365

Skewed log normal distribution of 0.25-g gluten test results from 'wheat spiked' 50-g samples (when 100% is assessed)



R. Fritz, Y. Chen, V. Contreras. Gluten-containing grains skew gluten assessment in oats due to sample grind non-homogeneity. Food Chem, 2017, Vol 216, 170-175

Probability that a 0.25 g test result reads <20 ppm for various average contamination rates in the host sample.



R. Fritz, Y. Chen, V. Contreras. Gluten-containing grains skew gluten assessment in oats due to sample grind non-homogeneity. Food Chem, 2017, Vol 216, 170-175

Estimated gluten in a serving (40 g) of oats containing a kernel of North American varieties of wheat

	Hard Red Winter	Hard Red Spring	Soft Red Winter	Soft White	Northern Durum	Desert Durum
Thousand kernel weight (g)*	29.1	30.4	32.6	35.3	39.2	48
Weight/kernel (g)	0.0291	0.0304	0.0326	0.0353	0.0392	0.048
% Protein*	12.7	14.1	10	10	13.5	13.4
% Gluten level in protein†	80	80	80	80	80	80
Gluten content in 40 g of oats containing 1 wheat kernel (ppm)	74	86	65	71	106	129

*Five year average values reported in 2015 Crop Quality Report by US Wheat Associates, http://www.uswheat.org/cropQuality.
†Shewry (2009).

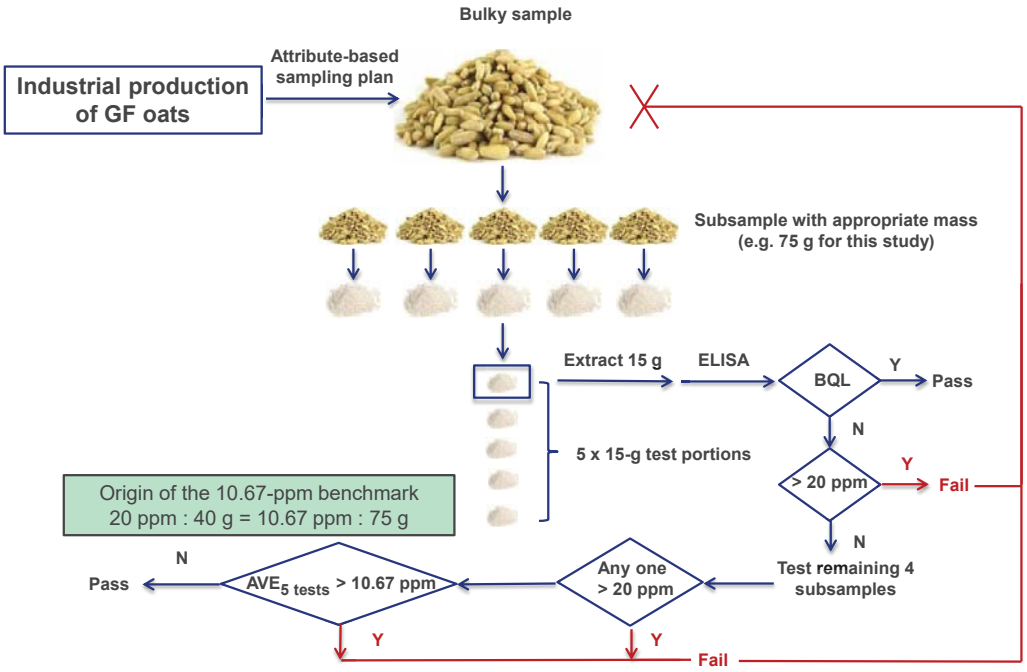
R. Fritz, Y. Chen. Kernel-based gluten contamination of gluten-free oatmeal complicates gluten assessment as it causes binary-like outcomes. Journal of Food Science and Technology, 2017, 52 (2), 359-365

Probabilities of randomly selecting one or more servings with a gluten-containing kernel

Assumed rate of servings with a gluten-containing kernel	Probability of selecting one or more contaminated servings in: 1 Try	2 Tries	3 Tries	4 Tries	5 Tries	10 Tries	25 Tries	50 Tries	# of servings' worth required to obtain 95% confidence defect rate to left is not exceeded (where all would need to be found 'clean') (found using attribute acceptance sampling)
1 in 10	0.1000	0.1900	0.2710	0.3439	0.4095	0.6513	0.9282	0.9948	29
1 in 15	0.0667	0.1289	0.1870	0.2412	0.2918	0.4984	0.8218	0.9682	44
1 in 25	0.0400	0.0784	0.1153	0.1507	0.1846	0.3352	0.6396	0.8701	74
1 in 50	0.0200	0.0396	0.0588	0.0776	0.0961	0.1829	0.3965	0.6358	149
1 in 100	0.0100	0.0199	0.0297	0.0394	0.0490	0.0956	0.2222	0.3950	298
1 in 200	0.0050	0.0100	0.0149	0.0199	0.0248	0.0489	0.1178	0.2217	599
1 in 500	0.0020	0.0040	0.0060	0.0080	0.0100	0.0198	0.0488	0.0953	1496
1 in 1000	0.0010	0.0020	0.0030	0.0040	0.0050	0.0100	0.0247	0.0488	2994

R. Fritz, Y. Chen. Kernel-based gluten contamination of gluten-free oatmeal complicates gluten assessment as it causes binary-like outcomes. Journal of Food Science and Technology, 2017, 52 (2), 359-365

A stepwise, 'test-all-positive' methodology to assess gluten-kernel contamination at serving-size level in gluten-free (GF) oat production



Information published @ https://www.glutenfreewatchdog.org/news/quaker-gluten-free-oatmeal-take-two/

References

1. R. Fritz, Y. Chen. Kernel-based gluten contamination of gluten-free oatmeal complicates gluten assessment as it causes binary-like outcomes. Journal of Food Science and Technology, 2017, 52 (2), 359-365

2. R. Fritz, Y. Chen, V. Contreras. Gluten-containing grains skew gluten assessment in oats due to sample grind non-homogeneity. Food Chem, 2017, Vol 216, 170-175

3. https://www.glutenfreewatchdog.org/news/quaker-gluten-free-oatmeal-take-two/

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Terry Koerner, Ph.D.
Health Canada
Regulatory Update

Labelling Regulations for Gluten Sources in Canada



Prepared by:

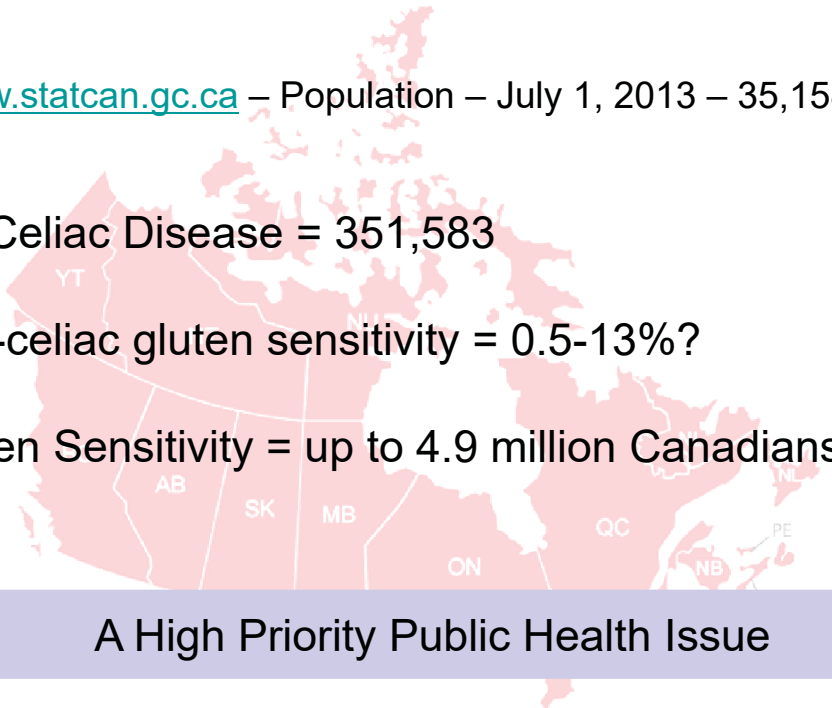
Michael Abbott and Terry Koerner
Bureau of Chemical Safety
Food Directorate
Health Products and Food Branch

Canada

Prevalence - Canadian Perspective

http://www.statcan.gc.ca – Population – July 1, 2013 – 35,158,300

- 1% Celiac Disease = 351,583
- Non-celiac gluten sensitivity = 0.5-13%?
- Gluten Sensitivity = up to 4.9 million Canadians



A High Priority Public Health Issue




Regulatory Amendments

"Gluten" Defined

Any gluten protein from the grain of any of the following cereals or the grain of a hybridized strain created from at least one of the following cereals:

- Wheat
- Oats
- Barley
- Rye
- Triticale

Section B.01.010.1 (1) of the Canadian *Food and Drug Regulations*

Gluten-Free Regulation

- In Canada there is a specific section of the *Food and Drug Regulations* that deals with gluten-free claims
- Section B.24.018 of the Canadian *Food and Drug Regulations*
- This section was updated as part of the development of the enhanced labelling requirements for allergens, gluten sources and added sulphites

Gluten-Free Regulation

- Section B.24.018 was first enacted in Canada in 1995

- The initial gluten-free regulations in Canada read as follows :

No person shall label, package, sell or advertise a food in a manner likely to create an impression that it is a "gluten-free" food unless the food does not contain wheat, including spelt and kamut, or oats, barley, rye, triticale or any part thereof.

Gluten-Free Regulation

- Section B.24.018 was amended to the following :

It is prohibited to label, package, sell or advertise a food in a manner likely to create an impression that it is a gluten-free food if the food contains any gluten protein or modified gluten protein, including any gluten protein fraction, referred to in the definition of "gluten" in subsection B.01.010(1)

  - Under B.01.010(1) "gluten" means any gluten protein from the grain of any of the following cereals or from the grain of a hybridized strain that is created from at least one of the following cereals

    Barley, oats, rye, triticale, wheat

Gluten-free Regulation

Update to Section B.24.018 of FDR

- The new regulations are specific to the presence of gluten protein since it is known that it is the gluten protein that causes adverse reactions in people with celiac disease.
- Better reflects the current internationally adopted scientific description of ‘gluten’ (i.e.: CODEX)
- Allows companies that manufacture products that do not contain gluten protein to have the option of labelling them as gluten-free in Canada, even if sourced from gluten-containing grains
- A benefit to celiac patients, since it could lead to further expansion of the availability of healthy food choices for this group

Gluten-Free Regulation (Level)

Based on the available scientific evidence, Health Canada considers that gluten-free foods, prepared under good manufacturing practices, which contain levels of gluten not exceeding 20 ppm as a result of cross-contamination, meet the health and safety intent of B.24.018 when a gluten-free claim is made.

Does this level of 20 ppm gluten provide a safe level for Canadians with Celiac disease?

Scientific Basis for Gluten Policy

A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease[1–3]

Carlo Catassi, Elisabetta Fabiani, Giuseppe Iacono, Cinzia D'Agate, Ruggiero Francavilla, Federico Biagi, Umberto Volta, Salvatore Accomando, Antonio Picarelli, Italo De Vitis, Giovanna Pianelli, Rosaria Gesuita, Flavia Carle, Alessandra Mandolesi, Italo Bearzi, and Alessio Fasano

Am J Clin Nutr 2007;85:160–6. Printed in USA. © 2007 American Society for Nutrition

Conclusions: The ingestion of contaminating gluten should be kept lower than 50 mg/d in the treatment of CD. *Am J Clin Nutr* 2007; 85:160–6.

Scientific Basis for Gluten Policy

Alimentary Pharmacology & Therapeutics

Systematic review: tolerable amount of gluten for people with coeliac disease

A. K. AKOBENG & A. G. THOMAS

Conclusions

The amount of tolerable gluten varies among people with coeliac disease. Although there is no evidence to suggest a single definitive threshold, a daily gluten intake of <10 mg is unlikely to cause significant histological abnormalities.

Aliment Pharmacol Ther 27, 1044–1052

Scientific Basis for Gluten Policy

- Based on the best available information, it is anticipated that the majority of people with celiac disease will not be negatively affected if they limit their gluten intake to less than 10 mg per day.

- Exposure modelling was done based on 20 ppm gluten and a 10 mg per day limit.

Modelling Daily Gluten Exposure in a Gluten-Free Diet

- No information available on a “typical” gluten-free diet
- Instead, information on a typical Canadian diet was used
  - collected as part of “The Canadian Community Health Survey – Cycle 2.2 on Nutrition, Statistics Canada, 2004”
  - Used 24 hour dietary recall with 10786 respondents
  - Data was split into a variety of age/sex categories to reflect the fact that these groups typically eat different amounts
- The amount of gluten-containing grain products in the typical Canadian diet was analyzed using hypothetical levels of 5, 10, 20 and 50 ppm of gluten

Daily Gluten Exposure Estimate at 20 ppm Gluten

Table 1: Estimated Percentiles of Gluten Exposure (mg) assuming Gluten Contamination at a Level of 20ppm

Age-Sex Groups	24-hour Intake		Usual Intake	
	50th percentile	97th percentile	50th percentile	97th percentile
1 to 3 years	1.59	4.60	1.73	3.65
4 to 8 years	2.71	6.90	2.94	4.67
M - 9 to 13 years	3.30	8.48	3.75	5.66
F - 9 to 13 years	2.88	6.86	3.19	5.06
M - 14 to 18 years	3.67	10.12	4.13	7.12
F - 14 to 18 years	2.75	7.58	2.95	5.33
M - 19 to 30 years	3.35	10.31	3.71	6.30
F - 19 to 30 years	2.34	6.80	2.49	4.28
M – 31 to 50 years	2.89	8.95	3.31	6.47
F – 31 to 50 years	2.23	6.33	2.43	4.14
M – 51 to 70 years	2.70	7.10	2.86	5.36
F – 51 to 70 years	2.06	5.88	2.22	4.01
M – 71 or more years	2.51	6.75	2.72	4.81
F – 71 or more years	2.03	4.83	2.16	3.83
Overall	2.54	7.72	3.03	5.64

Conclusions

Modelling Gluten Exposure in a Gluten-Free Diet

- 20 ppm gluten as an action level for substitute foods labelled as gluten-free should support restriction of usual gluten intake to less than 10 mg per day.

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Article

Estimated Levels of Gluten Incidentally Present in a Canadian Gluten-Free Diet

Sébastien La Vieille [1,*], Sheila Dubois [2], Stephen Hayward [3] and Terence B. Koerner [4]

Marketing Authorization for Gluten-Free Oats

Safety of "Gluten-Free" Oats

- In 2007, Health Canada published a review of scientific literature which concluded that most people with celiac disease could safely consume limited amounts of gluten-free oats which did not contain gluten from wheat, rye and barley, or their hybridized strains. This position was reviewed and updated in 2015 with a conclusion that there is no requirement to limit daily consumption of gluten-free oats to specific amounts.
- Oats are a nutritious source of proteins, carbohydrates and especially fibre. Eating foods made with gluten-free oats provides a wider choice of grain and cereal-type foods for people with celiac disease. Sticking to a gluten-free diet can be a challenge because of limited food choices. Introducing oats to a gluten-free diet could help people better cope with this challenge.

Oats and Gluten-Free in Canada

- Oats are included in the list of gluten sources in Canada, under section B.01.010.1 of the Regulations (B.24.018 refers back to B.01.010.1)
- Until recently, products made with pure oats (that have been specially grown or processed to ensure there is no wheat, rye or barley in them) could not carry a gluten-free claim in Canada
  - Regular oats are generally contaminated by gluten-containing grains because of harvesting/transportation practices, etc.
- Pure oats have been allowed to make gluten-free claims in some countries
- CODEX includes oats in its list of gluten sources but also notes decisions on whether to allow gluten-free claims for pure oats can be made at the national level

MA for gluten-free claims on oats

- MAs are ministerial regulations which enable the Minister to implement certain food safety decisions by exempting from prohibitions found in either the *Food and Drugs Act* or the *Food and Drug Regulations*.
- Health Canada's Marketing Authorization (MA) permits the use of gluten-free claims for gluten-free oats (that do not contain more than 20 ppm of gluten from wheat, rye, barley, or their hybridized strains) and for foods containing these oats as ingredients, under certain conditions.

MA for gluten-free claims on oats

- The MA provides an exemption from specific sections of the *Food and Drug Regulations* (including the list of gluten sources in B.01.010.1 and B.24.018) provided that:
  - The food contains no oats other than specially produced "gluten-free oats";
  - The finished product does not contain greater than 20 ppm of gluten from wheat, rye, barley or their hybridized strains;
  - The food contains no intentionally added gluten from wheat, rye, barley, or their hybridized strains; and
  - The "gluten-free oats" are clearly identified as such in all cases where 'oats' are referenced, including in the list of ingredients.

Links on Gluten-Free Oats

Announcement of MA for gluten-free oats

http://www.hc-sc.gc.ca/fn-an/securit/allerg/cel-coe/avoine-gluten-oats-eng.php

Actual MA published in the *Canada Gazette* Part II

http://gazette.gc.ca/rp-pr/p2/2015/2015-06-03/html/sor-dors114-eng.php

Merci beaucoup

Thank You

お疲れ様

Danke

Gracias

Grazie

Dank u

谢谢你

Obrigado

Thanks




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INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Joe Boison, Ph.D.

Fitness-for-Purpose Discussion/Development

AOAC INTERNATIONAL
Food Allergens Assays "Egg" Working Group
SMPR Comments (as of February 3, 2017)

Item	Line		Comment	Response
Section: General				
1	-	Masahiro Shoji, Morinaga Institute	In order to interpret the analysis result, it will be useful to know the target protein and antibody information. For instance, casein test and Beta-lacto albumin test is not necessarily identical in milk analysis.	
2	-	Paul Wehling General Mills, Inc.	The concept of trueness for an ELISA method is difficult to define, let alone experimentally estimate. We did discuss this on the last call. I think it is important for the developer to evaluate and describe the protein sequences that their antibodies (or other agents) bind to.	
3		Diana Kavolais, Hershey	OMA Appendix M is a great tool. Let us use it to indicate where we need validation to answer the three basic challenges for ELISA for any allergen -	
4	-	Paul Wehling General Mills, Inc.	In terms of measuring trueness, there should be some attempt made to evaluate this.	
Section: Title				
5	4	Melanie Downs. Univ of Neb	By inserting "chicken" to describe the source of egg, it may unintentionally disqualify ELISA methods that detect chicken egg but also react with other bird eggs (e.g. duck, turkey, etc.). While the primary purpose of the methods would be to detect and quantify chicken egg, the SMPR should perhaps address what types and/or levels of cross-reactivity with other species will be acceptable.	The scope of the is chicken egg. Inserting the term "chicken" will not disqualify assays that can measure eggs from other species. The developer can still characterize the specificity of the assay and it's performance vis a vie other species of egg (e.g. turkey...)
6		Melanie Downs. Univ of Neb	The use of "whole" may imply that egg white and egg yolk should be detected equivalently. Most methods, however, would primarily detect egg white proteins, even when whole dried egg is use as the method calibrant. It may be beneficial to discuss and describe the extent to which methods are required to detect egg yolk and egg white fractions independently.	The objective pursued is the quantification of chicken egg regardless of the marker chosen by the assay to lead to such quantification. The antibody characterization will indicate the target(s).
7		Markus Lacorn, R-Biopharm	The title mentions ELISA but chapter 3. Analytical Techniques also mention “other binding based technologies”. Furthermore, we always detect proteins and but not allergens in all cases; these proteins may be allergens to sensitized customers; National legislations demand to declare “egg” and not egg allergens. Change title: Quantitation of whole chicken egg proteins by immunochemical methods	see section 4 – Definitions - Enzyme-linked immunosorbent assay (ELISA). This section will be expanded to include other binding technologies.
8		Sefat E Khuda, FDA	My suggestion: Quantitation of chicken whole Egg proteins by Antibody-based or Immunochemical Methods Because under analytical techniques, there are different techniques like ELISA or other binding based technologies. Guessing that, other binding based technologies are also utilizing antibodies like ELISA. Regulation requires labeling of egg based on the presence of egg proteins in food. ELISA against whole chicken egg detects both allergenic and non-allergenic proteins from egg.	The objective is to quantify the commodity chicken egg without being restrictive to the allergenic nature of the markers. Specifying ELISA-based markers leave the scope open using this technology using any marker of chicken egg see section 4 – Definitions - Enzyme-linked immunosorbent assay (ELISA)
9		Markus Lacorn, R-Biopharm	Why “whole” egg proteins? One method provider could also measure ovalbumin and recalculate this to whole egg.	The scope of the is chicken egg. Inserting the term "chicken" will not disqualify assays that can measure eggs from other species. The developer can still characterize the specificity of the assay and it's performance vis a vie other species of egg (e.g. turkey...)
10		Girdhari Sharma, US FDA	The title should be modified to reflect the analytical technique section. Also since ELISA is a binding-based assay, it may not be specified separately if using a broader definition such as binding-based methods. Is the SMPR meant for protein binding-based or covers other techniques such as PCR as well?	This SMPR is specific to ELISA-based technologies including other binding assays. It does not include PCR (which is another technology based on other principles), which may be addressed through another SMPR.
Section: Intended Use				
11	6	Michael Farrow, Abbott	Would it be necessary to strike Reference from intended use section? Wouldn't reference status be at the discretion of AOAC committees once a novel method is up for review?	The working group discussed and agreed to remove the term "reference" from the intended use section. Comment has been accepted and incorporated in the revised version.
12			Add “in the Food Industry.”	The working group discussed and agreed to add " . . . "food manufacturing" the intended use section.

Section: Applicabililty				
13		Terry Koerner, Health Canada	Considering that clinical results will be expressed in mg of total egg protein, the results of the assays should be in total chicken egg protein.	The SMPR will require to state a conversion factor in the reporting unit (table 1) between protein and commodity.
14		Laura Allred, GFCO/GIG	Section 2 lines 18-19. Change this section to read "Quantitation of whole chicken egg protein in selected food products and ingredients." Protein would be a more achievable and more easily standardized target than allergens, of which there may be many, and some of which may be unknown. This would remove the difficulty of defining "Allergen" as listed on line 38, and would lead to the removal of the statement that allergen should be reported by dry weight at the bottom of Table 1.	The objective is to quantify the commodity chicken egg without being restrictive to the allergenic nature of the markers. Commodity and mg of protein are both important units as such, though the SMPR is describing things in units of commodity it is also requiring that the method developer provide a clear and difinitive conversion factor so the end user can express the results the units in their own chosing. Consensus was achieved to draft the SMPR in commodity based concentration units.
15		Girdhari Sharma, US FDA	Protein may be more appropriate than allergens, OR quantifying the egg as a commodity. It may be easier to convert between total protein and total egg based on known protein content in egg. One of the problem with quantifying allergens is to separate non-allergens from total proteins since the reference material will most likely have total proteins. This makes it difficult to calculate recovery of allergens. If changes made, it would also be need to be in Applicability section and Table 1.	
16		Laura Allred, GFCO/GIG	While we may want to recommend priority matrices, we may not want to tell assay developers that they must validate their kit for a fixed set of matrices, so perhaps we could omit the reference to Table 2, or rename Table 2 as a list of priority matrices.	Line 106 counsels method developers to provide data for **claimed matrixes.** Method developers are not required to claim all matrices.
17	18	Laura Allred, GFCO/GIG	A section should be added to all validations that describes the method limitations. For instance, if a kit manufacturer realizes their egg assay works well for raw egg but not cooked, in addition to only validating the kit for unheated foods, there should also be a statement that the kit is not suitable for testing cooked products. Similarly if the kit works well in some matrices but not others, this should be stated in the validation, since labs tend to take AOAC methods and use them for every situation.	Statement will be inserted in the validation guidance to require submission of data on raw and/or cooked eggs.
18		Laura Allred, GFCO/GIG	Some current ELISA methods have been shown to have difficulty detecting or accurately quantitating cooked egg material. Do we want to allow manufacturers the option to validate their kit for one or the other? Or do we want to say it must be validated for both? That might mean changing the wording here to "Quantitation of cooked and raw whole chicken egg protein in selected food products and ingredients."	Statement will be inserted in section 8 - validation guidance to require submission of data on raw and/or cooked eggs.
19		Markus Lacorn, R-Biopharm	Applicability: If surfaces and cleaning in place solutions should be included this need to taken into account in the whole document Change: to be discussed by the group	It was agreed that this SMPR is restricted to food matrices another SMPR may be developed for other matrices related to sanitation practices (fluids…)
20		Michael Farrow, Abbott	Section 2 Line 18: Add environmental samples; This is vital as ELISA-based quantitative technologies are often part of the method validations for qualitative technologies such as lateral flow devices. It may be pertinent to validate cleaning through demonstrating an X-fold reduction in the specific antigens used at a facility. Surfaces with and without dilute cleaning solutions can be problematic matrices for antibody-based assays.	
21		Yumin Chen, PepsiCo	Cover environmental and sanitation samples (Proposed for group discussion) i. Because quantatitive ELISA will be used to calibrate the analyte used to validate qualitative method (Agreed by several group members). ii. If a food contains food allergen, the label should describe that. The most valuable use of an allergen method is acutally to assess allergen footprint in order to clear a production line to run a second non-allergen containing product.	
22		Virginie Barrere, Université Laval	If environmental samples are not mentioned, the intended use has to be changed to Method for food testing for example. If the intended use is for cGMPs compliance, environmental samples have to be included in this SMPR. cGMPs involve sanitation, cleaning and control of cross contamination.	

Section: Analytical Technique and ELISA Definition				
23	23	Michael Farrow, Abbott	Section 3 Line 23: Strike "based assays" and include "with consideration of other ligand binding technologies."	The working group discussed and agreed to add " . . . or related binding based techologies." Section revised.
		Laura Allred, GFCO/GIG	Section 3 lines 23-24. The group has agreed to open up this SMPR to include other binding-based assays. In Section 4 lines 29-30, we have defined ELISA as encompassing other ligand binding assays, but in line 29 we have kept the requirement for color change as being part of an ELISA method. There are other reporter systems, such as fluorescent markers, that are non-enzymatic and non-color based, but assays that use these reporters are still commonly called ELISAs. Do we want to either widen the definition of an ELISA, or alternatively remove it altogether and state that the applicability is for "Protein binding assays, such as ELISA"?	
24	29	Markus Lacorn, R-Biopharm	Definition “ELISA”: Several formats are possible for ELISA: 1. Sandwich: analyte ligating agent is bound on surface and second analyte-ligating reagent is coupled to an enzyme: sandwich format 2. Analyte from calibrator or sample is coupled to surfaces (by the user) and ligating agent is coupled to enzyme: calibration curve like sandwich curve 3. Fixed amount of analyte is bound to surface and competes with free analyte in solution for ligating binding sites; this ligand is labelled to a marker (not only an enzyme); competitive format 4. Ligating reagent is coupled to surface: free analyte (from calibrator or sample) is competing with a fixed amount of labelled analyte for binging sites: competitive format These are only examples for microtiterplate based methods; LFD devices are even more complex and other systems may also be Change: Delete ELISA and insert new definition: “Binding-based assays: Antigen or ligand based methods where one of the components is attached to a surface. The measurement signal is directly or inversely proportional to the amount of measurand. ” This will also include quantitative LFDs or dip-sticks.	Definition was amended to be broader.
25	29	Michael Farrow, Abbott	Rewrite ELISA as follows: An assay that uses an immobilized solid phase component, antigen-antibody interactions, and color change to identify a substance. (Strike the rest); (4) Ligand-Binding Assay (definition to be determined)	
26	39	Markus Lacorn, R-Biopharm	Definition “allergens”: Do not define allergens because this is quite broad (they may derive from food but also from dust) but explain for “whole egg” that egg constituents may be allergenic to consumers for an individual extent	It was agreed that allergens will not be the target of methods. "Whole" was removed from the target analyte.
27	44	Melanie Downs. Univ of Neb	It would be useful to give some thought as to whether definitions for both "allergens" and "commodities" are necessary. If "allergens" are to be defined as allergenic source foods (similar to how most regulations define food allergens), then a definition for commodities may create additional confusion.	Both definitions were removed. Rely on dictionary definition for "allergy". The term "commodities" is not used in the new version of the SMPR.
28	44	Markus Lacorn, R-Biopharm	Definitions “commodities”: If surfaces and CIP water are included, commodities would not be sufficient as a definition; you may call them matrices but at the end we need to define matrices (food, CIP water) and surfaces	
29	76	Terry Koerner, Health Canada	Considering this is for egg should we be more specific in our definition?	The definition for "egg" was clarified.
Section: Definition- LOQ / LOD				
30	46	Paul Wehling General Mills, Inc.	Per LOD/LOQ definitions in the SMPR - I note that Appendix M includes these definitions. We should harmonize the SMPR to Appendix M. LOD is defined as the lowest concentration or mass of analyte in a test sample that can be distinguished from a true blank sample at a specified probability level. LOQ is the lowest level of analyte in a test sample that can be reasonably quantified at a specified level of precision.	Agreed and amended according to Appendix M. Specific requirements are set for recovery in Table 1 For a detailed discuss of false positive & false negative probability (see reference 10 in Appendix M)

31		Markus Lacorn, R-Biopharm	Definiton: LoQ is not defined in an acceptable way since sufficient precision and acceptable recovery should be mentioned. The terms “sufficient” and “acceptable” depends on the method developer and shall be stated with numbers. Change: to be discussed by the group	The Appendix M definition was used for LOQ.
32		Girdhari Sharma, US FDA	Line 50, LOD: would this be 90 or 95% certainty? LOD calculation is presented in Appendix M. Can the false-positive at minimum concentration of analyte be distinguishable from true-positive expected at the LOD concentration? The false-positive would be due to matrix interference.	Appendix M guidance was used, the certainty level will be specified in Table 1
32	50	Lisa Monteroso (3M)	Include proposed LOD text and strike MDL;	Agreed.
33		Terry Koerner, Health Canada	Same as LOQ. Be more specific to total egg protein	Egg comment was revised to be more specific
34		Lisa Monteroso (3M)	The statement on allowing spikes at no less than LLA x2 or less is confusing when you truly need to have the spiking done at the LOD and LOQ to confirm the LOD and LOQ.	LOD/LOQ were revised in accordance with Appendix M.
35		Lisa Monteroso (3M)	LOD should be estimated by a statistical analysis of the calibration data according to the ISO	
36		Lisa Monteroso (3M)	Add LOD ISO references: standard ISO 11843-2 (6) for linear data, or ISO 11843-5 (7) (Cut and paste from Appendix M)	
Section: Definition - Reproducibility				
37	67	Markus Lacorn, R-Biopharm	Definitions: mention that reproducibility is only characterized when a collaborative test was performed Change: include collaborative tests in the definition	There are actually other ways to collect reproducibility data other than collaorative study. RSDR can be calculated using proficiency data or a combination of collaborative and proficiency data.
Section: Definition - Recovery				
38	69	Markus Lacorn, R-Biopharm	Definition “recovery”: Recovery may be characterized by spiking experiments because incurred materials are not available; this SMPR should allow spiked samples if there is no other possibilities; incurred should be preferred in any case	Addressed in section 8 - validation guidance
39		Yumin Chen, PepsiCo	Spiking – According to the AOAC appendix M, the best spiking samp	
Section: Definition - Whole Egg				
40	76	Melanie Downs. Univ of Neb	The definition given in this section seems too specific to a particular product for the purposes of this SMPR. The definition given is that of refrigerated liquid whole eggs, as defined by the USDA FSIS. Given the complicated regulatory authority for eggs in the United States (i.e. the FDA regulates in shell eggs, while the USDA FSIS regulates egg products), it may be difficult to apply a regulatory definition of whole egg for the purposes of this SMPR. (The FDA also does not have a regulatory definition for "eggs", per 21 CFR 160.100.) It would be beneficial for this working group to agree upon a simple definition for egg that suits the purposes of the SMPR.	The definition was adjusted to refer to consider egg powder as the basis for reference material used in practical food testing
41		Terry Koerner, Health Canada	We should be clear on how this whole egg definition, which comes from a food inspection service will carry over to the reporting units of total egg protein.	Agreed and considered in the requirement set for Table 1.
42		Virginie Barrere, Université Laval	Yes. FAO CXP_015e Pasteurization – a microbiocidal control measure where eggs or egg products are subjected to a process, using heat to reduce the load of pathogenic microorganisms to an acceptable level to ensure safety.	The working group agreed to remove the term "pasterurized".
43				
Section: System Suitability				
44	81	Markus Lacorn, R-Biopharm	System suitability: Quantitative ELISA systems always contain calibrators therefore it is not necessary to deliver an additional check sample; instead: It is recommended that every user of these kits establish his own control samples that fits his needs best.	Agreed, draft amended accordingly.
45	84	Terry Koerner, Health Canada	Appendix M is clear about what the testing levels should be.	Agreed, draft amended accordingly.
46	85	Laura Allred, GFCO/GIG	Section 6 (line 85). Would this section be a good place to list required cross-reactivity checks, perhaps by referencing Table 1 of Appendix M?	Agreed, draft amended accordingly.
Section: Reference Materials				
47	85	Markus Lacorn, R-Biopharm	Reference Materials: Delete LGC materials since they are produced by a lab which is NOT ACCREDITED according to ISO Guides! Please refer to the “certificates” this lab delivers (only ISO 9001 is mentioned). Certifcates are available on request. The NIST materials are valuable.	Agreed, draft amended accordingly.

Section: Validation Guidance				
48		Girdhari Sharma, US FDA	Validation Guidance: As discussed in the meeting, there should be provision for additional matrices if desired.	This document does not prohibit a method developer from designing assays for additional matrices.
49		Terry Koerner, Health Canada	Appendix M requires two or three matrices in the initial study.	Performance will be reported based on matrices claimed.
50		Masahiro Shoji, Morinaga Institute	AOAC food allergen activities have already lots of archives (wisdoms of ancestors) as Appendix D, F & M. We shall utilize them as the basis because these were the results of intensive discussion of the experts. What we concentrate to do is to unify them, harmonize with them and input the most recent information.	Agreed, documents was amended accordingly.
51		Diana Kavolais, Hershey	Also the extent of cross reactivity needs to be reported in the test kit instructions.	Agreed, documents was amended accordingly see section 6.
52		Diana Kavolais, Hershey	Somewhere in this document we need to provide method instruction requirements.	Document was amended to state requirements related to system suitability that characterized the assay as per Appendix M. Instruction requirements are not included in the SMPR/OMA.
53	101	Diana Kavolais, Hershey	Some test kit manufacturers have reported cross reactivity results but they either do not test full strength extracts (they dilute 1:10, and then say there is none there) or, they consider no cross reactivity if it is below the LOQ, however it is above the LOD. That is a problem we have to address with this SMPR.	Cross reactivity requirments are set per Appendix M guidance.
54		Diana Kavolais, Hershey	The method performance is influenced as much by the clarity of instructions as well as the supporting validation data that needs to be used in to interpret results.	Agreed.
55		Diana Kavolais, Hershey	Validation study design should have standard data packet disclosure requirements and should be provided to customers prior to purchase of the kit.	Package insert review not requirement in OMA.
56		Diana Kavolais, Hershey	Spiking with purified proteins is a practice that ELISA kit manufacturers use, but foods are not purified protein which makes those studies somewhat misleading. That therefore means the requirements for kit instructions must include the details of the spiking design not just the results.	Appendix M used as guidance for recovery criteria
57		Diana Kavolais, Hershey	Appendix M indicates that validation be done in replicate on matrices without egg (zero), at the lower end of the calibration curve (this would include the region between the zero standard and the lowest positive standard) and evenly distributed throughout the range of the calibration.	Appendix M used as guidance for recovery criteria
Section: Maximum Time to Results				
58	124	Diana Kavolais, Hershey	Expect result within 4 hours of starting sample extraction time.	The working group thought that this is more of a marketing issue then a performance issue and agreed to remove the section.
59		Markus Lacorn, R-Biopharm	Maximum Time-to-Result: Customers will not accept an assay e.g. with incubation times over night.	
Section: Table 1 General				
60	126	Lisa Monteroso (3M)	Is whole egg referred to as allergen here (Note)? If not, reporting allergen from the material used in incurred samples would be difficult and vary depending on the antibody specificity.	The scope of the methods should target any marker of egg. Draft amended to be more precise as to the method reporting.
61		Lisa Monteroso (3M)	Incurred vs spiked?	Followed recommendation of Appendix M; incurred.
Section: Table 1 Matrixes				
62	126	Girdhari Sharma, US FDA	Table 1: What is Matrix X and why separated from other matrices?	"Matrix X" was removed.
63		Yumin Chen, PepsiCo	Create a category of alcohol containing beverage so that wine and eggnog can both go under	Agreed, category for wine was created.
64		Markus Lacorn, R-Biopharm	Table 1. Instead of defining commodities we could separate the table into low-processed samples (e.g. salad dressing, dough, ice cream) and high-processed samples (e.g. bread, cookies, pasta): not fixed number decision by method developer New Table 1 will be sent separately to Delia	Followed guidance from Appendix M for matrices.
64		Melanie Downs. Univ of Neb	Table 2: Adjust matrix types to general food categories: e.g. Baked Goods, Beverages (Non-alcoholic and alcoholic), Environmental Samples; Meats and Processed Foods, etc. Include examples within each category.	SMPR restricted to food matrices.

Section: Table 1 LOD /LOQ				
65		Girdhari Sharma, US FDA	As pointed in the meeting, LOD is typically lower than LOQ.	LOD/LOQ definitions adjusted as per Appendix M. Values set were identified to be suitable in the context of egg testing.
66		Yasutaka Nishiyama, NH Foods Ltd.	Because LOD is usually lower than LOQ, minimum acceptance criteria should be "<5", rather than "<10". In parameter column, "MDL" should be "LOD".	
67		Paul Wehling General Mills, Inc.	For LOD/LOQ, LOD criteria should be lower than the LOQ criterion. I'd expect the procedures described in Appx M to be used.	
68		Diana Kavolais, Hershey	The LLA (lowest level of application) term used in Appendix M suggests that the kit manufacturer can say that the kit is not applicable at the LOD and LOQ, but if that is the case, then the LOD and LOQ should be changed to match the LLA.	
69	126	Diana Kavolais, Hershey	I suggest the importance of LOD (MDL) should have it be at the top of the parameter list. Some food manufacturing companies look there and no further for most allergen methods. While LOQ and Range of Quantitation are important, LOD determines whether the assay is sensitive enough and applies to the matrices.	LOD/LOQ definitions adjusted as per Appendix M. Values set were identified to be suitable in the context of egg testing.
70		Melanie Downs. Univ of Neb	MDL should be changed to LOD, and the minimum value should be changed. (The LOD should be less than the LOQ.) It may also be worthwhile to discuss the actual utility and applicability of requiring an LOD for a quantitative method. Results below the LOQ and above the LOD often cause additional confusion for end users, as it then becomes difficult to interpret the information and evaluate the risk associated with such a result when quantitative information is lacking.	LOD/LOQ definitions adjusted as per Appendix M. Values set were identified to be suitable in the context of egg testing.
71		Terry Koerner, Health Canada	In my opinion we need to assess the level requirements differently based on consumption information and known clinical information. A 5 ppm level may be fine for some matrices, but it may be inadequate for others (bread, drinks, etc...)	LOD/LOQ definitions adjusted as per Appendix M. Values set were identified to be suitable in the context of egg testing.
Section: Table 1 Recovery Criteria				
72		Yasutaka Nishiyama, NH Foods Ltd.	According to Appendeix M, "recoveries between 50 and 150% will be considered acceptable" for incurred samples.	Appendix M used to define.
73		Laura Allred, GFCO/GIG	Table 1. Acceptable recovery % is skewed towards false negatives, which would not be preferable for public safety. I didn't see it in Appendix M, but i believe the Abbott paper recommended a recovery range of 50-150%, and many manufacturers have operated based on this. It would be nice to tighten this range, recognizing that it can be difficult to get excellent recovery across multiple matrices with a kit that has one extraction buffer and extraction protocol. Can we review recent PTMs and OMAs for ELISA methods and see if recoveries closer to 75-120% are realistic?	
74	126	Paul Wehling General Mills, Inc.	Criterion for recovery should be symmetrical about 100%, e.g., 60-140%	Appendix M used to define.
75		Markus Lacorn, R-Biopharm	Why do we need an analytical range? A possible user may decide if an analytical range is broad enough. At the moment the LoQ (or sometimes also LoD) is of most interest since we are only interested in presence or absence. This may change when threshold values will be installed (comparable to gluten). Change: Delete the analytical range from the table	Appendix M used to define.
76			Change "recovery" to "mean recovery" otherwise precision would not be necessary any longer	
Table 1: Units				
77	126	Melanie Downs. Univ of Neb	Table 1: The concentration units in this table should be much more specific. The note at the bottom of the table is more confusing than helpful in this regard. The units of "ppm" really must be clearly described somewhere, for example: "ppm indicates mg whole dried egg per kg product". The note at the bottom of the table could be interpreted to mean that the units should be expressed on a dry weight basis (i.e. mg whole dried egg per kg dry weight product), which we would not want. There should be some requirements added regarding specificity and cross-reactivity. (Referring to OMA Appendix M may be sufficient.)	Reporting requirements were adjusted.
78		Michael Farrow, Abbott	Part per million (ppm): microgram of detected food antigen per gram of protein.	Reporting requirements were adjusted.

Section: Table 1: Analytical Range				
79	126	Laura Allred, GFCO/GIG	Table 1. Analytical range should be more in the range of 5 ppm whole egg protein for most products (with special requirements as needed for other matrices such as wine). Most kits on the market now have an LOQ below 1 ppm egg protein, and this range would be more in scale with the VITAL reference dose/action level system. Do we want to provide conversions here to whole liquid egg, liquid egg whites or egg white protein (e.g.	Analytical range was set based on practical application of egg testing System suitability mandated the provision of all the required information related to correlation between reported levels and egg proteins
80		Melanie Downs. Univ of Neb	For the top end of the analytical range, it might be more clear to state something like, "at least ten times the LOQ", rather than just ">10", which could lead to somewhat narrow analytical ranges (e.g. 3-13 ppm whole dried egg).	The minimum requirement as set allow for a flexible analytical range.
81		Michael Farrow, Abbott	Table 1: Analytical Range: 0.5-500 ppm; LOQ: 0.5 ppm; LOD: 0.1 ppm; Recovery: 60-140%; Small r RSD: 15% ALSO these values should be adjusted to the food matrices that are being considered and should be adjusted for typical serving sizes. (Multiple tables may be necessary especially with the inclusion of environmental samples)	Minimum requirements set for these parameters satisfy egg testing needs. SMPR has been amended to require the kit manufacturer to clearly state the intended use and claimed matrices.
Section: Table 1: Specificity / Crossreactivity				
82		Virginie Barrere, Université Laval	If specifity is added, should cross reactivity be tested as well and a list of food matrices be added for this purpose?	system suitability section 6, now requires to list cross reactivity of the assay as per Appendix M recommendations
83		Michael Farrow, Abbott	Where would information on Cross-Reactivity be captured?	system suitability section 6, now requires to list cross reactivity of the assay as per Appendix M recommendations
84	126	Markus Lacorn, R-Biopharm	Table 2: Chicken is a possible cross-reacting commodity that needs to be characterized (see also AOAC Guidelines by Abbott et al.). To be discussed in the group if the list stated in the Abbott paper is sufficient.	Current requirements are minimum requirements and could be expanded upon
85	126	Diana Kavolais, Hershey	Add specificity/cross reactivity as a method performance requirement. The ELISA kit should report that versus Table 1 or a more relevant subset thereof for egg. Appendix M indicates to test based on full-strength extracts.	system suitability section 6, now requires to list cross reactivity of the assay as per Appendix M recommendations
86	27	Yumin Chen, PepsiCo	Definition – Specificity: need to connected to the binding technology that implied by the ELISA definition.	Captured in amendments to system suitability section 6 per Appendix M
87		Lisa Monteroso (3M)	Add a table 3 for cross-reactivity test materials.	system suitability section 6, now requires to list cross reactivity of the assay as per Appendix M recommendations
88		Paul Wehling General Mills, Inc.	Per discussion of including cross-reactivity in the SMPR. Appendix M does discuss this. Perhaps good language in the SMPR would be such like - "Cross-reactivity has been investigated as per Appendix M. Method developers should submit cross-reactivity data and include any notable observations." or something like that.	System suitability section 6, now requires to list cross reactivity of the assay as per Appendix M recommendations
Section: Table 2: Matrices				
89	131	Yasutaka Nishiyama, NH Foods Ltd.	These matirices are acceptable to be included in the table.	Matrices listed in table 2 are examples of recommended matrices for egg testing as per guidance from Appendix M (kit manufacturers can select amongst these matrices or beyond as needed)
90	131	Markus Lacorn, R-Biopharm	Delete Table 2 and include examples in Table 1 as described in another comment	Matrices listed in table 2 are examples of recommended matrices for egg testing as per guidance from Appendix M (kit manufacturers can select amongst these matrices or beyond as needed)
91	131	Diana Kavolais, Hershey	Can we combine some of M table 2 into categories as well as add new categories? 1) Raw and Cooked Baked Goods - (dough, bread, cookies), 2) Raw and cooked Meat - chicken, sausage. 3) Pasta - dry and fresh (refrigerated). 4) Confectionery - Dark Chocolate Ovomaltine (high tannin) - an ingredient used in confections, cookies, beverages - used worldwide - and formulations may or may not contain egg, nougat, marizpan. marshmellow), Ice Cream, 5) Fluids - Salad dressing, Milk beverages (soy).	Matrices listed in table 2 are examples of recommended matrices for egg testing as per guidance from Appendix M (kit manufacturers can select amongst these matrices or beyond as needed)
92		Diana Kavolais, Hershey	Expanding to include matrices that answer to Appendix M's statement on factors that influence test results (1) interactions with compounds in food (polyphenols and tannins). Raw and cooked - address	System suitability section 6, now requires to list cross reactivity of the assay and information on matrices (difficulties encountered) as per Appendix M recommendations

DRAFT AOAC Allergen SMPR Version 6; March 13, 2017

Quantitation of Chicken Egg by ELISA-based* Methods

Intended Use: Method for quantitation of chicken egg in the context of food manufacturing.

1. **Purpose:** AOAC SMPRs describe the minimum recommended performance characteristics to be used during the evaluation of a method. The evaluation may be an on-site verification, a single-laboratory validation, or a multi-site collaborative study. SMPRs are written and adopted by AOAC Stakeholder Panels composed of representatives from the industry, regulatory organizations, contract laboratories, test kit manufacturers, and academic institutions. AOAC SMPRs are used by AOAC Expert Review Panels in their evaluation of validation study data for method being considered for *Performance Tested Methods* or AOAC *Official Methods of Analysis*, and can be used as acceptance criteria for verification at user laboratories.

2. **Applicability**:
   Quantitation of chicken egg in one or more food(s) such as those listed in OMA Appendix M (see Table 3). Add Journal reference

3. **Analytical Technique**:
   Enzyme-linked immunosorbent assay (ELISA)-based assays (see definition in section 4).

4. **Definitions**:

   Enzyme-linked immunosorbent assay (ELISA)
   For the purposes of this document, ELISA is defined as "an analytical procedure characterized by the recognition and binding of specific antigens by antibodies"[1]. This definition is not meant to be restrictive and encompasses other related binding based technologies.

   Limit of Detection (LOD) (reference Appendix M)
   LOD is defined as the lowest concentration or mass of analyte in a test sample that can be distinguished from a true blank sample at a specified probability level**.**

   Limit of Quantitation (LOQ) (reference Appendix M)
   LOQ is the lowest level of analyte in a test sample that can be quantified at a specified level of precision.

   Limit of Application (LLA) (reference Appendix M)
   A level below which the method developer does not support or recommend use of the method.

[1] AOAC Appendix M

*See section 4 – Definitions - Enzyme-linked immunosorbent assay (ELISA)

Repeatability (reference ISO 5725)

Variation arising when all efforts are made to keep conditions constant by using the same instrument and operator (in the same laboratory) and repeating during a short time period. Expressed as the repeatability standard deviation (SD_r); or % repeatability relative standard deviation (%RSD_r).

Reproducibility

Variation arising when identical test materials are analyzed in different laboratory by different operators on different instruments. The standard deviation or relative standard deviation calculated from among-laboratory data. Expressed as the reproducibility standard deviation (SD_R); or % reproducibility relative standard deviation (% RSD_R).

Recovery

The fraction or percentage of analyte that is recovered when the test sample is analyzed using the entire method.

Egg

A combination of [chicken] egg whites and egg yolks in their entirety, in natural proportions.[2] For the purposes of this SMPR, egg is referred to in its dry form as represented by existing reference materials

5. Method Performance Requirements:

See table 1.

6. System suitability tests and/or analytical quality control:

See antibody information, cross reactivity, information on calibrators and information on matrices in section "Required Allergen-Specific Information to be Provided on the ELISA Method" of Appendix M.

Method developers should clearly identify what component(s) of the egg they are measuring and provide the conversion factor used to equate to dried egg.

Method developers should provide applicability statement for intended use and claimed matrices.

7. Reference Material(s):

Refer to Annex F: *Development and Use of In-House Reference Materials* in Appendix F: *Guidelines for Standard Method Performance Requirements*, 20th Edition of the AOAC INTERNATIONAL Official Methods of Analysis (2016). Available at: http://www.eoma.aoac.org/app_f.pdf

Chicken Egg

- NIST 8445 (Spray dried whole egg for allergen detection)

[2] Introduction to Egg Products, USDA Food Safety and Inspection Service, website: http://www.fsis.usda.gov/wps/wcm/connect/c5c85914-5055-4f09-8098-1a179a1c6e14/EPT_Introduction.pdf?MOD=AJPERES, accessed 12/15/2015.

8. Validation Guidance:

Method developers must provide data for method performance in all the claimed matrices.

Method developers must provide recovery data using incurred samples for all claimed matrices.

Add appropriate Appendix reference for PTM requirement (Appendix J).

Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis; 19th Edition of the AOAC INTERNATIONAL Official Methods of Analysis (2012). Available at: http://www.eoma.aoac.org/app_d.pdf

Appendix F: Guidelines for Standard Method Performance Requirements; 19th Edition of the AOAC INTERNATIONAL Official Methods of Analysis (2012). Available at: http://www.eoma.aoac.org/app_f.pdf

Appendix M: Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices; 19th Edition of the AOAC INTERNATIONAL Official Methods of Analysis (2012). Available at: http://www.eoma.aoac.org/app_m.pdf

Table 1: Method performance requirements (deleted wine section)

Parameter	**Minimum Acceptance Criteria for target matrix**
Analytical Range (ppm)	≤ 5 - ≥ 10
LOQ (ppm)	≤5
LOD (ppm)	≤5
Recovery (%)*	50-150%
% RSD_r	≤20 %
% RSD_R	≤ 30%
Note: ppm dried egg. *Using incurred samples (acceptance criteria in Appendix M).	

Quantitation of Chicken Egg by ELISA-based* Methods

Intended Use: Method for quantitation of chicken egg in the context of food manufacturing.

1. **Purpose:** AOAC SMPRs describe the minimum recommended performance characteristics to be used during the evaluation of a method. The evaluation may be an on-site verification, a single-laboratory validation, or a multi-site collaborative study. SMPRs are written and adopted by AOAC Stakeholder Panels composed of representatives from the industry, regulatory organizations, contract laboratories, test kit manufacturers, and academic institutions. AOAC SMPRs are used by AOAC Expert Review Panels in their evaluation of validation study data for method being considered for *Performance Tested Methods* or AOAC *Official Methods of Analysis*, and can be used as acceptance criteria for verification at user laboratories.

2. **Applicability**:
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4. **Definitions**:

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   A level below which the method developer does not support or recommend use of the method.

[1] AOAC Appendix M

*See section 4 – Definitions - Enzyme-linked immunosorbent assay (ELISA)

Repeatability (reference ISO 5725)
Variation arising when all efforts are made to keep conditions constant by using the same instrument and operator (in the same laboratory) and repeating during a short time period. Expressed as the repeatability standard deviation (SD_r); or % repeatability relative standard deviation ($\%RSD_r$).

Reproducibility
Variation arising when identical test materials are analyzed in different laboratory by different operators on different instruments. The standard deviation or relative standard deviation calculated from among-laboratory data. Expressed as the reproducibility standard deviation (SD_R); or % reproducibility relative standard deviation (% RSD_R).

Recovery
The fraction or percentage of analyte that is recovered when the test sample is analyzed using the entire method.

Egg
A combination of [chicken] egg whites and egg yolks in their entirety, in natural proportions.[2] For the purposes of this SMPR, egg is referred to in its dry form as represented by existing reference materials~~.~~

~~6.~~5. Method Performance Requirements:
See table 1.

~~7.~~6. System suitability tests and/or analytical quality control:

See antibody information, cross reactivity, information on calibrators and information on matrices in section "Required Allergen-Specific Information to be Provided on the ELISA Method" of Appendix M.

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~~8.~~7. Reference Material(s):

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[2] Introduction to Egg Products, USDA Food Safety and Inspection Service, website: http://www.fsis.usda.gov/wps/wcm/connect/c5c85914-5055-4f09-8098-1a179a1c6e14/EPT_Introduction.pdf?MOD=AJPERES, accessed 12/15/2015.

Chicken Egg

- NIST 8445 (Spray dried whole egg for allergen detection)

~~9.~~8. Validation Guidance:

Method developers ~~should~~ must provide data for method performance in all the claimed ~~matrixes~~matrices.

~~-~~Method developers must provide recovery data using incurred samples for all claimed matrices.

Add appropriate Appendix reference for PTM requirement (Appendix J)

Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis; 19th Edition of the AOAC INTERNATIONAL Official Methods of Analysis (2012). Available at: http://www.eoma.aoac.org/app_d.pdf

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Table 1: Method performance requirements (deleted wine section)

Parameter	**Minimum Acceptance Criteria for target matrix** ~~Cookies, Bread, Dough, Salad Dressing~~
Analytical Range (ppm)	≤~~<~~ 5 ~~-~~ - ≥~~>~~ 10
LOQ ~~-~~(ppm)	≤5
LOD (ppm)	≤5
Recovery (%)*	50-150%
% RSD_r	≤20 %

% RSD_R	≤ 30%
Note: ~~mg/kg~~ppm dried egg. ~~Reporting units for wine as mg/L.~~ *Using incurred samples (acceptance criteria in Appendix M).	

(DELETE TABLE 2)

Table 2: Selected Finished Food Products and Ingredients

cookies
salad dressing
wine
chicken
ice cream
pasta
soy milk

INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS (ISPAM)

Scott Coates, Ph.D.
AOAC INTERNATIONAL

Scott Coates is a 21-year AOAC veteran now serving as the AOAC Chief Scientific Officer. Dr. Coates joined AOAC in 1992 as a manager for the AOAC Research Institute, and ran the *Performance Method Tested* program until 2009 when he was promoted to become AOAC's Chief Scientific Officer (CSO). As CSO, Coates is involved in every major AOAC project, ranging from biological threat agent detection, food/environmental microbiology, and nutritional chemistry of infant formula. Coates was the lead author of the *Guideline for Standard Methods Performance Requirements* in the 19th edition of the *Official Methods of Analysis.* Coates is a University of Maryland alumni has a B.S. in microbiology and a M.S. in Biotechnology Management.

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INTERNATIONAL STAKEHOLDER PANEL ON ALTERNATIVE METHODS(ISPAM)

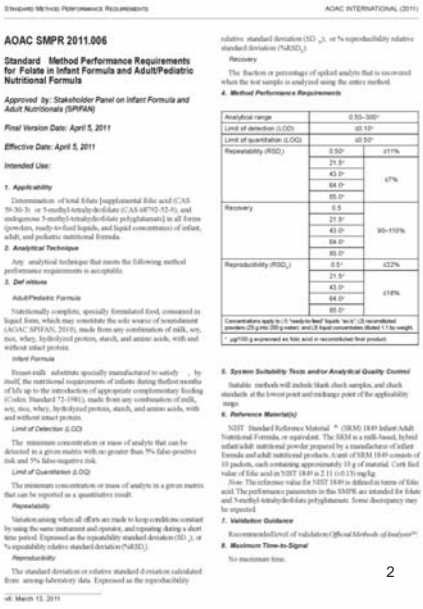
Standard Method Performance Requirements (SMPRs)

Scott Coates (CSO)
Gaithersburg, MD
March 14, 2017

The Scientific Association Dedicated to Analytical Excellence®

Standard Methods Performance Requirements

- Commonly referred to as:
  - SMPRs
  - "Smipper"s



AOAC SMPR 2011.006

Standard Method Performance Requirements for Folate in Infant Formula and Adult/Pediatric Nutritional Formula

Approved by: Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN)

Final Version Date: April 5, 2011

Effective Date: April 5, 2011

Intended Use:

1. Applicability

2. Analytical Technique

3. Definitions

4. Method Performance Requirements

5. System Suitability Tests and/or Analytical Quality Control

6. Reference Material(s)

7. Validation Guidance

8. Maximum Time-to-Signal

SMPRs

- documents a community's analytical method needs.
- very detailed description of the analytical requirements.
- includes method acceptance requirements.
- published as a standard.

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Uses of SMPRs

- Basis for method acceptance and approval.
- Guidance to method developers for the development of new methods.
- Advance the state-of-the-art in a particular direction.
- Address specific analytical needs.
- Allow AOAC to reach a broader community of method developers and users.

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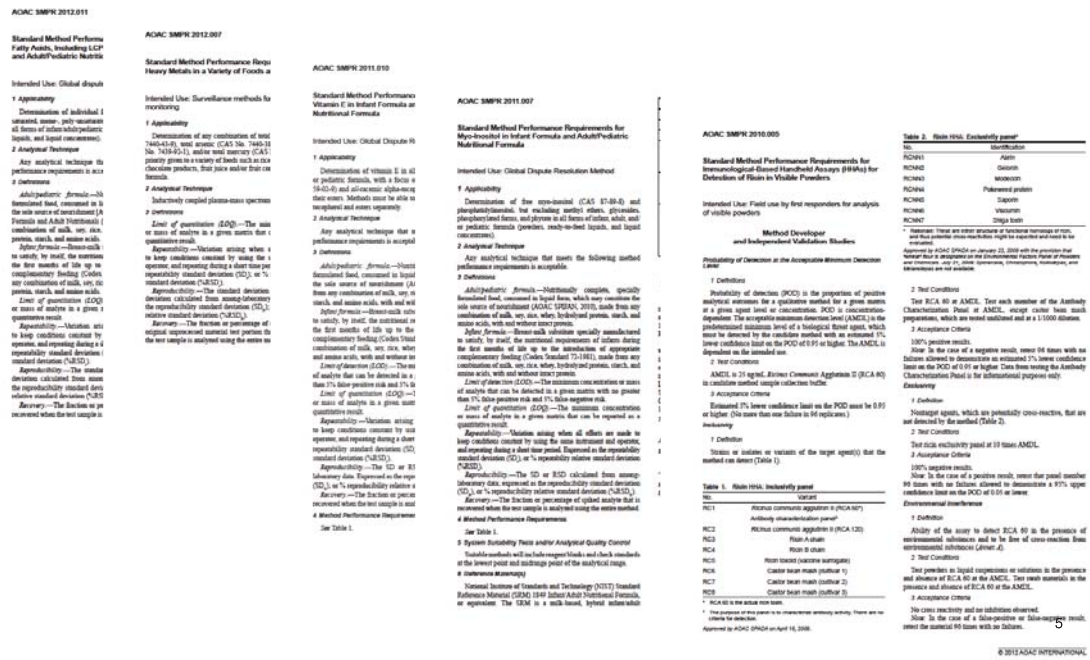
AOAC has adopted 70+ SMPRs



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When are SMPRS helpful?

- SPDS – no reference methods and very complex analytical issues.
- SPIFAN - *community* decides to modernize methods.
- SPSFAM – new analytical issues and new application.
- SPADA – new applications and guidance.

Example

- SMPR: Identification of Venezuelan Equine Encephalitis Virus (VEEV)

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Identification of Venezuelan Equine Encephalitis Virus (VEEV)

- **Intended Use**: Laboratory or field use by Department of Defense trained operators.
- **Applicability**: ***Identification*** of VEEV in liquid samples from aerosol collectors. The preferential method would be a field-deployable assay.
- **Analytical Technique**: Molecular methods of detecting target-specific viral component(s).

Identification of Venezuelan Equine Encephalitis Virus (VEEV)

Parameter	Minimum Performance Requirement
AMIL	5000 genome copies / mL
POI at AMIL within sample collection buffer	≥ 0.95
POI at AMIL in an aerosol environmental matrix	≥ 0.95
System False-Negative Rate using spiked aerosol environmental matrix	≤ 5%
System False-Positive Rate using aerosol environmental matrix	≤ 5%
Inclusivity panel purified DNA	All inclusivity strains (Table II) must be correctly identified at 2x the AMIL†
Exclusivity panel purified DNA	All exclusivity strains (Table III and Annex IV; part 2) must test negative at 10x the AMIL†

• Official Methods of Analysis of AOAC INTERNATIONAL (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, APPENDIX I; also on-line at http://www.eoma.aoac.or

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Identification of Venezuelan Equine Encephalitis Virus (VEEV)

Inclusivity Panel

VIRUS	Serotype / Variant	Representative Strain (s)
VEEV	VEE-IAB	Trinidad Donkey
		MF-8
	VEE-IC	ICVE93, ICVE95
	VEE-IE	IEMX63, IEPA62
	VEE-ID	1DPA61, 1DPE98, IDPE06

Identification of Venezuelan Equine Encephalitis Virus (VEEV)

Exclusivity Panel

VIRUS	Representative Strain (s)
Mosso das Pedras	78V 3531
Everglades	Fe-3-7c
Mucambo	A
	C (strain 71D-1252)
	D
Tonate	Tonate
Pixuna	Pixuna
Cabassou	Cabassou
Rio Negro	AG 80-663
EEE WEE	PE6 CBA87

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Potential Interferants

Compounds		Potential Theaters of Operation
group 1: petroleum-based	JP-8	airfield
	JP-5	naval
	diesel/gasoline mixture	ground
	fog oil (standard grade fuel number 2)	naval, ground
	burning rubber	ground, airfield
group 2: exhaust	gasoline exhaust	ground
	jet exhaust	naval, airfield
	diesel exhaust	ground
group 3: obscurants	terephthalic acid	ground
	zinc chloride smoke	ground
	solvent yellow 33	ground
group 4: environmental	burning vegetation	ground, airfield
	road dust	ground
	sea water (sea spray)	naval
group 5: Chemicals	brake fluid	all

Food Microbiology

- We don't need no stinkin' SMPRs.
- We got:
  - reference methods.
  - international guidelines.

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When are SMPRS helpful?

- No universally recognized reference methods and very complex analytical issues.
- Community decides to upgrade methods.
- New analytical issues and new application.
- New applications and guidance.

Potential Food Micro SMPR

- MALDI–TOF-MS
- Proteomics
- Application of *in silico* analysis
- New pathogens
- Viruses

Summary

- SMPR have been extremely useful to many other communities.
- AOAC is offering this process to the food microbiology community.

Discussion

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Thank You!

Medallion Labs

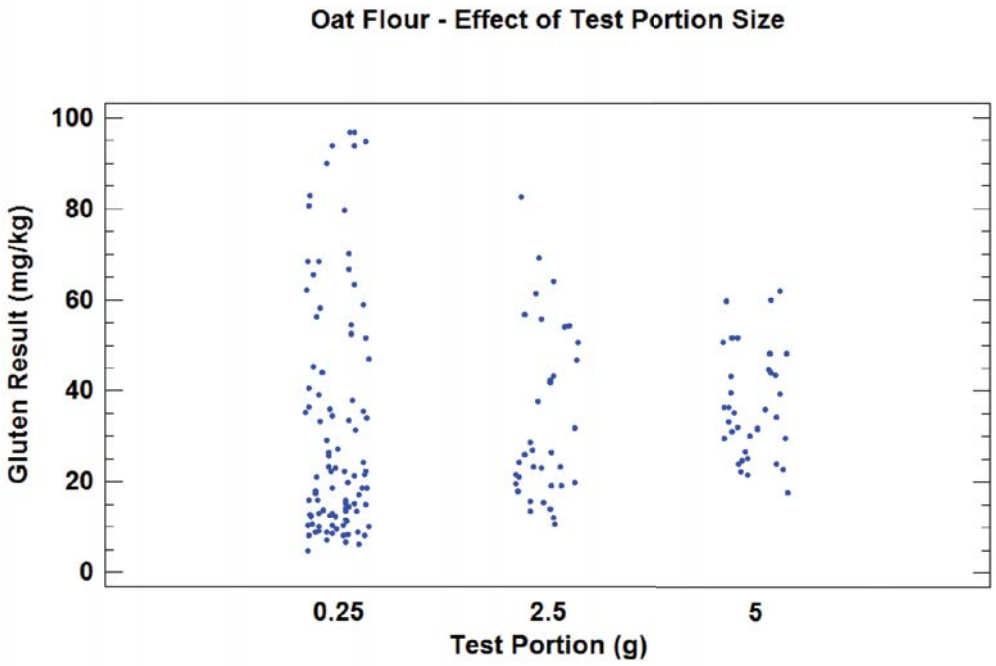
ISPAM GLUTEN IN OATS PROJECT

WORKING GROUP PRESENTATION

MEASUREMENT VARIATION

- Trace amounts of gluten can be present in a sample.
- The gluten in the sample is randomly distributed, but tends to be found in microscopic “flecks.”
- This microscopic inhomogeneity can result in abnormally high test variation due to sampling probabilities, especially at small test portions.
- Efforts to significantly reduce sample non-homogeneity through grinding have not been effective.
- Lack of sample homogeneity coupled with small test portions leads to significant test variation.

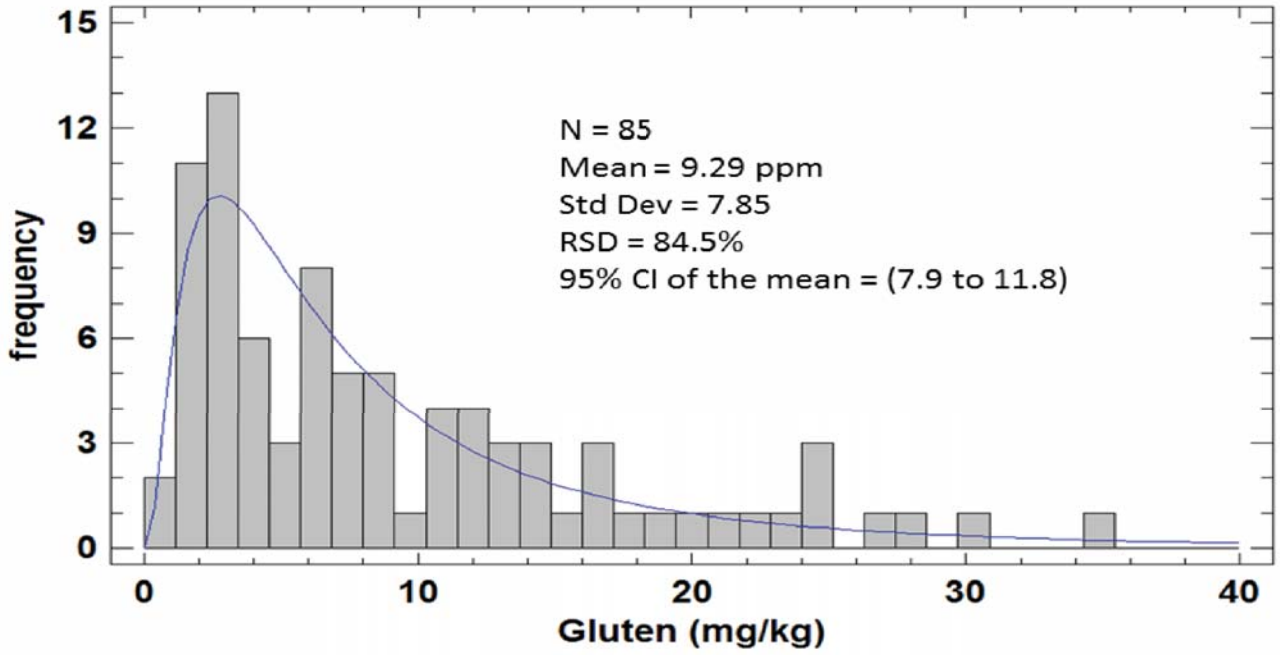
EFFECT OF TEST PORTION SIZE



RSD DEPENDS ON TEST PORTION – OAT FLOUR

Test Portion	0.25 g	2.5 g	5.0 g
N	98	36	36
Mean (mg/kg)	31.9	33.9	36.8
Std Dev (mg/kg)	29.6	20.3	12.1
RSD (%)	93.0	60.0	32.9

Histogram Flour Gluten Estimation - 5 g TP



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RSD DEPENDS ON SAMPLE TYPE AND LEVEL

Sample	Flour	Flour	Cereal	Cereal
Test Portion	5.0 g	5.0 g	1.0 g	0.25 g
N	36	85	108	461
Mean (mg/kg)	36.8	9.29	28.2	10.96
Std Dev (mg/kg)	12.1	7.85	9.50	13.6
RSD (%)	32.9	84.5	33.7	124

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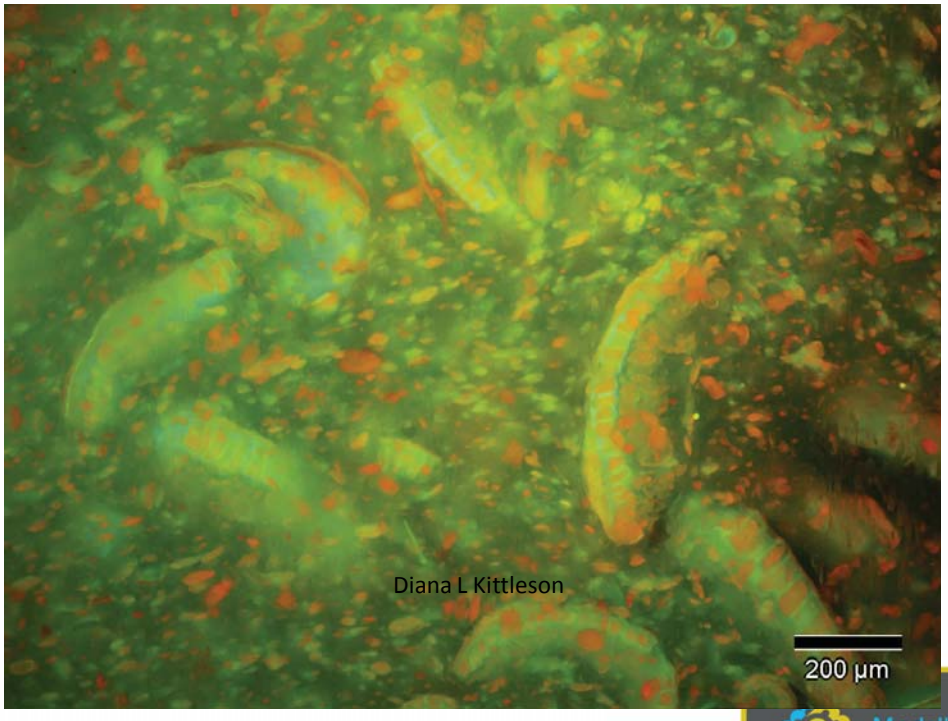
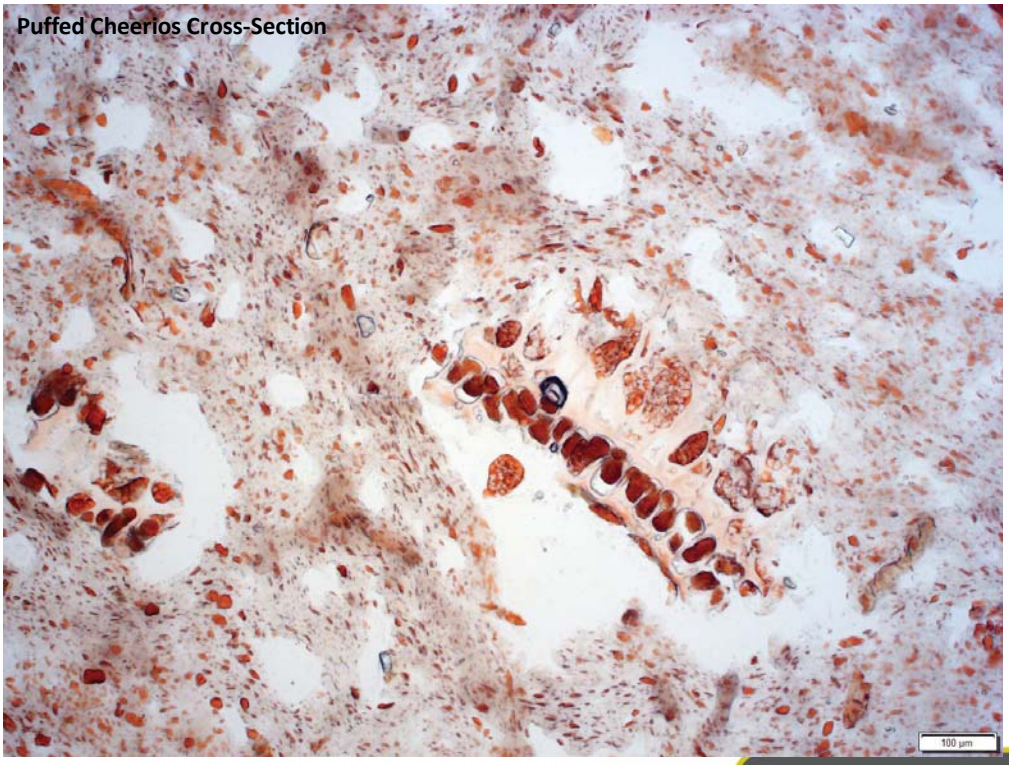
STRATEGIES FOR REDUCING VARIATION

- Larger TP sizes
- Replication
  - Finished Product: we use n=6 reps at 1 g TP
  - Oat Flour: We use n =18 reps at 5 g TP
  - React to mean of results with no removal of high “outliers” or censoring low results below LOQ

Wide Field Fluorescence Microscopy
Oats Cooked by Farinograph #2



Puffed Cheerios Cross-Section



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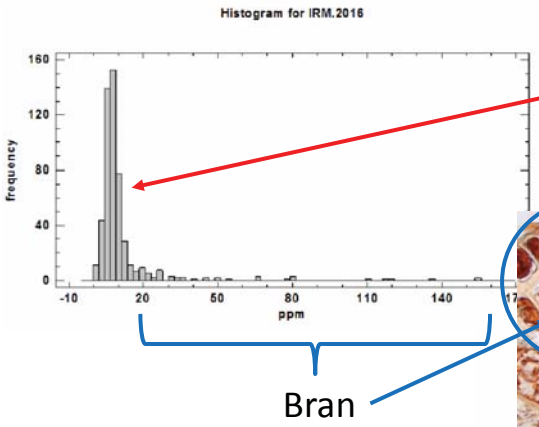
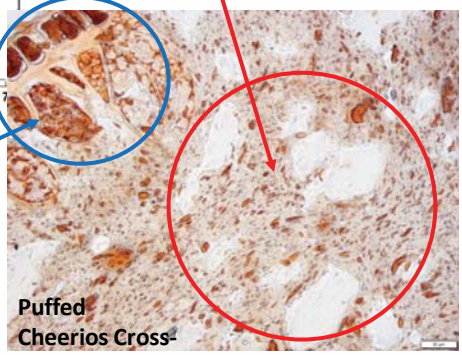
Measurement Variation
Bran vs Endosperm





Puffed Cheerios Cross-Section

*Red stain depicts protein - ~1/10,000 of the protein flecks are gluten

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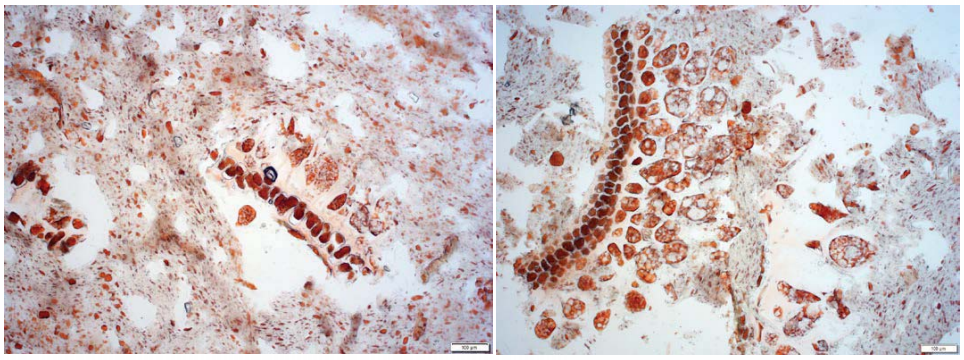
Measurement Variation & Sample Preparation

Microscopic analysis shows that the bran particles have not been significantly reduced in size by grinding.



Unground Cheerios

Ground Cheerios

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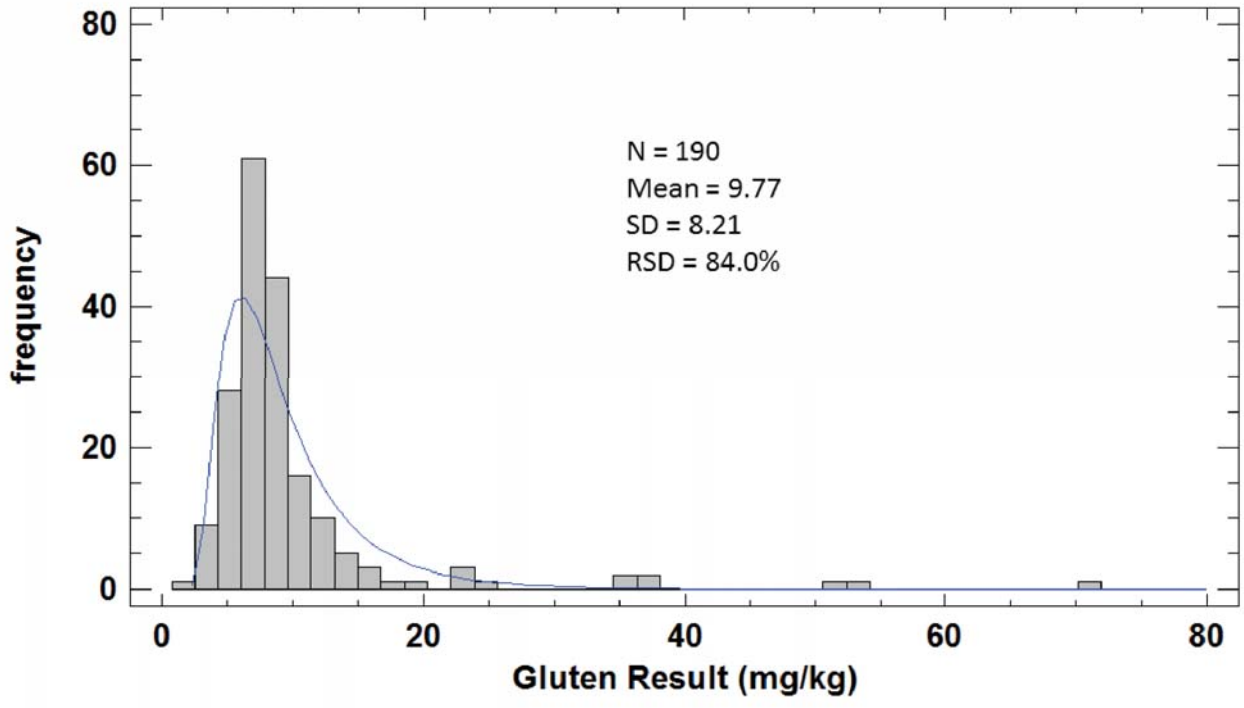
Histogram Puffed Cereal Gluten Measurement Variation - 0.25 g TP



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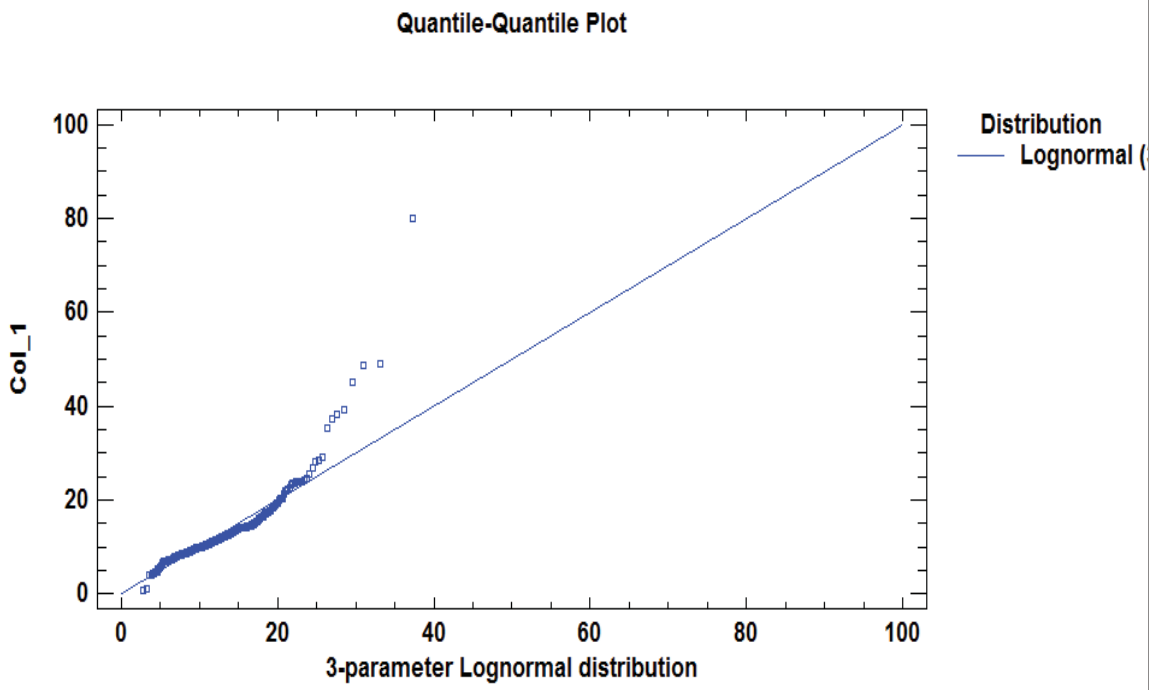
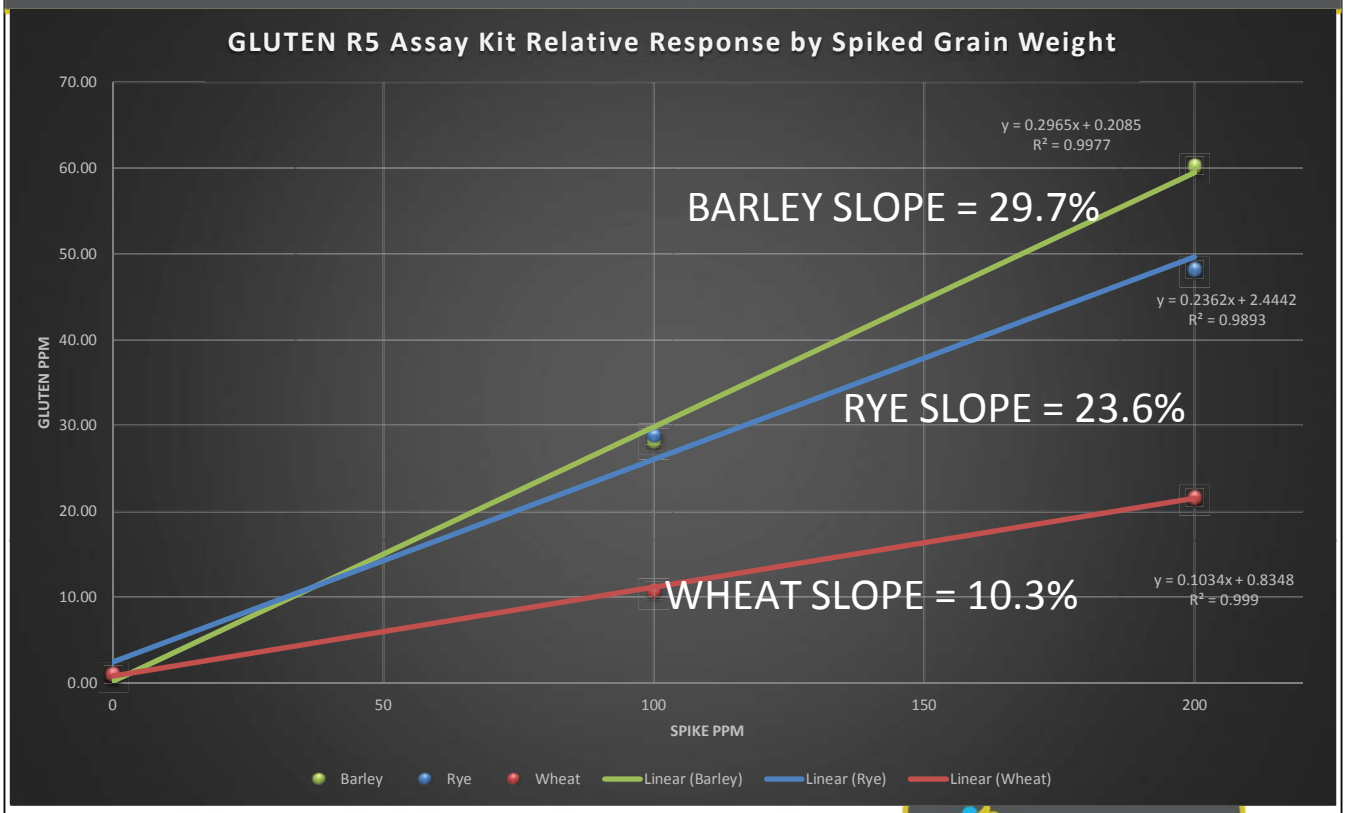
OLD CEREAL CHECK SAMPLE



ACCURACY ESTIMATES

- Experimental approach to evaluating response of gluten methods with respect to wheat, rye and barley varieties
- Obtain samples of Oat Flour, wheat, rye, barley.
- Grind wheat, rye barley grains as best as possible
- Analyze wheat, rye and barley grains for gluten by some independent, non-ELISA method to obtain quantitative estimate of gluten level.
- Spike “uncontaminated” oat flour with other grains at various levels.
- Analyze spiked samples with replication to evaluate relative response of ELISA method to different grains. (n=18 per level)
- Compare slope of line to non-ELISA result as recovery.



ACCURACY STRATEGY

- Response of prospective method should be evaluated for 3 grains independently.
- Experimental evaluation should be performed on grain (flour) spiked into oat flour, and extracted as per proposed kit extraction method.
- Samples of spiked material could be made by 3rd party lab and made available to researchers, along with non-ELISA "reference" values.
- Results of these experiments have shown much lower RSDs than we see in practice, so this experiment will not be useful for estimating precision.
- Because of the uncertainty in everything, the acceptance criteria for this approach will need to be very wide.
- This approach is probably best done as part of single lab validation or single lab with independent lab verification, along with some sort of cross-reactivity study.

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Gluten-containing grains skew gluten assessment in oats due to sample grind non-homogeneity

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ABSTRACT

Oats are easily contaminated with gluten-rich kernels of wheat, rye and barley. These contaminants are like gluten 'pills', shown here to skew gluten analysis results. Using R-Biopharm R5 ELISA, we quantified gluten in gluten-free oatmeal servings from an in-market survey. For samples with a 5–20 ppm reading on a first test, replicate analyses provided results ranging <5 ppm to >160 ppm. This suggests sample grinding may inadequately disperse gluten to allow a single accurate gluten assessment. To ascertain this, and characterize the distribution of 0.25-g gluten test results for kernel contaminated oats, twelve 50 g samples of pure oats, each spiked with a wheat kernel, showed that 0.25 g test results followed log-normal-like distributions. With this, we estimate probabilities of mis-assessment for a 'single measure/sample' relative to the <20 ppm regulatory threshold, and derive an equation relating the probability of mis-assessment to sample average gluten content.

1. Introduction

Celiac disease (CD) is a genetic autoimmune disease that affects approximately 0.2–1.0% of the population worldwide (Catassi & Fasano, 2008; Ludvigsson et al., 2013; Mooney et al., 2016; Mustalahti et al., 2010; Sanders et al., 2003). Its prevalence has continued to increase (Ludvigsson et al., 2013; Rubio-Tapia & Murray, 2010). CD patients cannot tolerate the gluten proteins in wheat, barley and rye, which trigger autoimmune damage of the small intestinal mucosa (Janatuinen et al., 1995). Consequently, CD patients have to strictly observe a gluten-free (GF) diet in order to avoid adverse consequences. In addition to CD patients, GF diets are attracting increased numbers of consumers, being viewed as part of a healthy life style (Sharma, Pereira, & Williams, 2015). Consequently, food products with GF claims are becoming more popular in the marketplace (Sapone et al., 2012). To be valid for a GF claim, the gluten content of a food product has to be below a threshold level. One widely accepted GF threshold is 20 ppm, which is recognized by food regulatory agencies, such as Codex Alimentarius, the European Union, and the US Food and Drug Administration (Sharma et al., 2015).

Oats provide dietary fiber, B-complex vitamins (thiamin, niacin and riboflavin), iron and proteins (Comino, Moreno, & Sousa, 2015; Rebello, O'Neil, & Greenway, 2016). They have a long history of human consumption and are considered one of the most important whole grains in one's diet (Jacobs & Gallaher, 2004; Slavin, Martini, Jacobs, & Marquart, 1999). There is abundant evidence to support that the consumption of oats or oat products provides health benefits (Cerio, Dohil, Magina, Mahé, & Stratigos, 2010; Kale, Hamaker, & Bordenave, 2014; Rebello, O'Neil, & Greenway, 2016). Regarding oat's suitability for CD patient consumption, there has been debate. For instance, although avenins, the storage proteins in oats, lack the well-recognized epitopes found in the corresponding gluten proteins of wheat, rye and barley (that can trigger autoimmune conditions (Londono et al., 2013)), there has been discussion whether certain amino acid sequences harbored in oat avenins pose potential risks to CD patients (as they show some degrees of similarity to the gluten epitopes (Comino et al., 2011, 2015; Londono et al., 2013)). Increasing amounts of clinical data however show that most CD patients can tolerate oats in their diets (Lundin et al., 2003; Tapsas, Fälth-Magnusson, Högberg, Hammersjö, & Hollén, 2014; Thompson, 2003). This has been demonstrated in multiple studies where moderate inclusion of oats in gluten-free diets (for both adult and child CD patients) has caused no adverse effects (Janatuinen et al., 1995; Tapsas et al., 2014). In fact, a recent clinical study using a daily oat consumption of 100 g indicates that the amount of pure oats commonly consumed does not trigger clinical relapse in celiac disease patients (Hardy et al., 2015). This supports results found in previous long-term feeding studies that

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oats are safe for most CD patients (Janatuinen et al., 1995; Lundin et al., 2003; Tapsas et al., 2014; Thompson, 2003). As a consequence, inclusion of oats in a GF diet may expand the dietary options and improve the nutritional status of GF consumers (Comino et al., 2015).

So, although pure oats, which are free of any non-oat cereal contaminants, are safe for most CD patients (Janatuinen et al., 1995; Lundin et al., 2003; Tapsas et al., 2014; Thompson, 2003), oats can be easily contaminated with gluten-containing kernels of wheat, rye and barley. This can occur in the field, during transportation, in storage and during processing (Hernando, Mujico, Mena, Lombardia, & Mendez, 2008; Koerner et al., 2011; Thompson, 2004; Thompson, Lee, & Grace, 2010). Removal of these contaminant kernels seems a conceptually straightforward way to produce gluten-free oats, but our in-market survey suggests this is not a simple task to accomplish or assess. We have paid attention to gluten analysis of oats at serving-size level (a pouch or ~50 g), because this sample size is what GF consumers including CD patients may consume on a regular basis. As shown herein, non-compliant servings are getting onto store shelves and assessment issues related to kernel-based gluten contamination are a prime suspect for that. This is because contaminant kernels are hardened, 'pill like' pockets of concentrated gluten, not evenly distributed throughout oats and as we have found not easily distributed within a ground sample.

Gluten can be quantitatively analyzed via enzyme-linked immunosorbent assay (ELISA) (Moron et al., 2008; Valdes, Garcia, Llorente, & Mendez, 2003), mass spectrometry (Fiedler, McGrath, Callahan, & Ross, 2014; Simonato, Mainente, Tolin, & Pasini, 2011) and polymerized chain reaction (Dahinden, von Büren, & Lüthy, 2001; Zeltner, Glomb, & Maede, 2008). ELISA is the most widely utilized analytical method both in industry and amongst regulatory agencies to determine GF compliance (Sharma et al., 2015). Because of this, we report here the effects of gluten kernel contaminants on gluten ELISA analysis using the well accepted ELISA method (Koerner et al., 2011; Sharma et al., 2015; Thompson, 2004; Thompson et al., 2010), R-Biopharm R5 sandwich ELISA R7001.

There were two parts to this research, first was an 'in-market survey' where repeated measures of gluten positive yet compliant (i.e., <20 ppm) servings were conducted. Secondly we attempted to characterize the distribution of gluten in 0.25 g sub-samples (coming from a larger ground sample) given a gluten containing kernel existed in the ground sample.

2. Materials and methods

2.1. Materials

For the 'in-market survey', gluten-free oatmeal was acquired from the market by a third party sample acquisition company and then tested by a third party laboratory using the R-Biopharm R5 ELISA RIDASCREEN Gliadin (R7001) kit, purchased from R-Biopharm, Inc. (Washington, MO, USA).

For the 'within ground sample gluten distribution characterization of kernel contaminated oats' part of this research, clean, pure oat groats spiked with Hard Red Winter wheat kernels (Western Canada Origin, 2014 crop year) were prepared by hand-picking and provided by PepsiCo, Inc. These samples were also analyzed with the R-Biopharm R5 ELISA RIDASCREEN Gliadin (R7001) kit purchased from R-Biopharm, Inc. (Washington, MO, USA).

2.2. Gluten analysis specifics

To prepare a solid sample for gluten analysis with the R-Biopharm R5 ELISA RIDASCREEN Gliadin kit (R7001), the manufacturer's instruction recommends at least 5 g of sample be ground and 0.25 g of the ground sample be analyzed to assess gluten content. Since FDA has not provided an advisory procedure relating to sample grinding in terms of grinder type, sample size, and grinding time, analytical labs usually come up with their own procedures on the basis of the test kit manufacturer's instruction. In our study, sample grinding was performed by commercial labs X and Y according to their best practice, where, as mentioned a serving size of oatmeal (a pouch) or oat groats (50 g) were ground with household coffee grinder or food processor for two minutes. R-Biopharm R5 ELISA RIDASCREEN Gliadin kit (R7001) has a quantification range of 5–80 ppm. In case the gluten content was beyond the upper quantification range (i.e., >80 ppm) of the R-Biopharm kit, the sample extraction was appropriately diluted with 60% ethanol and was subjected to another round of ELISA assay to obtain a numerical gluten reading.

2.3. 'In-market survey' repeated measures

Six hundred thirty-six servings (e.g., a serving pouch) of gluten-free oatmeal which were produced by two large gluten-free oatmeal producers were acquired from store shelves by a third party sample acquisition company (14 date codes all 8/16/15 or later). The identifiers of the brand names and producers on the packages were covered by non-transparent tapes. The samples were shipped directly from the sample acquisition company to a well-recognized third party analytical lab, denoted as Lab Y. Lab Y ground each serving for two minutes using a Kitchen Aid coffee grinder. A clean grinding head and sample cup was used to grind each sample. Gluten content of each sample was analyzed by Lab Y and reported back to the authors at PepsiCo, Inc. The remainders of these 636 ground samples were retained at Lab Y where a portion was eventually selected for repeated analyses by Lab Y.

2.4. Gluten distribution in ground wheat-spiked oat groats

Twelve samples of pure oat groats (50 g each) were spiked with a wheat kernel (of approximately 0.027 g). Six of these spiked samples were sent to each of two recognized laboratories, denoted Labs X and Y. Lab X used an Osterizer food processor and Lab Y used a Kitchen Aid coffee grinder to do the grinding. In both labs, a sample was ground for two minutes with a clean grinding head and sample cup. After grinding, the gluten content of each sample was analyzed in triplicate (0.25 g per analysis). The remainders of the 12 ground samples were sent back to the authors, and were then aliquot into 0.25 g portions. Each aliquot was subjected to gluten analysis performed by the PepsiCo analytical team with nearly 2300 total analyses conducted.

2.5. Probability distribution

Data analysis and data fitting were performed in Excel, Microsoft Office 2013. The log-normal distribution of the test results of the spiking experiments was determined by chi-square goodness of fit tests (Snedecor & Cochran, 1989). Estimation of the confidence intervals for the mean of data following a lognormal distribution was performed with the Modified Cox Method (Olsson, 2005).

3. Results and discussion

3.1. 'In-market survey' repeated measures

In our 'in-market survey', 636 servings (e.g., a serving pouch) of gluten-free oatmeal were ground and tested for gluten content by

Table 1
Re-test of samples with >5 ppm and <20 ppm gluten content reveal non-homogeneous distributions of gluten post grinding.

	Retests of in-market oatmeal finished goods (For samples found positive for gluten on 1st '0.25 g Test' but compliant, i.e., >5 and <20 ppm)												
	Original 1st 0.25 g Test Result	1st Retest Result	2nd Retest Result	3rd Retest Result	4th Retest Result	5th Retest Result	6th Retest Result	7th Retest Result	8th Retest Result	9th Retest Result	10th Retest Result	Resultant Avg. (n = 11)	Range of Outcomes
1	6.5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	2	7
2	6.6	14	10	13	7	8	8	39	BLQ	15	36	15	36
3	6.8	BLQ	6.5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	7.5	3	8
4	7.5	13	14	13	14	48	20	13	13	42	>160	>33	>153
5	7.7	34	16	14	6	20	8	BLQ	9	6.5	10	12	34
6	8.5	BLQ	BLQ	BLQ	7.5	BLQ	BLQ	BLQ	>160	13	BLQ	>18	>160
7	9.0	8.5	6.5	9.5	6	7	9	14	9	13	19	10	13
8	9.7	63	79	29	30	>160	31	30	70	65	>80	>59	>150
9	10.0	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	3	10
10	10.0	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	3	10
11	10.0	17	8	9.5	13	9.5	13	9	7	12	20	12	13
12	10.5	14	21	18	61	19	13	8.5	16	38	9.5	21	53
13	11.0	17	28	9.5	14	9.5	78	9.5	>80	24	12	>27	>71
14	12.5	15	8	BLQ	10	9	9	11	9	14	>160	>24	>160
15	13.0	BLQ	BLQ	BLQ	33	13	6	107	146	BLQ	87	38	146
16	13.4	31	25	18	26	14	32	75	27	20	52	30	62
17	15.0	24	17	18	21	15	13	9	8	17	10	15	16
18	15.5	36	40	18	16	14	7.5	76	42	14	6.5	26	70
19	16.0	BLQ	BLQ	6.5	BLQ	BLQ	>160	BLQ	BLQ	BLQ	BLQ	>19	>160
20	18.0	54	26	24	118	>160	24	28	28	95	39	>56	>142

%Averaging ⩾ 20 ppm 45–55%

Lab Y. Among these 636 servings, ten samples had a gluten content of ⩾20 ppm, not in compliance with the FDA's gluten-free regulation. Another 20 samples had gluten content between 5 and 20 ppm. Those 20 samples appeared on a first test to be in compliance with the FDA's gluten-free regulation. However, when ten more aliquots (0.25 g) from each of those 20 'positive yet compliant' samples were analyzed, gluten contents of >20 ppm were readily detected (Table 1). Interestingly, four of the 20 samples yielded both a BLQ (below the limit of quantification) result as well as an ALQ (above the limit of quantification). This indicates the possibility for a BLQ outcome to be obtained when ALQ level sub-samples (of 0.25 g) also exist in the same ground serving sample. Additionally, when looking at the average of the 11 gluten values for each of those 20 samples, at least nine of them (and potentially as many as 11 had we tested beyond a 160 ppm maximum) averaged >20 ppm, therefore 'averaging' non-compliant relative to the FDA's gluten-free regulation (Table 1).

Gluten analysis of our in-market survey samples illustrates a single gluten test of an oatmeal serving may be inadequate to accurately reveal the gluten risk inherent in it (at least under current best grinding practice). Kernel-based gluten contamination is the prime suspect, since it increases the difficulty of a homogenous grind due to gluten initially being centralized within a single contaminant kernel. This pill like form then needs to be adequately ground and uniformly distributed for homogeneity to occur.

3.2. Gluten distribution in ground wheat-spiked oat groats

To test whether kernel-based contamination can lead to chronic sample prep non-homogeneity, we prepared twelve pure oat groat (50 g) samples each spiked with a wheat kernel (of approximately 0.027 g). Six of them were sent to each of two recognized laboratories, Labs X and Y. The samples were ground, and the gluten content of each sample was analyzed in triplicate (0.25 g per analysis). The remainders of the 12 ground samples were sent back to the authors, and were then completely aliquot into 0.25 g portions and 100% of them analyzed by the PepsiCo analytical team. Overall, nearly 2300 analyses were conducted, varying from 184 to 196 results for each of the 12 spiked samples (Supplemental material, Table 1). Based on 100% evaluation of these 12 samples, plots of the individual distributions were created (Fig. 1).

Fig. 1 shows the distributions of 0.25-g test results are 'skewed right', tending to follow log-normal distributions as determined via chi-square goodness of fit tests. The log-normal approximations are presented in Fig. 1 as dotted lines. This skewness suggests a non-homogenous distribution of gluten particles despite the spiked oat groat samples being ground under current 'best' practices by two well-established commercial analytical labs. The post grinding particles produced by the contaminant kernels were apparently not well dispersed throughout the ground samples but rather tended to remain more highly concentrated in a small subset of the possible 0.25 g test portions. We found the maximum 0.25-g test result obtained to vary from 12 to 57 times more than the minimum for the sample. This difference in sample-to-sample variation could be due to differences in gluten kernel hardness, gluten content in the kernel itself, oat kernel hardness, 'grind to grind' variability, and other influencers. Needless to say however, with highly skewed distributions like this, the determination of gluten content in oat groats via a single 0.25-g sample test becomes error prone. This is because a few of the 0.25-g samples possess large amounts of gluten while others have received just a fraction of it. This leads to the potential for misdiagnosis (i.e., concluding either a sample average is <20 ppm when it is not, or that all possible test results are <20 ppm when they are not, depending on one's interpretation of the 20 ppm regulatory threshold).

This data set of spiked samples is relatively small (12 evaluated), but it does allow for a rough estimation of probabilities of obtaining a single reading possessing a value of <20 ppm given various true average gluten contents (in a 50-g sample). This means we can roughly assess the potential for misdiagnosis, using the log-normal distributional fits of test results (Fig. 2). Doing so, based on these outcomes, if one desires ⩾95% confidence that a single compliant reading (i.e., <20 ppm) does not come from a sample whose average is actually >20 ppm, the true gluten sample average would need to be >60 ppm, as Fig. 2 shows that a sample with an average gluten content of 60 ppm has a probability of 0.05 (i.e., 5%) to yield a <20 ppm gluten test result. In other words, for samples where the true gluten average is high enough, i.e., >60 ppm, there is little risk

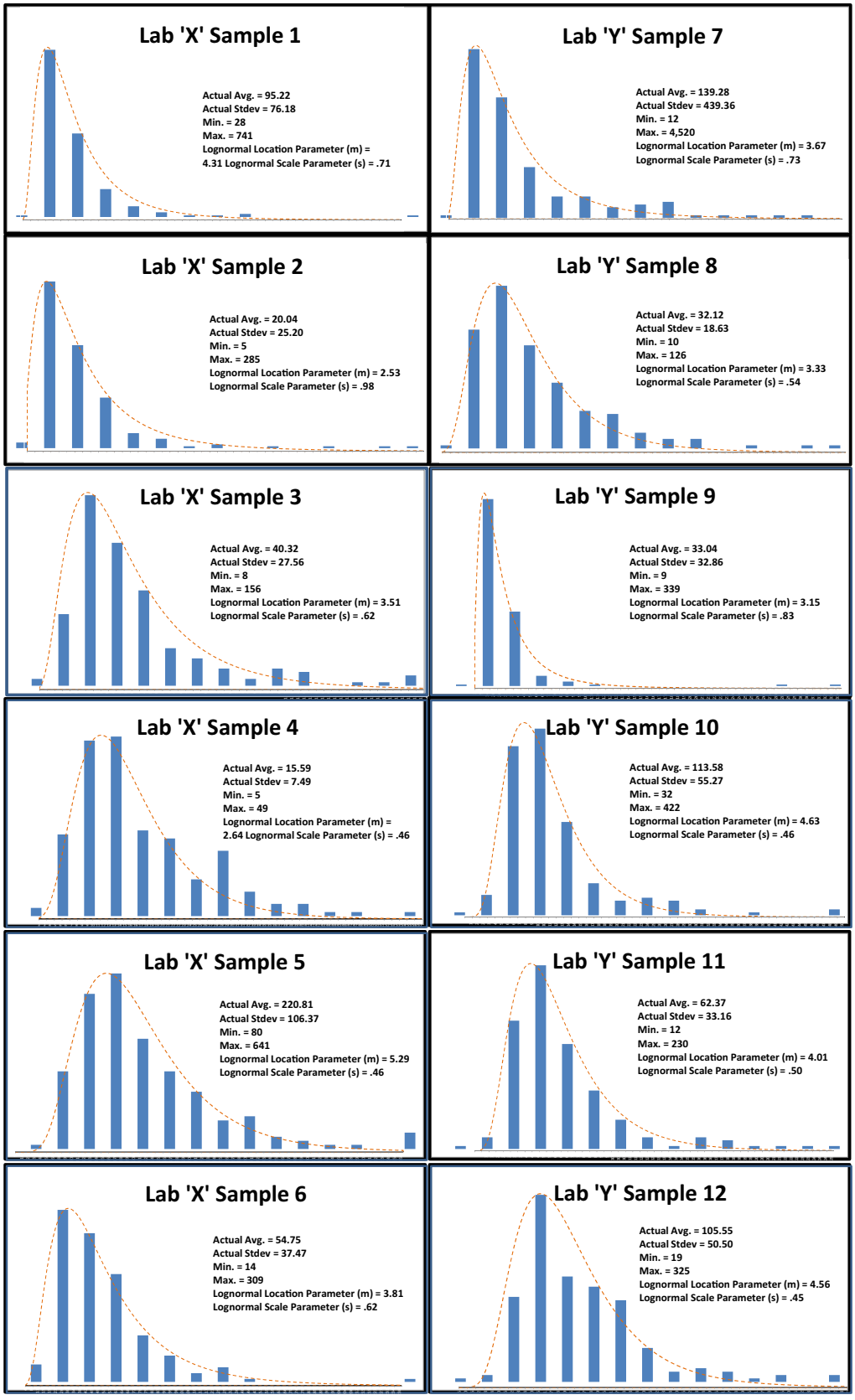

Fig. 1. Distributions of 0.25 g test results from 'wheat spiked' 50-g samples.

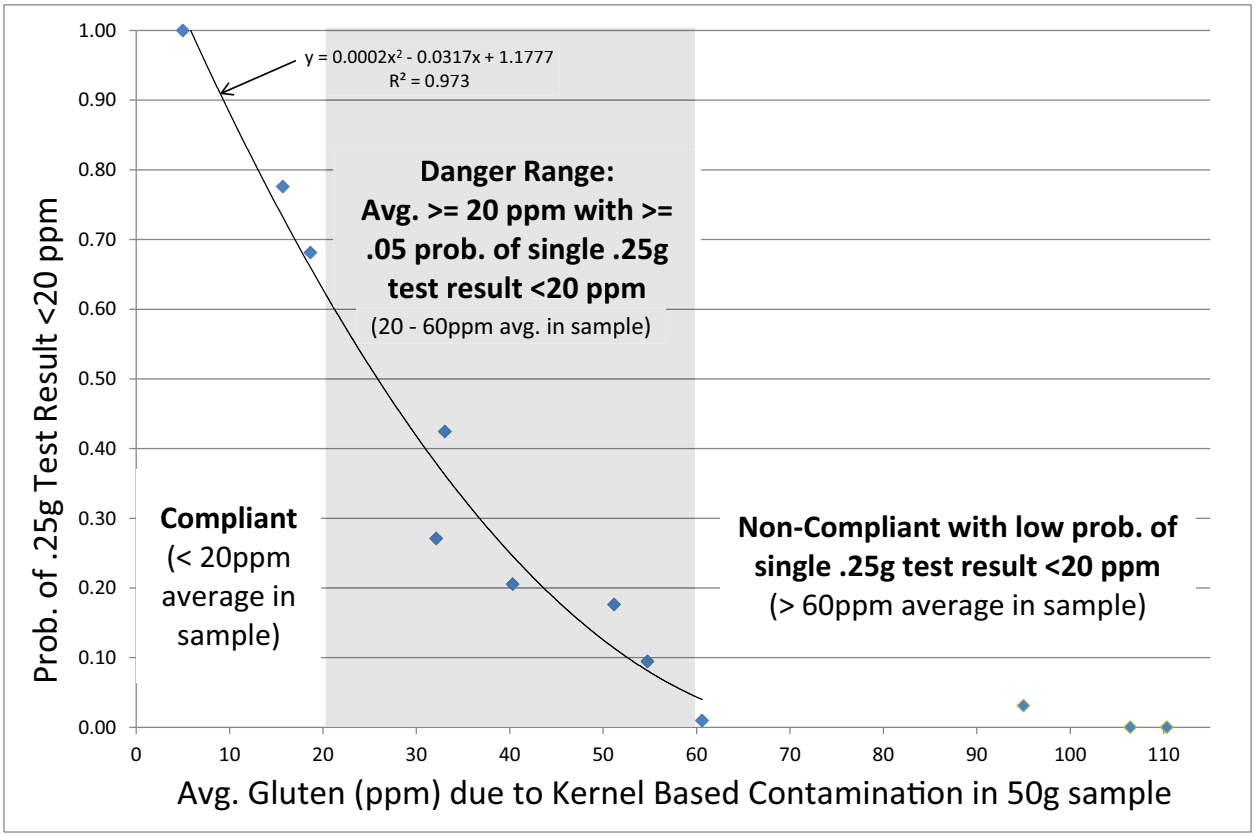

Fig. 2. Probability that a 0.25 g test result reads <20 ppm for various average contamination rates in the host sample.

of incorrectly deeming it compliant (i.e., <20) via a single reading. However for samples whose true gluten average spans from 20 ppm to 60 ppm, a 'danger zone' presents itself where the chance of incorrectly dispositioning it as compliant is above the often used 5% risk maximum.

An equation relating the probability of a single test reading being <20 ppm (i.e., compliant) relative to true sample average gluten content (for up to 60 ppm gluten) could then be defined as $Y = 0.000214X^2 - 0.0317X + 1.1777$, where Y = probability of getting a compliant reading (i.e., <20 ppm) via a single test outcome, and X = True average gluten in the sample. According to this equation, if the true sample average is 20 ppm, analysis with a single 0.25-g will have ~ 63% chance of getting a <20 ppm reading. So, when a gluten kernel exists and provides an overall average of 20 ppm gluten, roughly two out of three observations will end up less than 20 ppm (due to the non-homogenous grind and resultant skewed distribution characterized herein).

Additional tests per sample could improve the ability to accurately characterize a 'ground sample', but highly skewed distributions like this require a good amount of effort to get an 'accurate enough' estimate of the sample mean (compared to symmetrical ones like normally distributed data (Olsson, 2005)). For example, assuming we have a conforming sample that is log-normally distributed with a non-transformed average = 11.4 ppm and standard deviation = 4.0 ppm, it would take ~10 observations to gain 95% confidence the average of this sample does not exceed 20 ppm. This assessment was done via simulation, employing the Modified Cox Method for estimating confidence intervals for the mean of a lognormal distribution (Olsson, 2005).

Consequently, relatively large numbers of tests would tend to be needed to accurately assess gluten content given the non-homogeneity discovered in this research. And the poorer the grind, the worse this situation becomes since the contamination is then concentrated in fewer and fewer 0.25-g test amounts. Since additional observations is a costly way to deal with this, finding a solution to the non-homogeneity issue itself appears the more prudent alternative.

4. Conclusion

Our research has looked into the use of a single 0.25 g test amount to assess the gluten content of ground groats when a gluten-containing kernel is in a sample. The results indicate that a homogenous grind is difficult to attain and that resultant 0.25 g test results tend to be log-normally distributed. It appears this phenomenon is at play in finished goods as well (i.e., where whole grains have been cut and flaked), as our repetitive tests on 'gluten positive' servings from our 'in-market survey' suggest.

The log-normal distribution of gluten outcomes complicates the assessment task since a single observation (or even a number of them) may not accurately represent the rest of the sample (since a substantial range of outcomes is inherent in skewed distributions like this). Consequently, conventional use of a single 0.25 g test should be treated with caution, particularly when a positive compliant gluten reading has been obtained with that first reading.

Since it may be impractical in terms of cost to improve gluten assessment accuracy with multiple tests per sample, solving the non-homogenous grinding circumstance uncovered here is needed. Our research is underway in that direction. Parallel efforts from oat industry or GF industry should be encouraged.

Disclosure of competing interests

The authors declare that they have no competing interests. Ronald D. Fritz, Yumin Chen, and Veronica Contreras are salaried

employees of PepsiCo Inc. or Quaker Foods and Snacks (QFS), a subsidiary of PepsiCo, Inc., which funded this research. QFS has a commercial interest in gluten-free foods.

The views expressed in this manuscript are those of the authors and do not necessarily reflect the position or policy of PepsiCo Inc.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.08.031.

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Original article

Kernel-based gluten contamination of gluten-free oatmeal complicates gluten assessment as it causes binary-like test outcomes

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Summary Gluten-free (GF) foods, whose claim compliance is controlled at the 'serving level', hold better chances of protecting gluten-intolerant consumers. This is particularly true for GF oatmeal, as oats are easily contaminated with gluten-rich kernels of wheat, rye and barley, which remain intact to the spoon as pill-like flakes. A single contaminant kernel in otherwise pure oats results in GF labelling noncompliance, thereby posing a risk to patients with coeliac disease. Our in-market survey of 965 GF oatmeal servings uncovered that one in fifty-seven servings exceeded the GF labelling maximum of 20 mg kg^{-1} (i.e. 20 ppm). The noncompliance pattern was 'binary-like', with kernel-based contamination the suspected pass/fail driver. We have highlighted probabilities of misassessment for various sample sizes in light of oat's natural propensity for kernel-based contamination and proposed use of attribute-based sampling for compliance assessment, thereby providing a way to assess/manage/control 'rates of servings containing a contaminant kernel' within acceptable limits with high confidence.

Keywords Acceptance sampling, binomial, coeliac disease, ELISA, gluten, gluten-free, inference, in-market survey, oat.

Introduction

Coeliac disease (CD) is a lifelong, genetic, autoimmune intestinal disorder that affects approximately 0.2–1.0% of the world population (Sanders *et al.*, 2003; Catassi & Fasano, 2008; Mustalahti *et al.*, 2010; Ludvigsson *et al.*, 2013; Mooney *et al.*, 2016). Patients with CD have to abstain from dietary intake of gluten proteins found in wheat, barley and rye, as these proteins trigger autoimmune destruction of the mucosa of the small intestine (Janatuinen *et al.*, 1995). Members of a household that includes a patient with CD often choose to follow a GF diet as well, to avoid accidental consumption of gluten-containing food by the patient with CD. Increased numbers of consumers are also choosing to follow a GF diet (Sharma *et al.*, 2015). As a consequence, GF food products are getting more popular in the marketplace (Sapone *et al.*, 2012). To protect this growing number of consumers, food regulatory agencies have started to regulate gluten content in products with GF claims. For example, the US Food and Drug Administration (FDA) has decided that foods with GF claims should contain less than 20 ppm (i.e. mg kg^{-1}) of gluten (Sharma *et al.*, 2015). This has elevated the need for improved methodologies to determine whether grain-based product meets this standard.

Oats are recognised as one of the most important whole grain foods, being rich in dietary fibre, B-complex vitamins (thiamin, niacin and riboflavin), iron and proteins (Comino *et al.*, 2015; Rebello *et al.*, 2016). To expand the dietary options of patients with CD, as well as those following a GF diet, many researchers have investigated the suitability of dietary inclusion of oats (Lundin *et al.*, 2003; Thompson, 2003; Comino *et al.*, 2011, 2015; Londono *et al.*, 2013; Tapsas *et al.*, 2014). Although there has been debate whether oats present risks to patients with CD (Comino *et al.*, 2011, 2015; Londono *et al.*, 2013), increasing amounts of clinical data show that most patients with CD can tolerate dietary intake of oats (Lundin *et al.*, 2003; Thompson, 2003; Tapsas *et al.*, 2014). Therefore, inclusion of pure oats in a GF diet is considered safe (Janatuinen *et al.*, 1995; Lundin *et al.*, 2003; Thompson, 2003; Tapsas *et al.*, 2014) and viewed as a way to expand dietary options and improve nutritional status of GF conscious consumers (Comino *et al.*, 2015).

Pure oats, which are free of any nonoat cereal contaminants, are not easy to obtain though, as

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gluten-containing kernels of wheat, rye and barley can easily cross-contaminate oats in the field, during transport, in storage and during processing (Thompson, 2004; Hernando *et al*., 2008; Thompson *et al*., 2010; Koerner *et al*., 2011). These contaminant kernels act as 'pill-like' pockets of gluten, interspersed throughout otherwise GF pure oats. Removal of these contaminant kernels appears to be a straightforward way to produce GF product. However, if not effectively mitigated, these gluten 'pills' will be transformed into flakes, ultimately ending up in a pouch or comparable serving size. Consumption of such a serving presents a realistic risk to gluten-intolerant consumers, especially patients with CD. Because of this, it is felt that GF oatmeal claim compliance should be managed at the serving size level, as it holds a better chance to protect consumers from this form of kernel-based gluten contamination (than does assessing compliance at a 'higher' level like at the tote or batch level).

So, kernel-based contamination by its nature sets up a binary set of gluten test outcomes at the serving size evaluation level. One possible outcome is zero gluten in pure oats, and the other is high gluten in a kernel contaminated serving. This pass/fail circumstance highlights the need for a sampling approach tuned to this defect pattern. This contrasts with the use of 'variables' sampling, which assumes a continuous possible range of gluten content, serving to serving, and which may be subtly (and unintentionally) implied by the gluten regulatory threshold of 20 mg kg^{-1}, as this (or ('parts per million') is a continuous variable, appropriate for many nonwhole kernel GF foods, but not for those vulnerable to kernel-based contamination such as whole grain oat products.

We have investigated the dynamics and consequences of kernel-based gluten contamination in GF oatmeal herein, starting with the state of affairs of GF labelling compliance of GF oatmeal in the US market. The survey suggests shortcomings exist with producer outgoing quality inspection. This may be driven by an under appreciation of the subtle but important effects that kernel-based gluten contamination impose on process and lot acceptance sampling ability. The discussion is supplemented with probabilities of detection for various 'serving noncompliance rates' relative to the number of servings evaluated. We also provide guidelines, which prescribe sampling quantities to ensure with high confidence that various rates of nonconformance are not exceeded.

Materials and methods

Materials

GF oatmeal was acquired from the US marketplace by a third-party sample acquisition company. The R-Biopharm R5 ELISA RIDASCREEN Gliadin (R7001) kit was used for analyses, being purchased from R-Biopharm, Inc. (Washington, MO, USA).

Sample collection and gluten analysis for in-market survey

The GF oatmeal products acquired were produced by two large US producers and acquired from US store shelves by a third-party sample acquisition company. The identification of the brand names and producers on the packages were masked by the sample acquisition company and relabelled with sample numbers for subsequent tracking purposes. The oatmeal products collected from the market had two types of packages, 45 g in a serving pouch and 2 pounds in a bag. Three hundred and twenty-nine servings (e.g. either serving pouches or all 50-g oatmeal servings from a bag) were gathered in July 2014 and analysed at PepsiCo analytical laboratory. Before analysing the market survey samples though, a fit-for-purpose single laboratory validation of R-Biopharm R5 ELISA RIDASCREEN Gliadin (R7001) method was performed in our internal analytical laboratory. This was following the guideline provided by AOAC Official Method of Analysis, Appendix M. Accuracy and precision of the method both met the corresponding requirements listed AOAC Official Method Official Method of Analysis, Appendix M. The accuracy and precision values will not be disclosed because they are proprietary information. An additional six hundred thirty-six servings were then gathered in December 2014 and analysed at a well-recognised third-party laboratory with accreditation to ISO/IEC 17025:2005, which covers gluten ELISA analysis. We did not perform a multilaboratory gluten method validation due to the time strain on our research. However, before analysis of our in-market survey samples, split samples had been tested by the third-party contract analytical laboratory and PepsiCo analytical laboratory. No significant differences were found between these two laboratories. Each of the 965 servings (45 g directly from a serving pouch or every 50 g weighed out from 2-pound packages) was individually ground for 2 min using a magic bullet food processor (PepsiCo analytical laboratory), or a Kitchen Aid coffee grinder (third-party analytical laboratory). A clean grinding head and sample cup were used to grind each serving. Gluten extraction cocktail (R-Biopharm, R7006) was used to extract gluten from 0.25 g of each ground serving, and R-Biopharm R5 ELISA RIDASCREEN Gliadin kit (R7001) was used by both laboratories for gluten analyses. Only one 0.25 g of sample was tested for gluten for each ground serving. All analyses were conducted according to the manufacturer's instructions.

Statistical analysis

Data analysis was performed by custom macros using Excel, Microsoft Office 2013. Statistical significance was calculated at 95% confidence.

Results and discussion

In-market survey of GF oatmeal

The 'in-market' assessment of GF labelled oatmeal was conducted from July through December, 2014. Nine hundred and sixty-five servings (~45–50 g per serving) were acquired from store shelves. These were from two well-known US producers and spanned twenty-one different date codes. The number of servings obtained per date code varied from a low of ten up to a maximum of 108 servings.

The survey had two parts, divided by time and laboratory. The first was comprised of 329 servings acquired in the summer of 2014. All of these samples were tested at our internal analytical laboratory. This is referred to as the '7/14 Assessment' (Table 1). The second part was comprised of 636 additional servings acquired late 2014. These samples were tested at a recognised external laboratory. This is referred to as the '12/14 Assessment' (Table 1). The focus of our in-market survey was to examine whether the tested samples were in compliance with FDA gluten regulation (<20 mg kg^{-1}). So, once we knew the gluten content of a sample was above or below 20 ppm, we did not do further analysis. That was the reason that we did not further dilute sample extracts with >80 ppm readings (upper limit of the quantification curve of R-Biopharm R7001 ELISA kit) to obtain the exact gluten contents (Fig. 1 and Table 1).

The 965 total servings were acquired to characterise the overall state of affairs of GF oatmeal labelling compliance at the serving size level and to gain insight into differences/biases that exist between laboratories, time frames and producers. In this latter regard, statistical comparisons were made via chi-square contingency tests between laboratories/time frames ('7/14' vs. '12/14'), between producers ('Producer 1' vs. 'Producer 2'), between the two time frames for each producer (Producer 1 '7/14' vs. Producer 1 '12/14' and Producer 2 '7/14' vs. Producer 2 '12/14') and between date codes. No 'statistically significant' differences in 'proportion of servings found noncompliant' were found. Consequently, based on this data set, no evidence was found that the noncompliance rate at the serving size level differed between these two laboratories/time frames, producers or date codes.

As Table 1 shows, about 95% of the 965 servings were found to have a gluten content below the limit of quantification (BLQ) of the gluten testing kit, R-Biopharm 7001 (i.e. <5 ppm). It is worth mentioning that BLQ results do not equate to true zero results (and as gluten testing methodologies evolve, lower BLQ levels will no doubt be achieved). Continuing, 3.21% were found to be gluten positive but compliant, namely having a gluten content from 5 to <20 ppm, and 1.76% were found noncompliant, with a gluten content of ≥20 ppm. Additionally, 0.62% of the 965 were found >80 ppm, which is the quantification limit (ALQ) of the gluten testing kit. This equates to about one in every fifty-seven servings exceeding the FDA defined

Table 1 Oatmeal survey results

	Product	# of Date codes	Qty. of servings tested		BLQ (<5 ppm)	5–20 ppm	20–80 ppm	>80 ppm	Positives (>5 ppm)	Observed non conforming (≥20 ppm)
7/14 Assessment (Internal Laboratory tested)	Gluten-free oatmeal from Producer #1	5	228	Count	216	7	4	1	12	5
				% of Total	94.74%	3.07%	1.75%	0.44%	5.26%	**2.19%**
	Gluten-free oatmeal from Producer #2	2	101	Count	95	4	2	0	6	2
				% of Total	94.06%	3.96%	1.98%	0.00%	5.94%	**1.98%**
	Gluten-free oatmeal from both	7	329	Count	311	11	6	1	18	7
				% of Total	94.53%	3.34%	1.82%	0.30%	5.47%	**2.13%**
12/14 Assessment (indep. Laboratory tested)	Gluten-free oatmeal from Producer #1	6	316	Count	302	6	5	3	14	8
				% of Total	95.57%	1.90%	1.58%	0.95%	4.43%	**2.53%**
	Gluten-free oatmeal from Producer #2	8	320	Count	304	14	0	2	16	2
				% of Total	95.00%	4.38%	0.00%	0.63%	5.00%	**0.63%**
	Gluten-free oatmeal from both	14	636	Count	606	20	5	5	30	10
				% of Total	95.28%	3.14%	0.79%	0.79%	4.72%	**1.57%**
Both studies	Internal and external studies combined	21	965	Count	917	31	11	6	48	17
				% of Total	95.03%	3.21%	1.14%	0.62%	4.97%	**1.76%**

Bold values indicate % servings noncompliant.

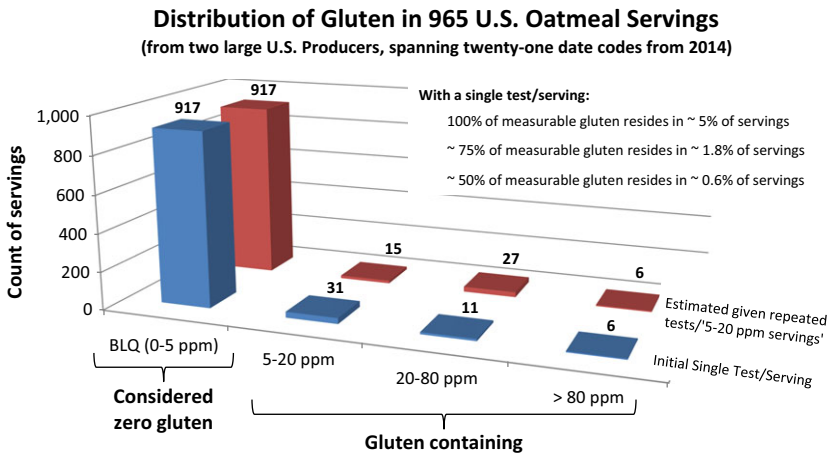

Figure 1 Distribution of gluten found in US 'in-market' survey of gluten-free oatmeal servings. (Both as observed with single results/serving and estimated given retests of '5 to <20 ppm' initial outcomes). [Colour figure can be viewed at wileyonlinelibrary.com]

labelling limit, and one in 161 being greater than four times that level.

Across the 965 servings, the overall average gluten is calculated to be 1.18 ppm per serving (i.e. 1.18 mg kg^{-1} per serving). This is assuming 0 mg kg^{-1} for BLQs and 80 mg kg^{-1} for ALQs. However, only forty-eight of the 965 servings contain any measurable gluten. Of those, if one conservatively assumes an AQL reading as 80 ppm, seventeen contain about 71% of total gluten observed, averaging about 48 ppm (so, about one in fifty-seven servings at this level).

It is important to note that the above referenced results were all single test outcomes per serving. Our recent research has shown that when a gluten-containing kernel exists in a serving of oatmeal, a single test result can underestimate overall serving gluten content (Fritz *et al.*, 2017). This is because gluten from the contaminate kernel tends to end up lognormally distributed in the ground sample (even with grinding performed under current best grinding practices). In other words, much of the gluten from the contaminant kernel remains concentrated in a few pockets within the serving after grinding, not being well dispersed. So consequently, a small test amount, randomly selected from the serving, is more likely than not to undercount overall gluten. With this insight, it was found that for samples initially testing 'gluten positive yet compliant' (i.e. 5 to <20 ppm), when ten additional tests per sample were conducted, about half ended up averaging noncompliant, that is ≥20 ppm (Fritz *et al.*, 2017). It is fair to assume that additional tests (beyond ten per serving for this 'positive yet compliant' group) could provide higher proportions of samples averaging >20 ppm, due to the lognormal distribution of gluten in these already deemed 'gluten-positive' ground samples.

Figure 1 provides a graphical depiction of the gluten distribution observed in the survey. It shows both the single test per serving outcomes and the assumed more accurate distribution if initially found 'positive yet compliant outcomes' were subjected to multiple tests per serving (using 50% of 5 to <20 ppm servings ending up in the 20–80 ppm category due to this).

The overall gluten per serving circumstance uncovered, that is where most servings measure BLQ and then are interspersed with occasional noncompliant servings, holds at the 'date code' level as well. Of the thirteen of twenty-one date codes possessing noncompliant servings, the maximum noncompliant serving rate was also 5% (like the overall data set). This maximum rate was not found statistically different from any other date code outcomes.

So it appears a bimodal-like noncompliance pattern (in terms of ppm) has been revealed in the GF oatmeal marketplace in terms of gluten per serving, where numerous BLQ servings are 'interrupted' by occasional noncompliant ones, some being several times the regulatory limit. These results indicate the GF oatmeal production processes of these two producers do not have sufficient capability to effectively mitigate gluten contamination to BLQ at the serving size level. The pattern of defects supports the premise that kernel-based gluten contamination is the cause, which produces high levels of gluten in oatmeal servings made from otherwise GF, pure oats. The outcomes encountered also suggest the inspection regimens used to assess overall process capability and lot acceptance are incapable of detecting the level of noncompliance observed.

This binary-like noncompliance pattern sheds light on interpretation of what some might view errantly as an attractive 1.18 ppm gluten average/serving found across these 965 servings. But despite a low ppm average per serving, the gluten is obviously not well dispersed across them (as an average errantly implies), but rather resides concentrated in a handful of servings, many being noncompliant in regard to GF labelling requirements.

The above survey insight has served as the genesis for this work, guiding the balance of the investigation.

Estimation of a wheat kernel's contribution to gluten content in pure oats

As mentioned, gluten-containing kernels of wheat, barley and rye are the predominant source of gluten contamination in oats (Thompson, 2004; Hernando *et al*., 2008; Thompson *et al*., 2010; Koerner *et al*., 2011). If not effectively mitigated, these kernel contaminants will survive the oatmeal production process intact (possibly being cut) and ultimately appear in a serving as indistinguishable flakes, consumed unknowingly by GF conscious consumers.

Using wheat as an example, we have estimated the gluten contribution from a single kernel in a typical serving size of 40 g of otherwise pure oats (Table 2). This is based on 'literature reported' wheat protein content (2015 Crop Quality Report by US Wheat Associates, http://www.uswheat.org/cropQuality) and wheat gluten content (Shewry, 2009). We found that for the six predominant North American wheat varieties, a single wheat kernel will bring on average 65–129 mg kg^{-1} of gluten to 40 g of pure oats.

This estimation suggests that gluten kernel contaminants, including a cut or broken kernel, can lead to noncompliance at a serving size level, thereby posing a risk to GF oatmeal consumers.

Sampling implications in assessment of kernel-based gluten contamination

The binary-like circumstance of gluten outcomes creates a sampling context similar to a pass/fail one. A serving fails when a gluten kernel or part of one exists in a serving, leading to noncompliance relative to gluten regulatory thresholds (e.g. >20 ppm by FDA), and passes when one does not. 'Attribute'-based sampling caters to binary type outcomes like this (Taylor, 1992). This type of sampling is in contrast to 'variable sampling', which assumes a few samples can provide information about the others around them. A key prerequisite for variable sampling therefore is the ability to pick a sample that is 'representative' of the rest (Taylor, 1992). The kind of distribution uncovered in this survey (Fig. 1) complicates doing so however, as randomly selecting some servings for analysis may not adequately provide a representative inference on the rest.

To investigate this, a sampling simulation was conducted where 10 000 samples of five, ten, twenty-five and fifty servings each were generated by randomly 'selecting' outcomes from the distribution of the 965 outcomes from the survey. Doing so, it was found that the probability of all servings selected being found compliant was 0.92, 0.84, 0.64 and 0.41 for samples of five, ten, twenty-five and fifty servings, respectively. So, with fifty servings evaluated for instance, about 40% of the time one will not get an indication of a compliance problem, getting all compliant outcomes. This probability increases when fewer servings are evaluated. So, with an underlying noncompliance rate of about one in fifty-seven servings, with ~1 in 161 being more than four times the regulatory maximum, sampling quantities in this range can fail to detect (with high confidence) inherent process and lot acceptance incapability. This is believed due to underlying statistical inferences that are being relied upon, which are undermined by the binary type distribution which kernel-based gluten contamination has been shown to cause.

Table 3 expands on this, showing the probability of selecting a contaminated serving in one to fifty tries for various rates of contamination present. This table is built on binomial distribution probabilities for pass/fail type outcomes (i.e. attribute-based sampling) and further shows how noncompliance can go undetected for a time when modest sampling efforts are employed. When nonconformance rates are as high as one in ten servings, examination of five servings under this scenario provides less than a 50/50 chance of randomly selecting a serving that contains a gluten kernel.

Attribute-based sampling guidelines

Sampling required to avoid this risk is also shown in Table 3. In the right most column are sample sizes

Table 2 Estimated gluten in 40 g of oats containing a kernel of North American varieties of wheat

	Hard Red Winter	Hard Red Spring	Soft Red Winter	Soft White	Northern Durum	Desert Durum
Thousand kernel weight (g)*	29.1	30.4	32.6	35.3	39.2	48
Weight/kernel (g)	0.0291	0.0304	0.0326	0.0353	0.0392	0.048
% Protein*	12.7	14.1	10	10	13.5	13.4
% Gluten level in protein†	80	80	80	80	80	80
Gluten content in 40 g of oats containing 1 wheat kernel (ppm)	74	86	65	71	106	129

*Five year average values reported in 2015 Crop Quality Report by US Wheat Associates, http://www.uswheat.org/cropQuality.
†Shewry (2009).

Table 3 Probabilities of randomly selecting one or more servings with a gluten-containing kernel

Assumed rate of servings with a gluten-containing kernel	Probability of selecting one or more contaminated servings in:								# of servings' worth required to obtain 95% confidence defect rate to left is not exceeded (where all would need to be found 'clean') (found using attribute acceptance sampling)
	1 Try	2 Tries	3 Tries	4 Tries	5 Tries	10 Tries	25 Tries	50 Tries	
1 in 10	0.1000	0.1900	0.2710	0.3439	0.4095	0.6513	0.9282	0.9948	29
1 in 15	0.0667	0.1289	0.1870	0.2412	0.2918	0.4984	0.8218	0.9682	44
1 in 25	0.0400	0.0784	0.1153	0.1507	0.1846	0.3352	0.6396	0.8701	74
1 in 50	0.0200	0.0396	0.0588	0.0776	0.0961	0.1829	0.3965	0.6358	149
1 in 100	0.0100	0.0199	0.0297	0.0394	0.0490	0.0956	0.2222	0.3950	298
1 in 200	0.0050	0.0100	0.0149	0.0199	0.0248	0.0489	0.1178	0.2217	599
1 in 500	0.0020	0.0040	0.0060	0.0080	0.0100	0.0198	0.0488	0.0953	1496
1 in 1000	0.0010	0.0020	0.0030	0.0040	0.0050	0.0100	0.0247	0.0488	2994

required to gain high confidence (i.e. 95% in this case) that various 'kernel-induced gluten noncompliance' rates are not being exceeded. These were derived using the same 'attribute-based acceptance sampling' as before (Taylor, 1992). These are large quantities, especially in comparison with continuous variable-based sampling, but provide high statistical confidence that products subject to 'kernel-based gluten' contamination are clean enough to be labelled gluten free.

For example, to affirm that the 'serving noncompliance rate' (i.e. rate of servings containing a gluten-containing kernel) is no greater than one in every 1000 servings with 95% confidence, one would have to look at 2994 servings and find them all clean to make that claim, doing so for a 'rationally defined' production lot. By 'rationally defined' is meant a lot that is relatively consistent in terms of the rate of kernel-based contamination, as might happen with oats from the same field potentially.

The extent of testing which attribute sampling requires is admittedly onerous, but appears necessary to accurately characterise the inherent capability to produce GF oatmeal at the serving level, and ensure outgoing quality is adequately controlled to protect CD consumers. More cost effective ways to accomplish this are clearly desirable, and research is underway in this direction.

Conclusion

We believe that GF foods, whose claim compliance is controlled at the 'serving level,' hold better chances to protect gluten-intolerant consumers and achieve brand differentiation. In that vein, our research here spotlights how wheat, rye and barley kernels act as 'gluten pills' in oatmeal, remaining intact to the spoon as indistinguishable flakes. And further, how this unique circumstance creates a binary-like set of possible gluten contamination outcomes at the serving level, namely servings with a contaminant kernel (being noncompliant) and those without (being compliant). Our investigation reveals how this situation impacts the sampling/assessment task, as extreme sets of outcomes like this undermine the commonly used sampling techniques of 'looking at a few' to 'draw inferences on the rest.' Findings suggest it prudent to consider a sampling/assessment task oriented towards characterising the 'rate of servings that possess gluten pills' instead of attempting to characterise 'mg kg^{-1} gluten' that might exist across 'representative' servings. The approach prescribed utilises attribute sampling. With this, one can gain high confidence that unacceptably high rates of gluten kernel contaminated servings are not getting onto store shelves, helping ensure processes are capably robust to the significant effects of kernel-based gluten contamination. But this assurance comes at a price in terms of sampling vigilance required, especially compared to what one could do given a more homogenously dispersed type of contamination like gluten dust or flour.

This situation has relevance as noncompliant gluten-free labelled products have been found on store shelves. This suggests incapable production processes are being viewed as capable, potentially due to this inferential nuance being overlooked. As we have seen, oversight of this can put CD consumers at risk, as they will occasionally ingest noncompliant servings measuring well over the FDA limit. It is the hope of this research to bring awareness, investigation, accounting and research to this subtle but important topic, and by doing so drive improvement towards higher integrity products for the growing gluten conscious marketplace. Furthermore, our consideration of measuring compliance at the serving size level may be instructive across other contamination-free claims in general, where kernel

contaminants are the source of contamination, for example GMO-free claims.

Conflicts of Interest

Ronald D. Fritz and Yumin Chen are salaried employees of PepsiCo, Inc. and have no competing interests. PepsiCo has funded this research and has a commercial interest in GF foods. However, the views expressed in this manuscript are those of the authors and do not necessarily reflect the position or policy of PepsiCo, Inc.

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FOOD COMPOSITION AND ADDITIVES

Validation Procedures for Quantitative Gluten ELISA Methods: AOAC Allergen Community Guidance and Best Practices

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The food allergen analytical community is endeavoring to create harmonized guidelines for the validation of food allergen ELISA methodologies to help protect food-sensitive individuals and promote consumer confidence. This document provides additional guidance to existing method validation publications for quantitative food allergen ELISA methods. The gluten-specific criterion provided in this document is divided into sections for information required by the method developer about the assay and information for the implementation of the multilaboratory validation study. Many of these recommendations and guidance are built upon the widely accepted Codex Alimentarius definitions and recommendations for gluten-free foods. The information in this document can be used as the basis of a harmonized validation protocol for any ELISA method for gluten, whether proprietary or nonproprietary, that will be submitted to AOAC and/or regulatory authorities or other bodies for status recognition. Future work is planned for the implementation of this guidance document for the validation of gluten methods and the creation of gluten reference materials.

Celiac disease occurs in genetically predisposed individuals, causing intolerance to storage proteins (gluten) of wheat, rye, barley, and possibly oats. It is estimated to affect approximately 1% of the population in developed countries (1), and scientific data indicate that its prevalence is on the rise (2). This intolerance to gluten is characterized by damage to the intestinal mucosa, resulting in malabsorption of nutrients (carbohydrates, protein, and fat), vitamins, and minerals. Celiac disease manifests as a complex array of symptoms, from classical intestinal irregularities to extraintestinal manifestations, such as iron-deficiency anemia, fatigue, osteoporosis, and peripheral neuropathy and ataxia (3, 4). Fortunately for most people, withdrawal of gluten-containing foods from the diet will reverse this damage and a significant recovery of the intestinal mucosa can be achieved (5). With the only prescription being avoidance of gluten in the diet, clear labeling of products to the presence of these proteins is essential for individuals to mitigate the risk and improve the quality of life for themselves and their families.

In order to help sensitive consumers identify products where gluten is absent, the Codex Alimentarius revised the Standard 118-1979 in 2008, which defines gluten and the requirements for use of a "gluten-free" statement (6). The standard states that below the level of 20 mg gluten/kg of food, a product can be labeled "gluten-free." Although many countries do not have regulated thresholds for gluten-free labeling, many follow this Codex Standard. For example, Canadian food regulatory authorities have proposed a threshold value that aligns with the Codex Alimentarius recommendation of 20 ppm of gluten protein (http://www.hc-sc.gc.ca/fn-an/securit/allerg/celcoe/gluten-position-eng.php) and the United States has recently done the same (http://www.gpo.gov/fdsys/pkg/FR-2013-08-05/pdf/2013-18813.pdf). Others like Australia consider gluten-free products those where gluten is non-detectable by current analytical methods and Brazil mandates the labeling of all food products either "gluten-free" or "contains gluten," in spite of

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not defining threshold levels or measures to ensure compliance. In the EU, Commission Regulation (EC) No. 41/2009, which is in part based on the Codex Standard, has been adopted (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:016:0003:0005:EN:PDF). All jurisdictions will require methods to confidently detect and quantify gluten in a variety of food matrixes in order to assess the validity of these "gluten-free" statements. In this regard, internationally accepted validation protocols will be important.

Gluten as it relates to celiac disease is defined in the Codex Alimentarius (6). From a legal point of view, "gluten is defined as a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 mol/L NaCl. Prolamins are defined as the fraction from gluten that can be extracted by 40–70% of ethanol. The prolamin from wheat is gliadin, from rye is secalin, from barley hordein, and from oats avenin. The prolamin content of gluten is generally taken as 50%." This definition shows that gluten is not an individual protein but a complex mixture of different, more or less related, proteins.

Numerous documents in the literature have addressed method validation in general and some address validation of ELISA methods for small molecules (7, 8). Recently, a document to address the specifics of food allergen analysis, including reference materials, spiking methods, and choice of matrix, has been published with an emphasis on milk and egg allergens (9). Methods for detecting the components of gluten have been available for approximately 20 years, and most of these methods use ELISA-based techniques to detect specific proteins of gluten in food matrixes (10, 11). The detection of gluten by ELISA is a unique analytical procedure characterized by the binding of specific antigens to antibodies. The specificity and sensitivity of commercial ELISA methods for gluten quantitation vary as they use different antibodies, which target different soluble protein types, rather than a specific protein or different epitopes on specific protein fractions. This mixture of target proteins will have diverse structural and chemical properties specific to the food matrix, and the ability of an ELISA method to detect gluten proteins in a test sample will be determined by a combination of the efficiency of these interactions and how well the proteins are extracted from the matrix. The fact that gluten-sensitive individuals react differently to soluble protein constituents of gluten further complicates the choice of targets (12). Furthermore, most food products are heat-treated in some fashion, which can have a significant influence on the solubility and extractability of the target proteins, as well as on the ability of the antibody or antibodies used in the ELISA to recognize them (13).

Considering all of these factors, there is a need to harmonize the validation of gluten methods in order to obtain confidence that a method is suitable for its intended purpose. Providing harmonized validation protocols for gluten will enable industry to use a validated method for their gluten control programs, labeling claims, and regulatory compliance. Important steps toward harmonization will require harmonized validation protocols, a commonly accepted gluten reference material, and at least one independent reference method to verify the routine methods. With all these measures in place, the harmonization of validation procedures for gluten ELISA methods will provide a level of confidence and acceptance of the results obtained from different methods.

This document is designed to accompany the AOAC Guidelines for *Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (14) and to provide specific guidance to the validation of quantitative ELISA-based methods for gluten. This protocol was designed to meet or exceed the minimum requirements set forth in Appendix D of the AOAC guidelines; it was developed with input from a wide range of experts in the area of gluten analysis, including the AOAC Food Allergens Community, the AOAC Gluten Working Group, and the Working Group on Prolamin Analysis and Toxicity. A recent publication provided some guidance for the validation of the performance characteristics of quantitative ELISA methods for food allergens (9) and was a guide for the development of this guidance document. This publication is intended to guide the collection of appropriate validation data for gluten ELISA methods that could be suitable for submission to AOAC INTERNATIONAL or regulatory bodies for scrutiny and recognition.

Required Information for Gluten ELISA Methods

Definition of Gluten

This document will provide guidance on the validation of ELISA methods for the quantification of gluten proteins in foods and raw materials. In this regard, it is essential to establish a practical definition of gluten so that subsequent sections and guidance can be developed. Many jurisdictions have adopted or are moving toward adopting the Codex recommendations to standardize gluten-free foods intended for people with celiac disease. Considering these efforts and the objective of this guidance document, the Codex definition of gluten will be used here, and is defined as a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 M NaCl (6). Additional information has been provided about the consumption of oats in a gluten-free diet. Based on current science, pure oats can be tolerated by the vast majority of people with celiac disease (15). Therefore, the allowance for uncontaminated oats (not contaminated with wheat, rye, or barley) in the dietary management of celiac disease and the need for methods to distinguish pure oats must be determined at the national level.

Antibodies

ELISA methods for gluten are based on the interactions of antibodies with certain gluten proteins or segments of gluten proteins, and some information should be supplied on these interactions. It should be known whether the antibody is mono- or polyclonal and if it targets a peptide sequence, a single protein, or multiple proteins. It should be known if the protein used to develop the antibodies was fractionated, modified, or synthesized in some way.

Information must also be supplied on the antibody's specificity to gluten-containing cereals, such as common wheat, barley, rye, and oats. Data on the cross-reactivity to other related grains, including but not limited to spelt wheat, khorasan wheat (Kamut™), triticale, durum wheat, einkorn wheat, and

Table 1. Items typically used in the manufacture of gluten-free products or products thought to be gluten-free[a]

Almond flour	Coffee	Guar gum	Potato flour/starch	Tapioca flour/starch
Amaranth flour	Corn starch/meal	Hazelnut flour	Quinoa flour	Tea
Arrowroot	Dried fruits	Lentil flour	Romano bean flour	White bean flour
Black bean flour	Egg powder	Lima bean flour	Sesame flour	White rice flour
Brown rice flour	Fava bean flour	Meats	Sorghum flour	Xanthan gum
Buckwheat flour	Flax seed flour/meal	Milk powder	Soya flour	Yellow pea flour
Chestnut flour	Garfava flour	Millet flour	Spices	
Coconut flour	Green pea flour	Oat flour	Sweet rice flour	

[a] These items should be tested for cross-reactivity as part of gluten ELISA development.

emmer wheat, may also be included. All of the cross-reactivity data needs to be expressed in relative terms or normalized to the response of common wheat because this will be the most important cereal used and calibrated against.

Cross-Reactivity

A positive response to a sample that does not contain any gluten is referred to as cross-reactivity, and the extent of cross-reactivity should be reported. ELISA developers must test their gluten methods for cross-reactivity in a selection of foods and ingredients, particularly those used in the production of gluten-free products. There is no recommended number of items that should be tested for cross-reactivity, but the more items tested the better the confidence in the assay. In addition, the testing should be on the products as they would normally be consumed (raw or cooked), and initially based on full-strength extracts, i.e., the extract obtained by applying the extraction procedure as prescribed by the manufacturer of the test kit. If a positive result is obtained, then dilutions should be performed to characterize the extent of cross-reactivity. Although there are a large variety of potential matrixes, a minimum list of food commodities that should be included in cross-reactivity testing for gluten is provided in Table 1.

Calibrators

The calibrators used to generate the calibration curve are a very important component of the quantitative ELISA and will be used to calculate the level of gluten in a sample, which will ultimately be used in making decisions. Ideally, the gluten community would accept a well-defined and characterized material for calibrating their assays or a material to reference their calibrators against. Gluten is a complex mixture of many proteins that have differing solubility, and the calibration standards must clearly define the target protein or fraction used to determine the level of gluten. Although there is some evidence to support the claim that both the prolamin and glutelin fractions of gluten are immuno-stimulatory in people with celiac disease (12), historically it has been the prolamin fraction that is extracted and used to indirectly measure total gluten content in a sample. It is known that the ratio of prolamin to glutelin in total gluten can be different depending on the cereal, and if the soluble fraction is being used, then it must be known how this calibrator relates to total gluten for the specific cereal (16).

A number of methods have been used to separate and characterize the different fractions of gluten and it should be known how the material used for the calibrator was characterized (17–19). Assay developers will need to specify what material they use as a calibrator: extract from flour, extract from purified gluten, purified protein fraction (prolamin or glutelin), or a peptide. If the assay is being validated for fermented or hydrolyzed food matrixes (e.g., beer, yogurt, soy sauce) the calibrator must be fit-for-purpose in regard to these types of hydrolyzed sources of gluten. The calibrators must represent the appropriate gluten fragment composition, with regard to the degree of hydrolysis, abundance, and length of the fragments for hydrolyzed gluten. All source information about the calibrator, whether for intact or hydrolyzed gluten, needs to be identified. This will include the grain(s) used, the cultivar(s), the commercial supplier, and the methods used to generate the calibrator(s). Another important component in any ELISA is the extraction buffer. The kit developer will need to describe any consequences in the analysis if there is a difference between the preparation of the calibrator(s) and the extraction of gluten using a proprietary extraction buffer.

Matrixes

Ideally, methods would be able to analyze all matrixes with equal reliability, but a method that is fit-for-purpose in one, or even several, matrixes may not be applicable in others. Gluten ELISA methods can be susceptible to matrix effects and have diminished performance due to interferences in some matrixes. This could be due to tannins in a sample or a certain degree of hydrolysis in some processes. Table 2 suggests matrixes that should be tested when a gluten ELISA method is being developed. The method developer should clearly identify which matrixes the method is fit-for-purpose on the basis of their in-house data, and identify any matrixes that diminish the method performance. Although incurred materials are preferred for validation studies (vide infra), a preliminary investigation before a multilaboratory study using spiked samples (direct spiking of gluten into the matrix) is acceptable due to the large number of materials tested. In these preliminary studies, it is important to determine whether the matrix has an influence on the measurement and, if so, the nature and magnitude of the effect.

LOQ, LOD, and Limit of Applicability (LLA)

Before conducting an interlaboratory study for gluten analysis, the LOD and the LOQ need to be determined. The

Table 2. Candidate food matrixes that could be tested when performing a multilaboratory validation study

Beer	Cereals	Energy/cereal bars	Oats	Sauce
Bread	Chips	Ice cream	Pasta	Soups
Breakfast cereals	Coated meat (baked)	Meat burger	Pies	Veggie burger
Cakes	Coated meat (fried)	Muffins	Salad dressing	Wine

LOD is defined as the lowest concentration of gluten that can be distinguished from a true blank. The LOD should be estimated by a statistical analysis of the calibration data according to the ISO standards for linear (20) and nonlinear data (21), with a default error probability of 0.05 for false positive (α) and false negative (β). The LOQ, on the other hand, is the lowest level of gluten in a sample that can be consistently quantified at a specific level of precision. Due to matrix, processing, and manufacturing variability, a kit developer may want to define the LLA rather than the LOQ. This will represent a level below which the method developer does not support or recommend the use of the method. Guidelines for single-laboratory method validations are available to assist determination of these parameters and any sources of possible variation (8). In order to obtain robust estimates for LOD/LOQ/LLA, it is recommended that data be collected in single-laboratory studies from at least three analysts over a minimum of 3 different days and preferably using at least two different instruments.

Ruggedness and Lot-to-Lot Variability

The determination of the ruggedness, or robustness, of an assay is a measure of its capacity to remain unaffected by small variations in procedural parameters. These types of experiments are investigated during method development and are reported in the assay documentation. Some parameters important to the end-user and final assay results will be the variation in reagent volumes, reagent concentrations (those prepared by end-user), extraction time and temperature, and incubation time and temperature. It is recommended that deviations for time and volume be investigated at ±5 to 10%, and incubation temperatures tried at ±3 to 5°C. Once the experimental variation that provides consistent results is known, the limits of these parameters must be included in the assay documentation. Other parameters that are important and must be tested and reported are the shelf life and stability of all reagents and components in the test kit, as well as their storage parameters. An expiration date for each of these components of the test kit, as well as of the kit itself, should be clearly indicated. A small number of test kits from each lot should be set aside for comparison with future lots to determine if any characteristics of the assay have changed. For example, a positive control sample, such as an incurred test sample or spiked sample, should be analyzed with each new lot to be sure that consistent results are achieved. Information on the lot-to-lot variability should be provided by the kit manufacturer as part of the data submission package.

Interlaboratory Validation Study

Key Elements for Laboratories and Samples

The key elements for an interlaboratory validation study have already been described for food allergen ELISA and will only be briefly detailed here in order to make specific recommendations for gluten analysis (9). It is important to obtain enough statistically relevant information from an interlaboratory study; a minimum of eight laboratories contributing usable data is required, but it is recommended that more laboratories are recruited so that enough usable data are available for the study. It is also recommended that no more than one-fourth of the total number of laboratories contributing data be from the same organization.

The initial interlaboratory validation study must evaluate the method for a minimum of two matrixes. Each matrix set must contain a blank and have four concentration levels, one level

Table 3. Theoretical raw data randomly generated in a collaborative study for a gluten ELISA with a stated LOQ of 5.0 mg/kg and an upper range of 100 mg/kg[a]

	0 mg/kg		2.5 mg/kg		10 mg/kg		40 mg/kg		80 mg/kg	
Lab	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B
1	–0.68	–0.19	2.84	3.07	8.11	11.63	46.99	36.94	73.52	72.18
2	–0.87	0.68	2.87	2.66	10.78	11.74	33.61	40.40	69.71	90.86
3	–0.66	–0.27	3.10	3.18	12.93	10.02	32.64	38.66	80.48	95.33
4	–0.62	–0.08	2.06	2.29	9.82	10.10	50.11	42.87	97.07	76.01
5	–0.60	–0.30	2.92	2.64	12.13	12.18	50.10	44.08	100.50	79.08
6	–0.82	–0.67	3.25	2.84	8.37	10.35	32.52	51.95	80.81	82.14
7	–0.68	0.44	2.12	2.18	10.74	9.36	47.49	49.72	65.96	81.96
8	0.52	–0.10	3.05	2.02	11.67	8.22	34.85	48.08	74.31	102.03
9	–0.09	0.05	2.88	1.95	9.04	9.80	33.22	48.27	76.06	89.94
10	–0.78	–0.53	2.28	2.41	10.68	11.25	36.36	34.37	84.29	94.59

[a] The study involved 10 laboratories each analyzing a blank and four concentration levels in duplicate (A and B) for a total of 10 samples.

Table 4. Calculated results for the hypothetical interlaboratory validation study in Table 3

	0 mg/kg	2.5 mg/kg	10 mg/kg	40 mg/kg	80 mg/kg
Total No.of laboratories	10	10	10	10	10
Total replicates	20	20	20	20	20
Overall mean	0.31	2.63	10.45	41.66	83.34
Repeatability SD (s_r)	0.50	0.34	1.42	7.10	12.03
Reproducibility SD (s_R)	0.46	0.43	1.38	7.09	10.74
Repeatability RSD (RSD_r)	158.7	13.0	13.6	17.1	14.4
Reproducability RSD (RSD_R)	147.5	16.4	13.2	17.0	12.9

of which should be below the LOQ or LLA concentration. One of the concentration levels should be at the lower end of the calibration curve, below two times the LOQ or LLA stated for the method. The remaining nonzero levels should be evenly distributed throughout the range of the calibration curve. For example, if a single-laboratory investigation (vive supra) determines the LOQ of the assay to be 5 mg/kg and the upper limit to be 100 mg/kg, then the recommended levels for the interlaboratory study would be 0, 2.5, 10, 40, and 80 mg/kg in each of the two matrixes chosen from Table 2. All samples will be tested as blind duplicates, so that each laboratory in the study will analyze 20 individual test portions (2 matrixes × 5 levels × 2 blind duplicates).

Data Analysis for Interlaboratory Studies

The ISO standards (22), AOAC *Official Methods of Analysis* (7), and a guidance document for the validation of food allergen ELISA methods (9) outline how to analyze the data from an interlaboratory study. In general, each matrix/level combination should be treated as a separate experiment and analyzed as such. Results obtained from blank samples should not be censored in any way. It is very important to evaluate the correct mean and distribution of the blank sample results in order to have an unbiased estimate for LOD and LOQ. As such, negative values should be treated unchanged and not censored to zero. Likewise, any thresholds, such as LOQ or LLA limits, should not be applied to collaborative study data sets. Participating laboratories should be instructed to report all results as calculated, and to not report as "ND" or "<LOQ."

The initial step in the workflow is to remove any outliers by sequential use of the Cochran and Grubbs tests, as indicated in AOAC *Official Methods of Analysis*, Appendix D (14). The mean, accuracy (if applicable), repeatability (S_r), reproducibility (S_R), RSD of repeatability (RSD_r), and RSD of reproducibility (RSD_R) should be calculated and reported. If the variance is found to be constant and normally distributed, then the LOD and LOQ can be estimated as 3.3 times and 10 times the SD of the distribution of blank results, respectively. If the variance is not constant with concentration, then a more accurate method of estimating the LOD and LOQ can be used (9). Table 3 gives an example of data generated in a hypothetical collaborative study. The levels were chosen based on the LOQ (5 mg/kg) and the upper limit of the calibration curve (100 mg/kg) determined from a pre-interlaboratory study. From these raw data, all the necessary information can be calculated in order to determine the LOD and LOQ for the assay in this hypothetical matrix (Table 4). Figure 1 shows that the reproducibility (S_R) in this study increases with the mean concentration, and an advanced formula is recommended to better estimate the LOD and LOQ. This formula uses the slope (0.131), the intercept (0.400), and the overall mean for the zero level (0.313) to calculate the LOD = ($\bar{x}$ (0) + 3.3 × intercept)/(1 – [1.65 × slope]), LOD = 2.20 mg/kg, and LOQ = 3 × LOD = 6.61 mg/kg.

This information can now be used to construct an operational curve for this assay and matrix combination. An example of such an operational curve is given in Figure 2, from which the probability of obtaining a result higher than the LOQ can be determined based on the concentration in the sample. For example, if the concentration of gluten in the sample was 10 mg/kg, there would be a 97.5% probability that the sample measurement could be higher than the estimated LOQ of 6.61 mg/kg.

Gluten-Specific Criteria

Reference Materials

The term "gluten" is used differently depending on the field of research. Historically, gluten is a highly complex mixture of proteins defined by their solubility. In 1907, T.B. Osborne defined gluten as being the protein fraction of the wheat kernel is not soluble in water or dilute salts, and this definition has carried through to this day. In terms of celiac disease, the Codex Alimentarius Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten (6), defines gluten as "a protein

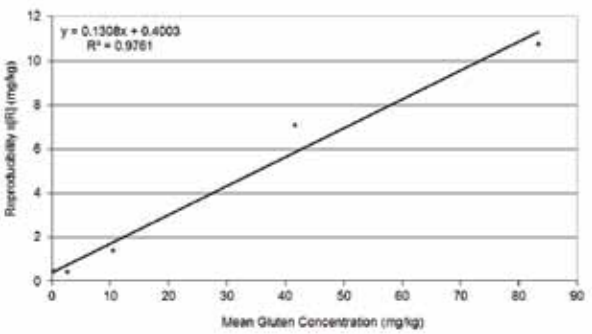

Figure 1. Plot of reproducibility versus the global mean observed gluten concentration for the hypothetical interlaboratory study.

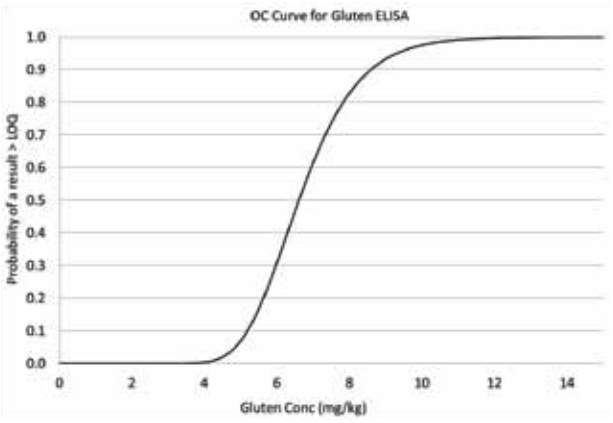

Figure 2. Operational curve calculated from the results of the theoretical interlaboratory validation study.

fraction from wheat, rye, barley, oats, or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 mol/L NaCl." Within the total gluten fraction, Osborne also defined two further protein subclasses referred to as the prolamins, which are soluble in aqueous alcohols, and glutelins, which are soluble in dilute acids, alkali, or in the presence of reducing agents. Although a good deal of clinical work has defined epitopes or fractions of the prolamin and glutelin proteins as contributing to the onset or exacerbation of gluten intolerance (celiac disease), the effects of all fractions are currently unknown.

To obtain the level of gluten in a sample, an assay will have a set of calibrators to generate a standard curve to which a response obtained on a sample can be related. These calibrators will be correlated to a gluten reference material in order to convert to a level of gluten in the sample. These reference materials may also be used to develop incurred materials or for spiking into blank food matrixes during a validation study. It would be recommended that a commercially available, well-characterized reference material be used for these purposes, but these materials are sometimes not available. To date the best characterized reference material is the so-called Prolamin Working Group-gliadin (19), which can be obtained from the Working Group on Prolamin Analysis and Toxicity (http://www.wgpat.com/index.html). Although this material is well-characterized, it represents only one fraction of total gluten and total cereal proteins. Currently, most commercial gluten ELISA methods detect the prolamin fraction. As the science around celiac disease is evolving, efforts should be made to develop and characterize an improved reference material containing all gluten protein fractions based on the Codex definition of gluten. This reference material would be of particular importance if ELISA methods able to detect both prolamins and glutelins become available.

Using an extracted gluten source instead of one minimally processed (e.g., cereal flour) can be controversial. Both materials are produced from a natural source and are subject to batch-to-batch variation, requiring a full characterization for every new batch. The extracted material might lack individual protein components present in a minimally processed sample. However, with respect to good laboratory practice, it would be desirable to have the reference material available in a soluble form to facilitate routine work. This soluble form could be achieved for an extracted gluten source but not for a native sample, which would require additional extraction and centrifugation steps before use. Obviously, the production of a common reference material for partially hydrolyzed gluten can be produced only from an extracted gluten source. Finally, a commonly accepted reference material should also be suitable for clinical studies to link toxicity of the material to a concentration.

For isolating the gluten fraction from wheat, the following protocol may be useful. Wheat flour is mixed to a dough, which is then washed with NaCl solution to remove starch and soluble proteins, leaving a gluten mass that can be dried and weighed after being tested for the absence of residual starch. Different organizations provide standard protocols for isolating wet and dry gluten, either by hand-washing or automatically by a Glutomatic machine (23–30). It has been suggested to remove the excess NaCl washing solution, which affects the final weight of the gluten, by additional washing with water. For the development of a gluten reference material this would not be recommended due to the potential loss of the ω-gliadin fraction. If only the weight of the gluten has to be determined, drying by heating can be carried out, but if the material is intended to be used as a reference, it should be dried by lyophilization. Because of the differences in gluten-containing cereals that are toxic to an individual with celiac disease, it will be necessary to characterize separate reference materials for wheat, barley, and rye.

Spiking Methods

In a validation study, the best source of information for the detection and quantification of the level of gluten will come from incurred samples. These samples will have a known amount of gluten incorporated into the product and will undergo processes similar to those used commercially. When incurred samples are included in a validation study, it is important that information be included about these materials. This information should include how the materials were prepared, including the recipe and preparation conditions, how they were characterized, homogeneity experiments, and analytical methodologies used. There are numerous matrixes, and it may be difficult to obtain incurred materials for gluten validation studies; therefore, spiked samples will be considered an acceptable way to prepare materials to generate performance data in a specific matrix. The preferred method of spiking will involve the fortification of a large batch of matrix with a high level of gluten followed by serial dilution with the blank matrix material. As with the incurred materials, the particulars of the preparation should be described in the validation, as well as the results of homogeneity studies. In some matrixes, burgers and sausages, for example, it will be difficult to prepare consistent serial dilutions from a master sample, and direct spiking of a gluten reference material may be required to determine how the matrix will affect the result.

Food Matrixes

The matrixes being analyzed can have a large impact on the performance of an ELISA method. When validating an ELISA method for gluten, it is advisable to determine how the kit performs with those matrixes that are important foods for

individuals following a gluten-free diet. Some of these foods are shown in Table 2, and represent potential products that could have cross-contaminated starting materials. This list is not exhaustive and there may be examples more appropriate for a specific jurisdiction.

Conclusions

Intolerance to gluten (celiac disease) requires the strict avoidance of gluten sources from wheat, barley, rye, or their crossbred species. Codex Alimentarius recommends that for a food to be labeled gluten-free, it must not contain more than 20 mg gluten/kg of food. The food allergen analytical community is endeavouring to create harmonized guidelines for food allergen ELISA methodologies to help protect food-sensitive consumers and promote consumer confidence. The guidance described here reflects a consensus reached, through input and collaboration, from various allergen analytical experts, and contains specific recommendations and requirements for the validation of ELISA methods for the detection and quantification of gluten proteins in food matrixes. Future work is planned for the implementation of this guidance document for the validation of gluten methods and the creation of gluten reference materials.

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Appendix F: Guidelines for *Standard Method Performance Requirements*

Contents

Introduction to *Standard Method Performance Requirements*

Standard Method Performance Requirements (SMPRs®) are a unique and novel concept for the analytical methods community. SMPRs are voluntary consensus standards, developed by stakeholders, that prescribe the minimum analytical performance requirements for classes of analytical methods. In the past, analytical methods were evaluated and the results compared to a "gold standard" method, or if a gold standard method did not exist, then reviewers would decide retrospectively if the analytical performance was acceptable. Frequently, method developers concentrated on the process of evaluating the performance parameters of a method, and rarely set acceptance criteria. However, as the *Eurachem Guide* points out: " . . . the judgment of method suitability for its intended use is equally important . . ." (1) to the evaluation process.

International Voluntary Consensus Standards

An SMPR is a form of an international, voluntary consensus standard. A standard is an agreed, repeatable way of doing something that is published as document that contains a technical specification or other precise criteria designed to be used consistently as a rule, guideline, or definition. SMPRs are a *consensus* standards developed by stakeholders in a very controlled process that ensures that users, research organizations, government departments, and consumers work together to create a standard that meets the demands of the analytical community and technology. SMPRs are also *voluntary* standards. AOAC cannot, and does not, impose the use of SMPRs. Users are free to use SMPRs as they see fit. AOAC is very careful to include participants from as many regions of the world as possible so that SMPRs are accepted as *international* standards.

Guidance for Standard Method Performance Requirements

Commonly known as the "SMPR Guidelines." The first version of the SMPR Guidelines were drafted in 2010 in response to the increasing use and popularity of SMPRs as a vehicle to describe the analytical requirements of a method. Several early "acceptance criteria" documents were prepared for publication in late 2009, but the format of the acceptance criteria documents diverged significantly from one another in basic format. AOAC realized that a guidance document was needed to promote uniformity.

An early version of the SMPR Guidelines were used for a project to define the analytical requirements for endocrine disruptors in potable water. The guidelines proved to be extremely useful in guiding the work of the experts and resulted in uniform SMPRs. Subsequent versions of the SMPR Guidelines were used in the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) project with very positive results. The SMPR Guidelines are published in the *Journal of AOAC INTERNATIONAL* and *Official Methods of Analysis*.

Users of the guidelines are advised that they are: (*1*) a *guidance* document, not a statute that users must conform to; and (*2*) a "living" document that is regularly updated, so users should check the AOAC website for the latest version before using these guidelines.

The SMPR Guidelines are intended to provide basic information for working groups assigned to prepare SMPRs. The guidelines consist of the standard format of an SMPR, followed by a series of informative tables and annexes.

SMPR Format

The general format for an SMPR is provided in *Annex A*.

Each SMPR is identified by a unique SMPR number consisting of the year followed by a sequential identification number (YYYY.XXX). An SMPR number is assigned when the standard is approved. By convention, the SMPR number indicates the year a standard is approved (as opposed to the year the standard is initiated). For example, SMPR 2010.003 indicates the third SMPR adopted in 2010.

The SMPR number is followed by a method name that must include the analyte(s), matrix(es), and analytical technique (unless the SMPR is truly intended to be independent of the analytical technology). The method name may also refer to a "common" name (e.g., "Kjeldahl" method).

The SMPR number and method name are followed by the name of the stakeholder panel or expert review panel that approved the SMPR, and the approval and effective dates.

Information about method requirements is itemized into nine categories: (*1*) intended use; (*2*) applicability; (*3*) analytical technique; (*4*) definitions; (*5*) method performance requirements; (*6*) system suitability; (*7*) reference materials; (*8*) validation guidance; and (*9*) maximum time-to-determination.

An SMPR for qualitative and/or identification methods may include up to three additional annexes: (*1*) inclusivity/selectivity panel; (*2*) exclusivity/cross-reactivity panel; and (*3*) environmental material panels. These annexes not required.

Informative tables.—The SMPR Guidelines contain seven informative tables that represent the distilled knowledge of many years of method evaluation, and are intended as guidance for SMPR working groups. The informative tables are not necessarily AOAC

policy. SMPR working groups are expected to apply their expertise in the development of SMPRs.

Table A1: Performance Requirements. Provides recommended performance parameters to be included into an SMPR. Table A1 is organized by five method classifications: (*1*) main component quantitative methods; (*2*) trace or contaminant quantitative methods; (*3*) main component qualitative methods; (*4*) trace or contaminant quantitative methods; and (*5*) identification methods. The table is designed to accommodate both microbiological and chemical methods. Alternate microbiological/chemical terms are provided for equivalent concepts.

Table A2: Recommended Definitions. Provides definitions for standard terms in the SMPR Guidelines. AOAC relies on *The International Vocabulary of Metrology Basic and General Concepts and Associated Terms* (VIM) and the International Organization for Standadization (ISO) for definition of terms not included in Table A2.

Table A3: Recommendations for Evaluation. Provides general guidance for evaluation of performance parameters. More detailed evaluation guidance can be found in *Appendix D, Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (2); *Appendix I, Guidelines for Validation of Biological Threat Agent Methods and/or Procedures* (3); *Appendix K, AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* (4); Codex Alimentarius Codex Procedure Manual (5); and ISO Standard 5725-1-1994 (6).

Table A4: Expected Precision (Repeatability) as a Function of Analyte Concentration. The precision of a method is the closeness of agreement between independent test results obtained under stipulated conditions. Precision is usually expressed in terms of imprecision and computed as a relative standard deviation (RSD) of the test results. The imprecision of a method increases as the concentration of the analyte decreases. This table provides target RSDs for a range of analyte concentrations.

Table A5: Expected Recovery as a Function of Analyte Concentration. Recovery is defined as the ratio of the observed mean test result to the true value. The range of the acceptable mean recovery expands as the concentration of the analyte decreases. This table provides target mean recovery ranges for analyte concentrations from 1 ppb to 100%.

Table A6: Predicted Relative Standard Deviation of Reproducibility ($PRSD_R$). This table provides the calculated $PRSD_R$ using the Horwitz formula:

$$PRSD_R = 2C^{-0.15}$$

where C is expressed as a mass fraction.

Table A7: POD and Number of Test Portions. This table provides the calculated probability of detection (POD) for given sample sizes and events (detections). A method developer can use this table to determine the number of analyses required to obtain a specific POD.

Informative annexes.—The SMPR Guidelines contain informative annexes on the topics of classification of methods, POD model, HorRat values, reference materials, and method accuracy and review. As with the informative tables, these annexes are intended to provide guidance and information to the working groups.

Initiation of an SMPR

See Figure 1 for a schematic flowchart diagram of the SMPR development process.

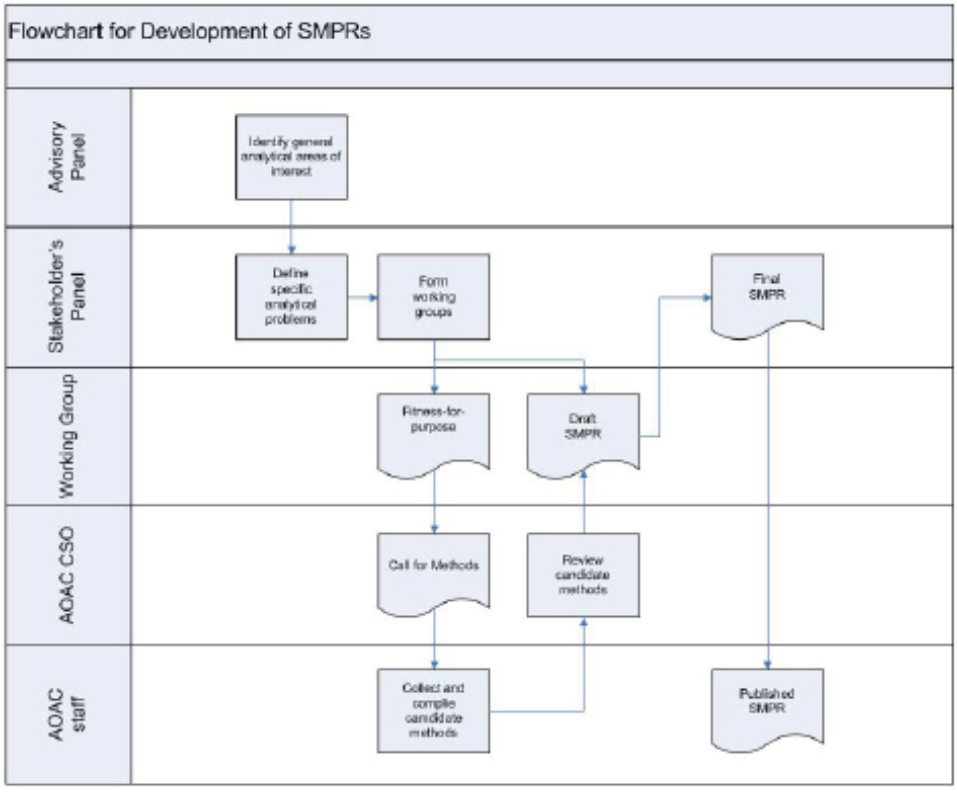

Figure 1. Schematic flowchart diagram of the SMPR development process.

Advisory panels.—Most commonly, an SMPR is created in response to an analytical need identified by an advisory panel. Advisory panels normally consist of sponsors and key stakeholders who have organized to address analytical problems. Usually, the advisory panel identifies general analytical problems, such as the need to update analytical methods for determination of nutrients in infant formula. An advisory panel, with the input of appropriate subject matter experts, also prioritizes the specific analytical problems within the general topic. This panel is critical in planning for the stakeholder panel meeting.

Stakeholder panels.—After an advisory panel has identified a general analytical problem, AOAC announces the standards development activity, identifies stakeholders, and organizes a stakeholder panel. Membership on a stakeholder panel is open to anyone materially affected by the proposed standard. AOAC recruits scientists to participate on stakeholder panels on the basis of their expertise with the analytical problem identified by the advisory panel. Experts are recruited from academia, government, nongovernmental organizations (such as ISO), industry, contract research organizations, method developers, and instrument/equipment manufacturers. AOAC employs a representative voting panel model to ensure balance with regards to stakeholder perspective, and to ensure that no particular stakeholder perspective dominates the proceedings of the stakeholder panel. All stakeholder candidates are reviewed by the AOAC Chief Scientific Officer (CSO) for relevant qualifications, and again by the Official Methods Board to ensure that the stakeholder panel is balanced and all stakeholders are fairly represented.

Stakeholder panels are extremely important as they serve several functions: (*1*) identify specific analytical topics within the general analytical problem described by the advisory panel; (*2*) form working groups to address the specific analytical topics; (*3*) identify additional subject matter experts needed for the working groups; (*4*) provide oversight of the SMPR development; and (*5*) formally adopt SMPRs originally drafted by working groups.

Working groups.—Working groups are formed by the stakeholder panel when a specific analytical topic has been identified. The primary purpose of a working group is to draft an SMPR. Working groups may also be formed to make general recommendations, such as developing a common definition to be used by multiple working groups. For example, SPIFAN formed a working group to create a definition for "infant formula" that could be shared and used by all of the SPIFAN working groups.

The process of drafting an SMPR usually requires several months, and several meetings and conference calls. An SMPR drafted by a working group is presented to a stakeholder panel. A stakeholder panel may revise, amend, or adopt a proposed SMPR on behalf of AOAC.

Fitness-for-Purpose Statement and Call for Methods

One of the first steps in organizing a project is creating a fitness-for-purpose statement. In AOAC, the fitness-for-purpose statement is a very general description of the methods needed. It is the responsibility of a working group chair to draft a fitness-for-purpose statement. A working group chair is also asked to prepare a presentation with background information about the analyte, matrix, and the nature of the analytical problem. A working group chair presents the background information and proposes a draft fitness-for-purpose statement to the presiding stakeholder panel. The stakeholder panel is asked to endorse the fitness-for-purpose statement.

The AOAC CSO prepares a call for methods based on the stakeholder panel-approved fitness-for-purpose statement. The call for methods is posted on the AOAC website and/or e-mailed to the AOAC membership and other known interested parties. AOAC staff collects and compiles candidate methods submitted in response to the call for methods. The CSO reviews and categorizes the methods.

Creating an SMPR

Starting the process of developing an SMPR can be a daunting challenge. In fact, drafting an SMPR should be a daunting challenge because the advisory panel has specifically identified an analytical problem that has yet to be resolved. Completing an SMPR can be a very rewarding experience because working group members will have worked with their colleagues through a tangle of problems and reached a consensus where before there were only questions.

It is advisable to have some representative candidate methods available for reference when a working group starts to develop an SMPR. These methods may have been submitted in response to the call for methods, or may be known to a working group member. In any case, whatever the origin of the method, candidate methods may assist working group members to determine reasonable performance requirements to be specified in the SMPR. The performance capabilities of exisiting analytical methodologies is a common question facing a working group.

Normally, a working chair and/or the AOAC CSO prepares a draft SMPR. A draft SMPR greatly facilitates the process and provides the working group with a structure from which to work.

Working group members are advised to first consider the "intended use" and "maximum time-to-determination" sections as this will greatly affect expectations for candidate methods. For example, methods intended to be used for surveillance probably need to be quick but do not require a great deal of precision, and false-positive results might be more tolerable. Whereas methods intended to be used for dispute resolution will require better accuracy, precision, and reproducibility, but time to determination is not as important.

Once a working group has agreed on the intended use of candidate methods, then it can begin to define the applicability of candidate methods. The applicability section of the SMPR is one of the most important, and sometimes most difficult, sections of the SMPR. The analyte(s) and matrixes must be explicitly identified. For chemical analytes, International Union of Pure and Applied Chemistry (IUPAC) nomenclature and/or Chemical Abstracts Service (CAS) registry numbers should be specified. Matrixes should be clearly identified including the form of the matrix such as raw, cooked, tablets, powders, etc. The nature of the matrix may affect the specific analyte. It may be advantageous to fully identify and describe the matrix before determining the specific analyte(s). It is not uncommon for working groups to revise the initial definition of the analyte(s) after the matrix(es) has been better defined.

Table 1. Example of method performance table for a single analyte

Analytical range	7.0–382.6 µg/mL	
Limit of quantitation (LOQ)	≤7.0 µg/mL	
Repeatability (RSD_r)	<10 µg/mL	≤8%
	≥10 µg/mL	≤6%

Table 2. Example of method performance table for multiple analytes

	Analyte 1		Analyte 2		Analyte 3	
Analytical range	10–20 µg/mL		100–200 µg/mL		200–500 µg/mL	
Limit of quantitation (LOQ)	≤10 µg/mL		≤100 µg/mL		≤200 µg/mL	
Repeatability (RSD_r)	<10 µg/mL	≤8%	<10 µg/mL	≤8%	<200 µg/mL	≤10%
	≥10 µg/mL	≤6%	≥10 µg/mL	≤6%	≥200 µg/mL	≤8%

For projects with multiple analytes, for example, vitamins A, D, E, and K in infant formula, it may be useful to organize a separate working group to fully describe the matrix(es) so that a common description of the matrix(es) can be applied to all of the analytes.

For single analyte SMPRs, it is most common to organize the method performance requirements into a table with 2–3 columns as illustrated in Table 1. For multiple analyte SMPRs, it is often convenient to present the requirements in an expanded table with analytes forming additional columns as illustrated in Table 2.

Once the intended use, analytical techniques, and method performance requirements have been determined, then a working group can proceed to consider the quality control parameters, such as the minimum validation requirements, system suitability procedures, and reference materials (if available). It is not uncommon that an appropriate reference material is not available. *Annex F* of the SMPR Guidelines provides comprehensive guidance for the development and use of in-house reference materials.

Most working groups are able to prepare a consensus SMPR in about 3 months.

Open Comment Period

Once a working group has produced a draft standard, AOAC opens a comment period for the standard. The comment period provides an opportunity for other stakeholders to state their perspective on the draft SMPR. All collected comments are reviewed by the AOAC CSO and the working group chair, and the comments are reconciled. If there are significant changes required to the draft standard as a result of the comments, the working group is convened to discuss and any unresolved issues will be presented for discussion at the stakeholder panel meeting.

Submission of Draft SMPRs to the Stakeholder Panel

Stakeholder panels meet several times a year at various locations. The working group chair (or designee) presents a draft SMPR to the stakeholder panel for review and discussion. A working group chair is expected to be able to explain the conclusions of the working group, discuss comments received, and to answer questions from the stakeholder panel. The members of the stakeholder panel may revise, amend, approve, or defer a decision on the proposed SMPR. A super majority of 2/3 or more of those voting is required to adopt an SMPR as an AOAC voluntary consensus standard.

Publication

Adopted SMPRs are prepared for publication by AOAC staff, and are published in the *Journal of AOAC INTERNATIONAL* and in the AOAC *Official Methods of Analysis*℠ compendium. Often, the AOAC CSO and working group chair prepare a companion article to introduce an SMPR and describe the analytical issues considered and resolved by the SMPR. An SMPR is usually published within 6 months of adoption.

Conclusion

SMPRs are a unique and novel concept for the analytical methods community. SMPRs are voluntary, consensus standards developed by stakeholders that prescribe the minimum analytical performance requirements for classes of analytical methods. The SMPR Guidelines provide a structure for working groups to use as they develop an SMPR. The guidelines have been employed in several AOAC projects and have been proven to be very useful. The guidelines are not a statute that users must conform to; they are a "living" document that is regularly updated, so users should check the AOAC website for the latest version before using the guidelines.

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(5) Codex Alimentarius Codex Procedure Manual

(6) International Organization for Standardization, Geneva, Switzlerland

ANNEX A
Format of a
Standard Method Performance Requirement

AOAC SMPR® YYYY.XXX
(YYYY = Year; XXX = sequential identification number)

Method Name: Must include the analyte(s), matrix(es), and analytical technique [unless the *Standard Method Performance Requirement* (SMPR®) is truly intended to be independent of the analytical technology]. The method name may refer to a "common" name (e.g., "Kjeldahl" method).

Approved By: Name of stakeholder panel or expert review panel

Final Version Date: Date

Effective Date: Date

1. Intended Use: Additional information about the method and conditions for use.

2. Applicability: List matrixes if more than one. Provide details on matrix such as specific species for biological analytes, or International Union of Pure and Applied Chemistry (IUPAC) nomenclature and Chemical Abstracts Service (CAS) registry number for chemical analytes. Specify the form of the matrix such as raw, cooked, tablets, powders, etc.

3. Analytical Technique: Provide a detailed description of the analytical technique if the SMPR is to apply to a specific analytical technique; or state that the SMPR applies to any method that meets the method performance requirements.

4. Definitions: List and define terms used in the performance parameter table (*see* Table A2 for list of standard terms).

5. Method Performance Requirements: List the performance parameters and acceptance criteria appropriate for each method/analyte/matrix. *See* Table A1 for appropriate performance requirements.

If more than one analyte/matrix, and if acceptance criteria differ for analyte/matrix combinations then organize a table listing each analyte/matrix combination and its minimum acceptance criteria for each performance criteria.

6. System Suitability Tests and/or Analytical Quality Control: Describe minimum system controls and QC procedures.

7. Reference Material(s): Identify the appropriate reference materials if they exist, or state that reference materials are not available. Refer to *Annex E* (*AOAC Method Accuracy Review*) for instructions on the use of reference materials in evaluations.

8. Validation Guidance: Recommendations for type of evaluation or validation program such as single-laboratory validation (SLV), *Official Methods of Analysis*℠ (OMA), or *Performance Tested Methods*℠ (PTM).

9. Maximum Time-to-Determination: Maximum allowable time to complete an analysis starting from the test portion preparation to final determination or measurement.

Annex I: Inclusivity/Selectivity Panel. Recommended for qualitative and identification method SMPRs.

Annex II: Exclusivity/Cross-Reactivity Panel. Recommended for qualitative and identification method SMPRs.

Annex III: Environmental Materials Panel. Recommended for qualitative and identification method SMPRs.

Table A1. Performance requirements

Classifications of methods[a]				
Quantitative method		Qualitative method		
Main component[b]	Trace or contaminant[c]	Main component[b]	Trace or contaminant[c]	Identification method
Parameter				
Single-laboratory validation				
Applicable range	Applicable range	Inclusivity/selectivity	Inclusivity/selectivity	Inclusivity/selectivity
Bias[d]	Bias[d]	Exclusivity/cross-reactivity	Exclusivity/cross-reactivity	Exclusivity/cross-reactivity
Precision	Precision	Environmental interference	Environmental interference	Environmental interference
Recovery	Recovery	Laboratory variance	Laboratory variance	
Limit of quantitation (LOQ)	LOQ			
		Probability of detection (POD)[e]	POD at AMDL[f]	Probability of identification (POI)
Reproducibility				
RSD_R or target measurement uncertainty	RSD_R or target measurement uncertainty	POD (0)	POD (0)	POI (c)
		POD (c)	POD (c)	
		Laboratory POD[g]	Laboratory POD[g]	Laboratory POI

[a] *See Annex B* for additional information on classification of methods.

[b] ≥100 g/kg.

[c] <100 g/kg.

[d] If a reference material is available.

[e] At a critical level.

[f] AMDL = Acceptable minimum detection level.

[g] LPOD = CPOD.

Table A2. Recommended definitions

Bias	Difference between the expectation of the test results and an accepted reference value. Bias is the total systematic error as contrasted to random error. There may be one or more systematic error components contributing to the bias.
Environmental interference	Ability of the assay to detect target organism in the presence of environmental substances and to be free of cross reaction from environmental substances.
Exclusivity	Strains or isolates or variants of the target agent(s) that the method must not detect.
Inclusivity	Strains or isolates or variants of the target agent(s) that the method can detect.
Laboratory probability of detection (POD)	Overall fractional response (mean POD = CPOD) for the method calculated from the pooled POD_j responses of the individual laboratories (j = 1, 2, ..., L).[a] *See Annex C*.
Limit of quantitation (LOQ)	Minimum concentration or mass of analyte in a given matrix that can be reported as a quantitative result.
POD (0)	Probability of the method giving a (+) response when the sample is truly without analyte.
POD (c)	Probability of the method giving a (–) response when the sample is truly without analyte.
POD	Proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. Consult *Annex C* for a full explanation.
Probability of identification (POI)	Expected or observed fraction of test portions at a given concentration that gives positive result when tested at a given concentration. Consult *Probability of Identification (POI): A Statistical Model for the Validation of Qualitative Botanical Identification Methods*.[c]
Precision	Closeness of agreement between independent test results obtained under stipulated conditions. The measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results.[d]
Recovery	Fraction or percentage of the analyte that is recovered when the test sample is analyzed using the entire method. There are two types of recovery: (*1*) Total recovery based on recovery of the native plus added analyte, and (*2*) marginal recovery based only on the added analyte (the native analyte is subtracted from both the numerator and denominator).[e]
Repeatability	Precision under repeatability conditions.
Repeatability conditions	Conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.
Reproducibility	Precision under reproducibility conditions.
Reproducibility conditions	Conditions where independent test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.
Relative standard deviation (RSD)	$RSD = s_i \times 100/\bar{x}$
Standard deviation (s_i)	$s_i = [\Sigma(x_i - \bar{x})^2/n]^{0.5}$

[a] *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures* (*Calculation of CPOD and dCPOD Values from Qualitative Method Collaborative Study Data*), *J. AOAC Int.* **94**, 1359(2011) and *Official Methods of Analysis of AOAC INTERNATIONAL* (current edition), *Appendix I*.

[b] *International Vocabulary of Metrology (VIM)—Basic and General Concepts and Associated Terms* (2008) JCGM 200:2008, Joint Committee for Guides in Metrology (JCGM), www.bipm.org.

[c] LaBudde, R.A., & Harnly, J.M. (2012) *J. AOAC Int.* **95**, 273–285.

[d] ISO 5725-1-1994.

[e] *Official Methods of Analysis* (current edition) *Appendix D* (*Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis*), AOAC INTERNATIONAL, Rockville, MD, USA.

Table A3. Recommendations for evaluation

Bias (if a reference material is available)	A minimum of five replicate analyses of a Certified Reference Material.[a]
Environmental interference	Analyze test portions containing a specified concentration of one environmental materials panel member. Materials may be pooled. Consult with AOAC statistician.
Exclusivity/cross-reactivity	Analyze one test portion containing a specified concentration of one exclusivity panel member. More replicates can be used. Consult with AOAC statistician.
Inclusivity/selectivity	Analyze one test portion containing a specified concentration of one inclusivity panel member. More replicates can be used. Consult with AOAC statistician.
Limit of quantitation (LOQ)	Estimate the LOQ = average (blank) + 10 × s_0 (blank). Measure blank samples with analyte at the estimated LOQ. Calculate the mean average and standard deviation of the results. Guidance[b]: For ML ≥ 100 ppb (0.1 mg/kg): LOD = ML × 1/5. For ML < 100 ppb (0.1 mg/kg): LOD = ML × 2/5.
Measurement uncertainty	Use ISO 21748: *Guidance for the use of repeatability, reproducibility, and trueness estimates in measurement uncertainty estimation to analyze data collected for bias, repeatability, and intermediate precision to estimate measurement uncertainty*.
POD(0)	Use data from collaborative study.
POD (c)	
Repeatability	Prepare and homogenize three unknown samples at different concentrations to represent the full, claimed range of the method. Analyze each unknown sample by the candidate method seven times, beginning each analysis from weighing out the test portion through to final result with no additional replication (unless stated to do so in the method). All of the analyses for one unknown sample should be performed within as short a period of time as is allowed by the method. The second and third unknowns may be analyzed in another short time period. Repeat for each claimed matrix.
Probability of detection (POD)	Determine the desired POD at a critical concentration. Consult with Table A7 to determine the number of test portions required to demonstrate the desired POD.
Probability of identification (POI)	Consult *Probability of Identification (POI): A Statistical Model for the Validation of Qualitative Botanical Identification Methods*[c].
Recovery	Determined from spiked blanks or samples with at least seven independent analyses per concentration level at a minimum of three concentration levels covering the analytical range. Independent means at least at different times. If no confirmed (natural) blank is available, the average inherent (naturally containing) level of the analyte should be determined on at least seven independent replicates. Marginal % recovery = $(C_f - C_u) \times 100/C_A$ Total % recovery = $100(C_f)/(C_u + C_A)$ where C_f = concentration of fortified samples, C_u = concentration of unfortified samples, and C_A = concentration of analyte added to the test sample.[d] Usually total recovery is used unless the native analyte is present in amounts greater than about 10% of the amount added, in which case use the method of addition.[e]
Reproducibility (collaborative or interlaboratory study)	Quantitative methods: Recruit 10–12 collaborators; must have eight valid data sets; two blind duplicate replicates at five concentrations for each analyte/matrix combination to each collaborator.
	Qualitative methods: Recruit 12–15 collaborators; must have 10 valid data sets; six replicates at five concentrations for each analyte/matrix combination to each collaborator.

[a] *Guidance for Industry for Bioanalytical Method Validation* (May 2001) U.S. Department of Health and Human Services, U.S. Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM).

[b] Codex Alimentarius Codex Procedure Manual.

[c] LaBudde, R.A., & Harnly, J.M. (2012) *J. AOAC Int.* **95**, 273–285.

[d] *Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (current edition) *Official Methods of Analysis*, *Appendix D,* AOAC INTERNATIONAL, Rockville, MD, USA.

[e] *AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* (current edition) *Official Methods of Analysis*, *Appendix K*, AOAC INTERNATIONAL, Rockville, MD, USA.

Table A4. Expected precision (repeatability) as a function of analyte concentration[a]

Analyte, %	Mass fraction (C)	Unit	RSD_r, %
100	1	100%	1.3
10	10^{-1}	10%	1.9
1	10^{-2}	1%	2.7
0.1	10^{-3}	0.1%	3.7
0.01	10^{-4}	100 ppm (mg/kg)	5.3
0.001	10^{-5}	10 ppm (mg/kg)	7.3
0.0001	10^{-6}	1 ppm (mg/kg)	11
0.00001	10^{-7}	100 ppb (µg/kg)	15
0.000001	10^{-8}	10 ppb (µg/kg)	21
0.0000001	10^{-9}	1 ppb (µg/kg)	30

[a] Table excerpted from *AOAC Peer-Verified Methods Program, Manual on Policies and Procedures* (1998) AOAC INTERNATIONAL, Rockville, MD, USA.

The precision of a method is the closeness of agreement between independent test results obtained under stipulated conditions. Precision is usually expressed in terms of imprecision and computed as a relative standard deviation of the test results. The imprecision of a method increases as the concentration of the analyte decreases. This table provides targets RSDs for a range of analyte concentrations.

Table A5. Expected recovery as a function of analyte concentration[a]

Analyte, %	Mass fraction (C)	Unit	Mean recovery, %
100	1	100%	98–102
10	10^{-1}	10%	
1	10^{-2}	1%	97–103
0.1	10^{-3}	0.1%	95–105
0.01	10^{-4}	100 ppm (mg/kg)	90–107
0.001	10^{-5}	10 ppm (mg/kg)	80–110
0.0001	10^{-6}	1 ppm (mg/kg)	
0.00001	10^{-7}	100 ppb (µg/kg)	
0.000001	10^{-8}	10 ppb (µg/kg)	60–115
0.0000001	10^{-9}	1 ppb (µg/kg)	40–120

[a] Table excerpted from *AOAC Peer-Verified Methods Program, Manual on Policies and Procedures* (1998) AOAC INTERNATIONAL, Rockville, MD, USA.

Recovery is defined as the ratio of the observed mean test result to the true value. The range of the acceptable mean recovery expands as the concentration of the analyte decreases. This table provides target mean recovery ranges for analyte concentrations from 100% to 1 ppb.

Table A6. Predicted relative standard deviation of reproducibility ($PRSD_R$)[a]

Analyte, %	Mass fraction (C)	Unit	RSD_R, %
100	1	100%	2
10	10^{-1}	10%	3
1	10^{-2}	1%	4
0.1	10^{-3}	0.1%	6
0.01	10^{-4}	100 ppm (mg/kg)	8
0.001	10^{-5}	10 ppm (mg/kg)	11
0.0001	10^{-6}	1 ppm (mg/kg)	16
0.00001	10^{-7}	100 ppb (µg/kg)	22
0.000001	10^{-8}	10 ppb (µg/kg)	32
0.0000001	10^{-9}	1 ppb (µg/kg)	45

[a] Table excerpted from *Definitions and Calculations of HorRat Values from Intralaboratory Data*, HorRat for SLV.doc, 2004-01-18, AOAC INTERNATIONAL, Rockville, MD, USA.

Predicted relative standard deviation or reproducibility = $PRSD_R$: Reproducibility relative standard deviation calculated from the Horwitz formula:

$$PRSD_R = 2C^{-0.15}$$

where C is expressed as a mass fraction.

This table provides the calculated $PRSD_R$ for a range of concentrations. *See* Annex D for additional information.

Table A7. POD and number of test portions[a,b]

	Sample size required for proportion						
Assume	1. Binary outcome (occur/not occur). 2. Constant probability rho of event occurring. 3. Independent trials (e.g., simple random sample). 4. Fixed number of trials (*N*)						
Inference	95% Confidence interval lies entirely at or above specified minimum rho						
Desired	Sample size *N* needed						
Minimum probability rho, %	Sample size (*N*)	Minimum No. events (*x*)	Maximum No. nonevents (*y*)	1-Sided lower confidence limit on rho[c], %	Expected lower confidence limit on rho, %	Expected upper confidence limit on rho, %	Effective AOQL[d] rho, %
50	3	3	0	52.6	43.8	100.0	71.9
50	10	8	2	54.1	49.0	94.3	71.7
50	20	14	6	51.6	48.1	85.5	66.8
50	40	26	14	52.0	49.5	77.9	63.7
50	80	48	32	50.8	49.0	70.0	59.5
55	4	4	0	59.7	51.0	100.0	75.5
55	10	9	1	65.2	59.6	100.0	79.8
55	20	15	5	56.8	53.1	88.8	71.0
55	40	28	12	57.1	54.6	81.9	68.2
55	80	52	28	55.9	54.1	74.5	64.3
60	5	5	0	64.9	56.5	100.0	78.3
60	10	9	1	65.2	59.6	100.0	79.8
60	20	16	4	62.2	58.4	91.9	75.2
60	40	30	10	62.4	59.8	85.8	72.8
60	80	56	24	61.0	59.2	78.9	69.1
65	6	6	0	68.9	61.0	100.0	80.5
65	10	9	1	65.2	59.6	100.0	79.8
65	20	17	3	67.8	64.0	94.8	79.4
65	40	31	9	65.1	62.5	87.7	75.1
65	80	59	21	65.0	63.2	82.1	72.7
70	7	7	0	72.1	64.6	100.0	82.3
70	10	10	0	78.7	72.2	100.0	86.1
70	20	18	2	73.8	69.9	97.2	83.6
70	40	33	7	70.7	68.0	91.3	79.7
70	80	63	17	70.4	68.6	86.3	77.4
75	9	9	0	76.9	70.1	100.0	85.0
75	10	10	0	78.7	72.2	100.0	86.1
75	20	19	1	80.4	76.4	100.0	88.2
75	40	35	5	76.5	73.9	94.5	84.2
75	80	67	13	75.9	74.2	90.3	82.2
80	11	11	0	80.3	74.1	100.0	87.1
80	20	19	1	80.4	76.4	100.0	88.2
80	40	37	3	82.7	80.1	97.4	88.8
80	80	70	10	80.2	78.5	93.1	85.8
85	20	20	0	88.1	83.9	100.0	91.9
85	40	38	2	86.0	83.5	98.6	91.1
85	80	74	6	86.1	84.6	96.5	90.6
90	40	40	0	93.7	91.2	100.0	95.6
90	60	58	2	90.4	88.6	99.1	93.9
90	80	77	3	91.0	89.5	98.7	94.1
95	60	60	0	95.7	94.0	100.0	97.0
95	80	80	0	96.7	95.4	100.0	97.7
95	90	89	1	95.2	94.0	100.0	97.0
95	96	95	1	95.5	94.3	100.0	97.2
98	130	130	0	98.0	97.1	100.0	98.6
98	240	239	1	98.2	97.7	100.0	98.8
99	280	280	0	99.0	98.6	100.0	99.3
99	480	479	1	99.1	98.8	100.0	99.4

[a] Table excerpted from Technical Report TR308, *Sampling plans to verify the proportion of an event exceeds or falls below a specified value*, LaBudde, R. (June 4, 2010) (not published). The table was produced as part of an informative report for the Working Group for Validation of Identity Methods for Botanical Raw Materials commissioned by the AOAC INTERNATIONAL Presidential Task Force on Dietary Supplements. The project was funded by the Office of Dietary Supplements, National Institutes of Health.

[b] Copyright 2010 by Least Cost Formulations, Ltd. All rights reserved.

[c] Based on modified Wilson score 1-sided confidence interval.

[d] AOQL = Average outgoing quality level.

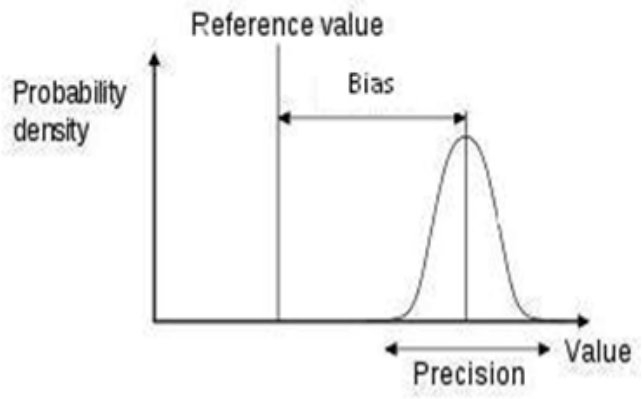


Figure A1. Relationship between precision versus bias (trueness). Trueness is reported as bias. Bias is defined as the difference between the test results and an accepted reference value.

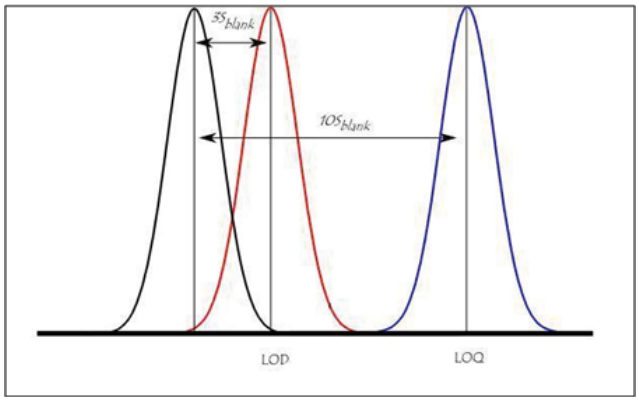


Figure A2. Relationship between LOD and LOQ. LOD is defined as the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit. LOQ is the level above which quantitative results may be obtained with a stated degree of confidence.

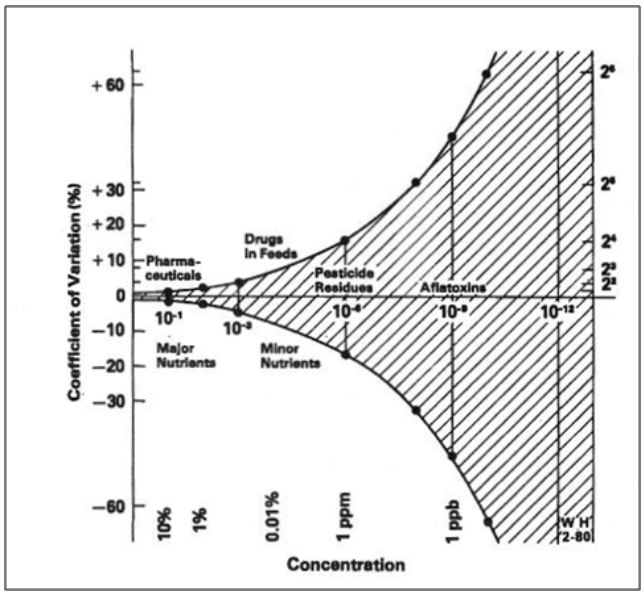


Figure A3. Horwitz Curve, illustrating the exponential increase in the coefficient of variation as the concentration of the analyte decreases [*J. AOAC Int.* 89, 1095(2006)].

ANNEX B
Classification of Methods

The following guidance may be used to determine which performance parameters in Table A1 apply to different classifications of methods. AOAC INTERNATIONAL does not recognize the term "semiquantitative" as a method classification. Methods that have been self-identified as semiquantitative will be classified into one of the following five types:

Type I: Quantitative Methods

Characteristics: Generates a continuous number as a result.

Recommendation: Use performance requirements specified for quantitative method (main or trace component). Use recovery range and maximum precision variation in Tables A4 and A5.

In some cases and for some purposes, methods with less accuracy and precision than recommended in Tables A4 and A5 may be acceptable. Method developers should consult with the appropriate method committee to determine if the recommendations in Tables A4 and A5 do or do not apply to their method.

Type II: Methods that Report Ranges

Characteristics: Generates a "range" indicator such as 0, low, moderate, and high.

Recommendation: Use performance requirements specified for qualitative methods (main component). Specify a range of POD for each range "range" indicator.

Type III: Methods with Cutoff Values

Characteristics: Method may generate a continuous number as an interim result (such as a CT value for a PCR method), which is not reported but converted to a qualitative result (presence/ absence) with the use of a cutoff value.

Recommendation: Use performance requirements specified for qualitative methods.

Type IV: Qualitative Methods

Characteristics: Method of analysis whose response is either the presence or absence of the analyte detected either directly or indirectly in a specified test portion.

Recommendation: Use performance requirements specified for qualitative methods.

Type V: Identification Methods

Characteristics: Method of analysis whose purpose is to determine the identity of an analyte.

Recommendation: Use performance requirements specified for identification methods.

ANNEX C
Understanding the POD Model

Excerpted from AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures, J. AOAC Int. ***94****, 1359(2011) and Official Methods of Analysis of AOAC INTERNATIONAL (current edition), Appendix I.*

The Probability of Detection (POD) model is a way of characterizing the performance of a qualitative (binary) method. A binary qualitative method is one that gives a result as one of two possible outcomes, either positive or negative, presence/absence, or +/–.

The single parameter of interest is the POD, which is defined as the probability at a given concentration of obtaining a positive response by the detection method. POD is assumed to be dependent on concentration, and generally, the probability of a positive response will increase as concentration increases.

For example, at very low concentration, the expectation is that the method will not be sensitive to the analyte, and at very high concentration, a high probability of obtaining a positive response is desired. The goal of method validation is to characterize how method response transitions from low concentration/low response to high concentration/high response.

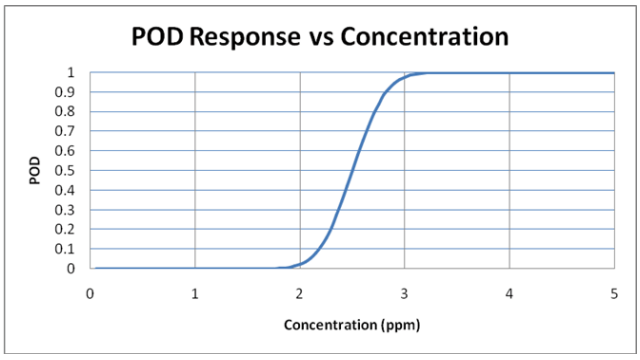


Figure C1. Theoretical POD curve for a qualitative detection method.

POD is always considered to be dependent upon analyte concentration. The POD curve is a graphical representation of method performance, where the probability is plotted as a function of concentration (*see*, for example, Figure C1).

The POD model is designed to allow an objective description of method response without consideration to an a priori expectation of the probabilities at given concentrations. The model is general enough to allow comparisons to any theoretical probability function.

The POD model is also designed to allow for an independent description of method response without consideration to the response of a reference method. The model is general enough to allow for comparisons between reference and candidate method responses, if desired.

Older validation models have used the terms "sensitivity," "specificity," "false positive," and "false negative" to describe method performance. The POD model incorporates all of the performance concepts of these systems into a single parameter, POD.

For example, false positive has been defined by some models as the probability of a positive response, given the sample is truly negative (concentration = 0). The equivalent point on the POD curve for this performance characteristic is the value of the curve at Conc = 0.

Similarly, false negative has sometimes been defined as the probability of a negative response when the sample is truly positive (concentration >0). In the POD curve, this would always be specific to a given sample concentration, but would be represented as the distance from the POD curve to the POD = 1 horizontal top axis at all concentrations except C = 0.

The POD model incorporates all these method characteristics into a single parameter, which is always assumed to vary by concentration. In other models, the terms "false positive," "false negative," "sensitivity," and "specificity" have been defined in a variety of ways, usually not conditional on concentration. For these reasons, these terms are obsolete under this model (*see* Table C1).

The terms "sensitivity," "specificity," "false positive," and "false negative" are obsolete under the POD model (*see* Figure C2).

Table C1. Terminology

Traditional terminology	Concept	POD equivalent	Comment
False positive	Probability of the method giving a (+) response when the sample is truly without analyte	POD(0) POD at conc = 0	POD curve value at conc = 0; "*Y*-intercept" of the POD curve
Specificity	Probability of the method giving a (-) response when the sample is truly without analyte	1-POD(0)	Distance along the POD axis from POD = 1 to the POD curve value
False negative (at a given concentration)	Probability of a (–) response at a given concentration	1-POD(c)	Distance from the POD curve to the POD = 1 "top axis" in the vertical direction
Sensitivity (at a given concentration)	Probability of a (+) response at a given concentration	POD(c)	Value of the POD curve at any given concentration
True negative	A sample that contains no analyte	C = 0	Point on concentration axis where c = 0
True positive	A sample that contains analyte at some positive concentration	C > 0	Range of concentration where c > 0

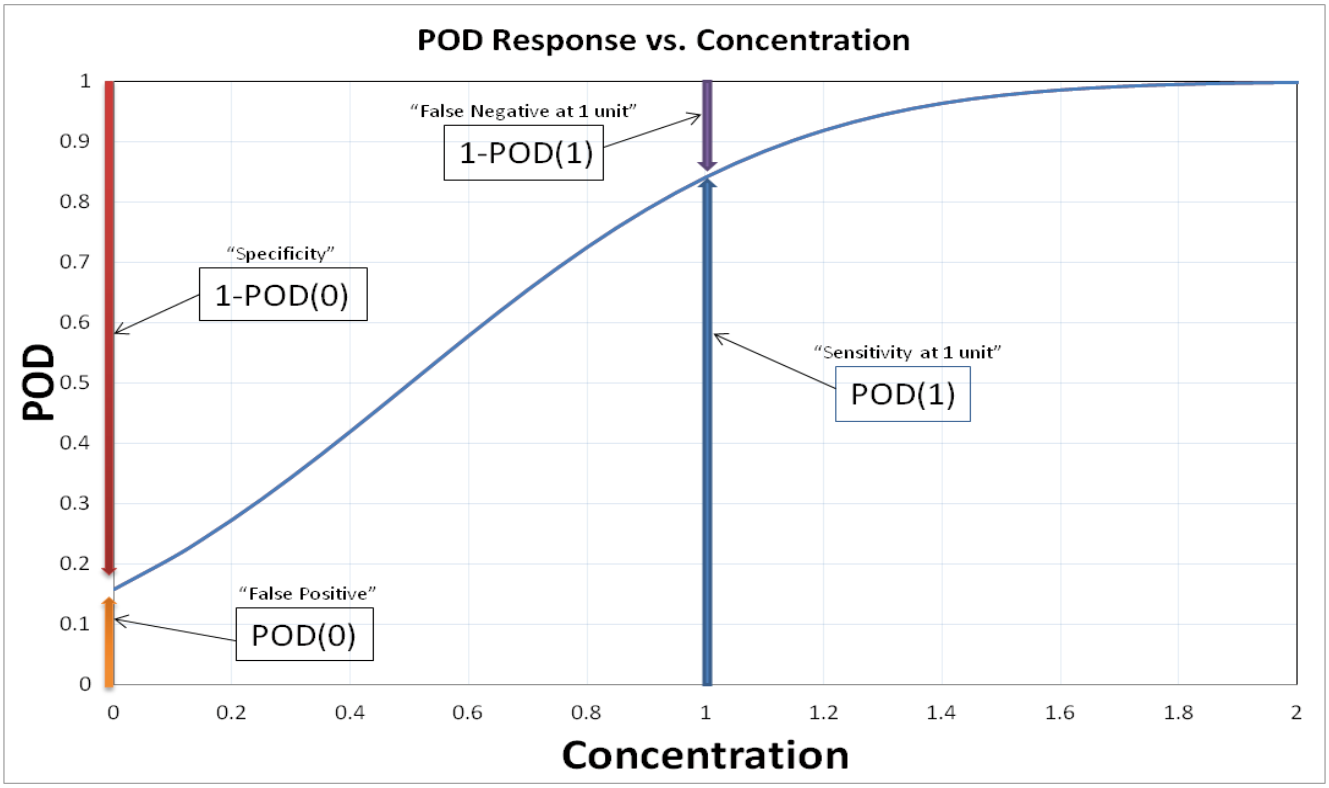


Figure C2. Comparison of POD model terminology to other obsolete terms.

ANNEX D
Definitions and Calculations of HorRat Values from Intralaboratory Data

Excerpted from Definitions and Calculations of HorRat Values from Intralaboratory Data, AOAC INTERNATIONAL, HorRat for SLV.doc, 2004-01-18.

1. Definitions

1.1 Replicate Data

Data developed under common conditions in the same laboratory: simultaneous performance, or, if necessary to obtain sufficient values, same series, same analyst, same day. Such data provides "repeatability statistical parameters."

1.2 Pooled Data

Replicate data developed in the same laboratory under different conditions but considered sufficiently similar that, for the purpose of statistical analysis, they may be considered together. These may include different runs, different instruments, different analysts, and different days.

1.3 Average

0 = Sum of the individual values, x_i, divided by the number of individual values, n.

$$0 = (\Sigma\, x_i)/n$$

1.4 Standard Deviation

$$s_i = [\Sigma(x_i - (\overline{x})^2/n]^{0.5}$$

1.5 Relative Standard Deviation

$$RSD = s_i \times 100/\overline{x}$$

1.5.1 Repeatability Relative Standard Deviation [RSD(r) or RSD_r]

The relative standard deviation calculated from within-laboratory data.

1.5.2 Reproducibility Relative Standard Deviation [RSD(R) or RSD_R]

The relative standard deviation calculated from among-laboratory data.

Table D1. Predicted relative standard deviations

Concentration (C)	Mass fraction (C)	$PRSD_R$, %
100%	1.0	2
1%	0.01	4
0.01%	0.0001	8
1 ppm	0.000001	16
10 ppb	0.00000001	32
1 ppb	0.000000001	45

1.6 Mass Fraction

Concentration, C, expressed as a decimal fraction. For calculating and reporting statistical parameters, data may be expressed in any convenient units (e.g., %, ppm, ppb, mg/g, µg/g; µg/kg; µg/L, µg/µL, etc.). For reporting HorRat values, data must be reported as a mass fraction where the units of the numerator and denominator are the same: e.g., for 100% (pure materials), the mass fraction C = 1.00; for 1 µg/g (ppm), C = 0.000001 = (E-6). *See* Table D1 for other examples.

1.7 Predicted Relative Standard Deviation [PRSD(R) or $PRSD_R$]

The reproducibility relative standard deviation calculated from the Horwitz formula:

$$PRSD(R) = 2C^{-0.15}$$

where C is expressed as a mass fraction. *See* Table D1.

In spreadsheet notation: PRSD(R) = 2 * C ^(–0.15).

1.8 HorRat Value

The ratio of the reproducibility relative standard deviation calculated from the data to the PRSD(R) calculated from the Horwitz formula:

$$HorRat = RSD(R)/PRSD(R)$$

To differentiate the usual HorRat value calculated from reproducibility data from the HorRat value calculated from repeatability data, attach an R for the former and an r for the latter. But note that the denominator always uses the PRSD(R) calculated from reproducibility data because this parameter is more predictable than the parameter calculated from repeatability data:

$$HorRat(R) = RSD_R/PRSD(R)$$

$$HorRat(r) = RSD_r/PRSD(R)$$

Some expected, predicted relative standard deviations are given in Table D1.

2 *Acceptable HorRat Values*

2.1 For Interlaboratory Studies

HorRat(R): The original data developed from interlaboratory (among-laboratory) studies assigned a HorRat value of 1.0 with limits of acceptability of 0.5 to 2.0. The corresponding within-laboratory relative standard deviations were found to be typically 1/2 to 2/3 the among-laboratory relative standard deviations.

Table D2. Predicted relative standard deviations

Concentration (C)	$PRSD_R$, %	$PRSD_r$, %
100%	2	1
1%	4	2
0.01%	8	4
1 ppm	16	8
10 ppb	32	16
1 ppb	45	22

2.1.1 Limitations

HorRat values do not apply to method-defined (empirical) analytes (moisture, ash, fiber, carbohydrates by difference, etc.), physical properties or physical methods (pH, viscosity, drained weight, etc.), and ill-defined analytes (polymers, products of enzyme reactions).

2.2 For Intralaboratory Studies

2.2.1 Repeatability

Within-laboratory acceptable predicted target values for repeatability are given in Table D2 at 1/2 of PRSD(R), which represents the best case.

2.2.2 HorRat(r)

Based on experience and for the purpose of exploring the extrapolation of HorRat values to SLV studies, take as the minimum acceptability 1/2 of the lower limit ($0.5 \times 0.5 \approx 0.3$) and as the maximum acceptability 2/3 of the upper limit ($0.67 \times 2.0 \approx 1.3$).

Calculate HorRat(r) from the SLV data:

$$HorRat(r) = RSD(r)/PRSD(R)$$

Acceptable HorRat(r) values are 0.3–1.3. Values at the extremes must be interpreted with caution. With a series of low values, check for unreported averaging or prior knowledge of the analyte content; with a series of high values, check for method deficiencies such as unrestricted times, temperatures, masses, volumes, and concentrations; unrecognized impurities (detergent residues on glassware, peroxides in ether); incomplete extractions and transfers and uncontrolled parameters in specific instrumental techniques.

2.3 Other Limitations and Extrapolations

The HorRat value is a very rough but useful summary of the precision in analytical chemistry. It overestimates the precision at the extremes, predicting more variability than observed at the high end of the scale (C > ca 0.1; i.e., >10%) and at the low end of the scale (C < E-8; i.e., 10 ng/g; 10 ppb).

ANNEX E
AOAC Method Accuracy Review

Accuracy of Method Based on Reference Material

Reference material (RM) used.—The use of RMs should be seen as integral to the process of method development, validation, and performance evaluation. RMs are not the only component of a quality system, but correct use of RMs is essential to appropriate quality management. RMs with or without assigned quantity values can be used for measurement precision control, whereas only RMs with assigned quantity values can be used for calibration or measurement trueness control. Method development and validation for matrices within the scope of the method is done to characterize attributes such as recovery, selectivity, "trueness" (accuracy, bias), precision (repeatability and reproducibility), uncertainty estimation, ruggedness, LOQ or LOD, and dynamic range. RMs should be chosen that are fit-for-purpose. When certified reference materials (CRMs) are available with matrices that match the method scope, much of the work involved in method development has already been completed, and that work is documented through the certificate. RMs with analyte values in the range of test samples, as well as "blank" matrix RMs, with values below or near detection limits, are needed.

Availability of RM.—Consideration needs to be given to the future availability of the chosen RM. Well-documented methods that cannot be verified in the future due to lack of material may lose credibility or be seen as inferior.

Fit to method scope.—Natural matrix CRMs provide the greatest assurance that the method is capable of producing accurate results for that matrix. When selecting an RM to perform a method validation, analysts should consider the method to material fit. An example of a good fit would be a method for specified organic molecules in infant formula and using an infant formula or powder milk RM. A poor fit would be a method for specified organic molecules in infant formula and using a sediment material.

Stability.—Providing a stable RM can be challenging where analytes are biologically active, easily oxidized, or interactive with other components of the matrix. CRM producers provide assurance of material stability, as well as homogeneity. CRMs are accompanied by a certificate that includes the following key criteria:

(*1*) Assigned values with measurement uncertainty and metrological traceability
(*2*) Homogeneity
(*3*) Stability, with the expiration date for the certificate
(*4*) Storage requirements
(*5*) Information on intended use
(*6*) Identity of matrix

For some RMs, such as botanical RMs, the source and/or authenticity can be a very important piece of information that should be included with the certificate. Even under ideal storage conditions, many analytes have some rate of change. Recertification may be done by the supplier, and a certificate reissued with a different expiration date and with certain analyte data updated or removed.

Definition of CRM.—Refer to the AOAC TDRM document for definitions from ISO Guide 30, Amd. 1 (2008), http://www.aoac.org/divisions/References.pdf.

Information on source of RM is available.—It is the responsibility of the material producer to provide reliable authentication of the RM and make a clear statement in the accompanying documentation. This should be an as detailed listing as possible, including handling of ingredients, identification of plant materials as completely as feasible (species, type, subtype, growing region), etc. This is comparable to other required information on an RM for judging its suitability for a specific application purpose (e.g., containing how much of the targeted analyte, stabilized by adding acid—therefore not suited for certain parameters/procedures, etc.).

Separate RM used for calibration and validation.—A single RM cannot be used for both calibration and validation of results in the same measurement procedure.

Blank RM used where appropriate.—Blank matrix RMs are useful for ensuring performance at or near the detection limits. These are particularly useful for routine quality control in methods measuring, for instance, trace levels of allergens, mycotoxins, or drug residues.

Storage requirements were maintained.—Method developers should maintain good documentation showing that the RM producer's recommended storage conditions were followed.

Cost.—The cost of ongoing method checks should be considered. Daily use of CRMs can be cost prohibitive. Monthly or quarterly analysis of these materials may be an option.

Concentration of analyte fits intended method.—Concentration of the analyte of interest is appropriate for *Standard Method Performance Requirements* (SMPRs®).

Uncertainty available.—Every measurement result has an uncertainty associated with it, and the individual contributions toward the combined uncertainty arise from multiple sources. Achieving the target measurement uncertainty set by the customer for his/her problem of interest is often one of the criteria used in selecting a method for a given application. Estimation of measurement uncertainty can be accomplished by different approaches, but the use of RMs greatly facilitates this part of a method validation.

Demonstration of Method Accuracy when No Reference Material Is Available

If an RM is not available, how is accuracy demonstrated?

There are many analytes for which a CRM with a suitable matrix is not available. This leaves the analyst with few options. For some methods, there may be proficiency testing programs that include a matrix of interest for the analyte. Proficiency testing allows an analyst to compare results with results from other laboratories, which may or may not be using similar methods. Spiking is another technique that may be used. When alternative methods are available, results may be compared between the different methods. These alternatives do not provide the same level of assurance that is gained through the use of a CRM.

Spike recovery.—In the absence of an available CRM, one technique that is sometimes used for assessing performance is the spiking of a matrix RM with a known quantity of the analyte. When this method is used, it cannot be assumed that the analyte is bound in the same way as it would be in a natural matrix. Nevertheless, a certified blank RM would be the preferred choice for constructing a spiked material.

When preparing reference solutions, the pure standards must be completely soluble in the solvent. For insoluble materials in a liquid suspension or for powdered forms of dry materials, validation is required to demonstrate that the analyte is homogeneously

The document, *AOAC Method Accuracy Review*, was prepared by the AOAC Technical Division on Reference Materials (TDRM) and approved by the AOAC Official Methods Board in June 2012.

distributed and that the response of the detection system to the analyte is not affected by the matrix or preparation technique. When a matrix material is selected for spiking, it should be reasonably characterized to determine that it is sufficiently representative of the matrix of interest. Spiked samples must be carried through all steps of the method. Many analytes are bound in a natural matrix and whether the spiked analyte will behave the same as the analyte in a natural matrix is unknown.

Other.—Use of a substitute RM involves the replacement of the CRM with an alternative matrix RM matching the matrix of interest as close as possible based on technical knowledge.

ANNEX F
Development and Use of In-House Reference Materials

The use of reference materials is a vital part of any analytical quality assurance program. However, you may have questions about their creation and use. The purpose of this document is to help answer many of these questions.

- What is a reference material?
- Why use reference materials?
- What certified reference materials (CRMs) are currently available?
- Why use an in-house reference material?
- How do I create an in-house reference material?
- How do I use the data from an in-house reference material?

What Is a Reference Material?

The International Organization for Standardization (ISO) defines a reference material as a "material or substance one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials" (1). In plain English, natural-matrix reference materials, such as those you might prepare for use in-house, can be used to validate an analytical method or for quality assurance while you're using your method to analyze your samples. (Natural-matrix materials are not generally used as calibrants because of the increased uncertainty that this would add to an analysis.) The assigned values for the target analytes of an in-house reference material can be used to establish the precision of your analytical method and, if used in conjunction with a CRM, to establish the accuracy of your method.

ISO defines a CRM as a "reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence" (1).

Why Use Reference Materials?

CRMs can be used across the entire scope of an analytical method and can provide traceability of results to the International System of Units (SI). During method development, CRMs can be used to optimize your method. During method validation, they can be used to ensure that your method is capable of producing the "right" answer, and to determine how close your result is to that answer. During routine use, they can be used to determine within-day and between-day repeatability, and so demonstrate that your method is in control and is producing accurate results every time it is used.

Natural-matrix reference materials should mimic the real samples that will be analyzed with a method. They should behave just as your samples would during a procedure, so if you obtain accurate and precise values for your reference material, you should obtain accurate and precise values for your samples as well.

What Certified Reference Materials Are Currently Available?

CRMs are available from a number of sources, including (but not limited to):

- American Association of Cereal Chemists (AACC)
- American Oil Chemists Society (AOCS)
- International Atomic Energy Agency (IAEA)
- Institute for Reference Materials and Measurements (IRMM)
- LGC Promochem
- National Institute of Standards and Technology (NIST)
- National Research Council Canada (NRC Canada)
- UK Food Analysis Proficiency Assessment Program (FAPAS)

A number of websites provide general overviews and catalogs of producers' and distributors' reference materials:

http://www.aocs.org/tech/crm/
http://www.comar.bam.de
http://www.erm-crm.org
http://www.iaea.org/oregrammeslaqcs
http://www.aaccnet.org/checksample
http://www.irmm·ire.be/mrm.html
http://www.lgcpromochem.com
http://www.naweb.iaea.org/nahu/nmrm/
http://www.nist.gov/srm
http://www.fapas.com/index. cfm
http://www.virm.net.

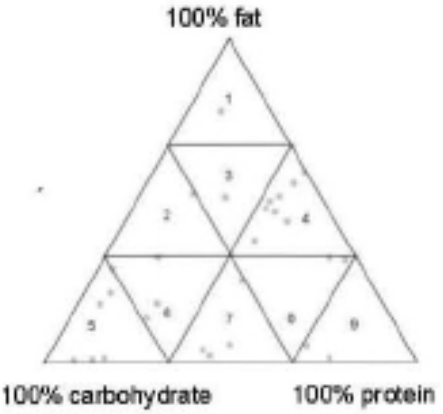
Because new reference materials are produced regularly, it is important to check these websites to determine what is currently available.

Why Use an In-House Reference Material?

There are many benefits to the use of a CRM. CRMs have been prepared to be homogeneous and, if stored under the proper conditions, stable. You are provided with a certified value as well as the statistical data for the concentration of your analyte; this is about as close as you can come to knowing the true value of the concentration of the analyte. The material has been tested by experienced analysts in leading laboratories, so you have the security of knowing that your method is generating values similar to those generated in other competent laboratories. The CRMs from the sources mentioned above are nationally and/or internationally recognized, so when you obtain acceptable results for a CRM using your analytical method, you give credibility to your methodology and traceability to your results.

But there are some drawbacks associated with CRMs. Unfortunately, many analyte/matrix combinations are not currently available. When testing food products for nutrient content, for example, a laboratory can be asked to analyze anything that might be found in a kitchen or grocery store. Reference materials that represent all of the types of foods that need to be tested are not available, and most CRMs are certified for a limited number of analytes. It is important to match the reference material matrix to your sample matrix. (Food examples dominate the discussion

Excerpted from *Development and Use of In-House Reference Materials*, Rev. 2, 2009. Copyright 2005 by the AOAC Technical Division on Reference Materials (TDRM).



below, but the same processes apply to the development of in-house reference materials in other areas of analytical chemistry.)

To demonstrate the applicability of an analytical method to a wide variety of food matrices, AOAC INTERNATIONAL's Task Force on Methods for Nutrition Labeling developed a triangle partitioned into sectors in which foods are placed based on their protein, fat, and carbohydrate content (2, 3). Since ash does not have a great impact on the performance of an analytical method for organic-material foods, and water can be added or removed, it can be assumed that the behavior of an analytical method is determined to large extent by the relative proportions of these proximates. AOAC INTERNATIONAL anticipated that one or two foods in a given sector would be representative of other foods in that sector and therefore would be useful for method assessment. Similarly, one or two reference materials in a given sector (or near each other in adjacent sectors) should be useful for quality assurance for analyses involving the other foods in the sector. The positions of many of the food-matrix CRMs from the sources listed above are shown in the triangle and are provided in the list.

These food-matrix reference materials are spread through all sectors of the triangle, thereby making it likely that you can find an appropriate CRM to match to your samples. Ultimately, however, the routine use of a CRM can be cost prohibitive, and is not really the purpose of CRMs. For example, in order to use NIST's Standard Reference Material (SRM) 2387 Peanut Butter for all mandatory nutrition labeling analyses, you could buy one sales unit (three jars, each containing 170 g material) for $649 (2009 price). If you charge your customer about $1000 for analysis of all mandatory nutrients in a test material, the control material would account for more than 60% of your fees. Therefore, many laboratories have found it more cost-effective to create in-house reference materials for routine quality control and characterize them in conjunction with the analysis of a CRM (4). You can prepare larger quantities of a reference material by preparing it in-house, and you have more flexibility in the types of matrices you can use. There are not many limitations on what can be purchased.

How Do I Create an In-House Reference Material?

There are basically three steps to preparing an in-house reference material: selection (including consideration of homogeneity and stability), preparation, and characterization. Additional guidance through these steps can be provided from the AOAC Technical Division on Reference Materials (TDRM), as well as in ISO Guides 34 (5) and 35 (6).

Sector	RM No.	Matrix
	NIST 1563	Coconut oil
1	NIST 3274	Fatty acids in botanical oils
1	NIST 3276	Carrot extract in oil
1	LGC 7104	Sterilized cream
2	NIST 2384	Baking chocolate
3	NIST 2387	Peanut butter
4	NIST 1546	Meat homogenate
4	LGC 7106	Processed cheese
4	LGC 7000	Beef/pork meat
4	LGC 7150	Processed meat
4	LGC 7151	Processed meat
4	LGC 7152	Processed meat
4	SMRD 2000	Fresh meat
4	LGC 7101	Mackerel paste
4	LGC QC1001	Meat paste 1
4	LGC QC1004	Fish paste 1
5	BCR-382	Wleat flour
5	BCR-381	Rye flour
5	LGC 7103	Sweet digestive biscuit
5	LGC 7107	Madeira cake
5	LGC QC1002	Flour 1
6	NIST 1544	Fatty acids
6	NIST 1548a	Typical diet
6	NIST 1849	Infant/adult nutritional formula
6	LGC 7105	Rice pudding
7	LGC 7001	Pork meat
7	NIST 1566b	Oyster tissue
7	NIST 1570a	Spinach leaves
7	NIST 2385	Spinach
8	NIST 1946	Lake trout
8	LGC 7176	Canned pet food
9	NIST 1974a	Mussel tissue
9	NIST 3244	Protein powder

References

(1) JCGM 200:2008, *International vocabulary of metrology—Basic and general concepts and associated terms (VIM)*, International Bureau of Weights and Measures (www.bipm.org)

(2) Wolf, W.R., & Andrews, K.W. (1995) *Fresenius' J. Anal. Chem*. **352**, 73–76

(3) Wolf, W.R. (1993) *Methods of Analysis for Nutrition Labeling*, D.R. Sullivan & D.E. Carpenter (Eds), AOAC INTERNATIONAL, Gaithersburg, MD

(4) European Reference Materials (2005) *Comparison of a Measurement Result with the Certified Value*, Application Note 1

(5) *ISO Guide 34 General Requirements for the Competence of Reference Material Producers* (2009) 2nd, International Organization for Standardization, Geneva, Switzerland

(6) *Guide 35 Certification of Reference Materials—General and Statistical Principles* (2006) International Organization for Standardization, Geneva, Switzerland

For more information about TDRM, visit http://aoac.org/divisions/tdrm.

Appendix M: Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices

Although there are a number of documents published on method validation (1, 2) which target analytical methods in general, and there are numerous publications on validation of ELISA methods for pesticides, these documents do not address specific areas of concern for food allergen analysis, such as reference materials, spiking methods, or choice of matrixes. In the absence of a universally recognized reference standard for food allergen ELISAs, many organizations and end-users use different validation protocols and different analytical standards. Such inconsistency and duplication inevitably has a negative economic impact on the food allergen community. This document is designed to accompany the *AOAC Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (1), and to provide guidance specific to the validation of quantitative ELISA-based methods for food allergens. This protocol was designed to meet or exceed the minimum requirements set forth in the AOAC guidelines; it was developed with input from a wide range of experts in the area of food allergens, working under the auspices of the AOAC Presidential Task Force on Food Allergens and with the active contribution of the Allergen Working Group, part of the MoniQA network of excellence. This document will focus on developing guidance on a method validation study protocol to validate the performance characteristics of quantitative food allergen ELISA methods. The practical protocol is intended to help method developers in designing a study to generate appropriate validation data that would be suitable for submission to AOAC INTERNATIONAL or regulatory bodies for recognition. Both the study design and data would be subject to scrutiny before acceptance by the AOAC or other authority.

Methods for detecting various food allergens have been available for a number of years. Many of these methods use ELISA-based techniques to detect specific protein markers in food matrixes. The detection of food allergens by ELISA is a unique analytical procedure characterized by the recognition and binding of specific antigens by antibodies. Food allergens are proteins, which are large and complex molecules with defined structures in their native forms, that can induce allergic reactions in sensitized consumers. From the analytical point of view, the integrity of the protein structure is critical to favor protein solubility and promote antibody-allergen binding. Although specificity of antibodies in commercial ELISAs for food allergens varies, in most cases, these methods target a complex mixture of soluble allergenic and nonallergenic proteins, rather than a specific protein. This mixture of target proteins will have diverse structural and chemical properties in the complex mixture of a food matrix. Some food commodities contain several allergenic proteins, e.g., at least eight peanut proteins, such as Ara h 1 and Ara h 2, can potentially cause an immunological response. But other commodities, such as fish, shellfish, and mollusks, contain only one major allergen; still others may consist mainly of allergenic proteins, e.g., all major milk proteins (caseins, β-lactoglobulin, α-lactalbumin, etc.) possess an allergenic capacity.

The ability of an ELISA method to detect food allergen proteins in a test sample is affected by the efficiency with which these proteins are extracted from the sample, as well as the efficiency with which the antibody or antibodies used in the ELISA detect these proteins in the sample extract. The overall performance of an ELISA-based method for the detection of food allergens is a function of these two parameters.

The fact that allergic individuals often react to different protein constituents of the allergenic food further complicates the choice of targets. Because most food products are heat-treated, food production processes like roasting and extrusion can have significant influence on the solubility and extractability of the target proteins, as well as on the ability of the antibody or antibodies used in the ELISA to recognize them. Factors that may influence the test results include: (*1*) interactions with compounds in a food matrix (e.g., polyphenols and tannins); (*2*) reduced solubility and reactivity of heat-denatured proteins; and (*3*) differences in the protein profile of a particular food allergen from different species, varieties, and geographic origins. These factors all contribute to the difficulty in finding appropriate reference materials for food allergens and explaining why the proteins in a sample extract might not be fully comparable to that of the calibrators included with a particular detection method. These topics have been extensively reviewed recently (3).

Availability of validated methods is critical for both method developers and end-users. For method developers, validation of an analytical procedure is used to demonstrate that it is suitable for its

This document provides supplemental guidance on specifications for the development and implementation of studies to validate the performance characteristics of quantitative ELISA methods for the determination of food allergens. It is intended as a companion document to other existing publications on method validation. The guidance is divided into two sections: information to be provided by the method developer on various characteristics of the method, and implementation of a multilaboratory validation study. Certain criteria included in the guidance are allergen-specific. Two food allergens, egg and milk, are used to demonstrate the criteria guidance. These recommendations will be the basis of the harmonized validation protocol for any food allergen ELISA method, whether proprietary or nonproprietary, that will be submitted to AOAC and/or regulatory authorities or other bodies for status recognition. Regulatory authorities may have their own particular requirements for data packages in addition to the guidance in this document. Future work planned for the implementation and validation of this guidance will include guidance specific to other priority allergens.

These guidance and best practices were completed by the AOAC Food Allergens Analytical Community and submitted to AOAC INTERNATIONAL for publication in 2009.

Reference: Abbott, M., Hayward, S., Ross, W., Godefroy, S.B., Ulberth, F., Van Hengel, A.J., Roberts, J., Akiyama, H., Popping, B., Yeung, J.M., Wehling, P., Taylor, S., Poms, R.E., & Delahaut, P. (2010) *J. AOAC Int*. **93**, 442–450

Table 1. Food commodities that should be included in cross-reactivity testing for ELISA methods targeting egg

Adzuki beans	Almond	Barley	Beef	Brazil nut
Buckwheat	Cashew	Chestnut	Chick peas	Chicken
Cocoa	Coconut	Corn	Crustacean/prawn/shrimp	Duck
Fish	Gelatin (bovine)	Hazelnut	Kidney beans	Kiwi
Lecithin	Lentils	Lima beans	Linseed	Macadamia nut
Milk	Oats	Octopus	Peanut	Peas
Pecans	Pine nut	Pistachio	Poppy seeds	Pork
Pumpkin seed	Rice—white and brown	Rye	Sesame	Soybean
Split peas	Sunflower seed	Turkey	Walnut	Wheat

intended purpose. For end-users, validated methods help to ensure reliability, repeatability, accuracy, and precision of the results generated using a particular method.

Method performance is documented using information and data provided by the method developer through interlaboratory validation studies. Minimum requirements for both information and data are included in this guidance, and may be applicable to any priority food allergen, as defined by the Codex Alimentarius Committee on Food Labeling in 1998 (4). However, due to the nature of food allergens, certain aspects, such as reference materials and spiking methods, would need to be addressed on a case-by-case basis. This document addresses these allergen-specific criteria for two food allergens, egg and milk. Further guidance for other priority allergens will be developed and communicated by the AOAC Presidential Task Force on Food Allergens and/or the Food Allergens Analytical Community under the auspices of the MoniQA network.

Required Allergen-Specific Information to be Provided on the ELISA Method

Information relating to the design of a method and its target analytes, as well as method performance characteristics, shall be provided by the method developer when submitting validation data for assessment. This information can be an important part of an overall package of information for evaluating a method. Proprietary information on antibody design or certain aspects of the method do not have to be disclosed. The AOAC guidelines (1) outline requirements for a final collaborative study manuscript. These allergen-specific requirements are additional recommendations that apply only to food allergen ELISA methods during method development and the final collaborative study.

The following information should be submitted along with the interlaboratory validation study data:

Antibody information.—Information on the antibody must include whether the antibody is monoclonal or polyclonal, whether it targets a single protein or multiple proteins, and whether the target protein used to generate the antibody was fractionated, modified, or synthesized in some way. Method developers are encouraged to include as much additional information about the antibody as possible. It is not necessary to reveal proprietary information. An example of antibody characterization for ELISA methods was discussed in a previous communication, specifically targeting mycotoxin/phycotoxin analysis (5). This approach could be adapted for allergen-specific antibodies.

Cross-reactivity.—Cross-reactivity is defined as a positive response to a sample that does not contain any of the target analyte. Method developers must test their allergen detection method for cross-reactivity for the target allergen in a variety of food commodities, which will vary for different target analytes and will depend on a number of factors. Food commodities tested for cross-reactivity should include a wide selection of foods and ingredients, particularly those that are genetically similar to the target allergenic commodity and that are likely to be analyzed for the presence of the target food allergen. The greater the number of items tested for cross-reactivity the better. In general, food items tested for cross-reactivity should be prepared as they would normally be consumed (raw or cooked).

Cross-reactivity testing should be based on the full-strength extracts, i.e., a sample of the item being tested for cross-reactivity should be extracted using the extraction buffer and procedure outlined in the method instructions, then analyzed at full strength to determine if it leads to a positive result. If a positive result is obtained, the extract must be diluted and rerun to characterize the extent of the cross-reactivity.

A minimum list of food commodities that should be included in cross-reactivity testing for egg and milk is provided in Tables 1 and 2, respectively. Many of these commodities will be the same for

Table 2. Food commodities that should be included in cross-reactivity testing for ELISA methods targeting milk

Almond	Barley	Brazil nut	Beef	Buckwheat
Cashew	Chick peas	Cocoa	Corn meal	Crustacean/prawn
Egg	Fish	Hazelnut	Lecithin	Lima bean
Oats	Peas	Peanut	Pecan	Pine nut
Pistachio	Poppy seed	Pumpkin seed	Rice–white and brown	Rye
Sesame seed	Soy bean	Split peas	Sunflower seed	Walnut
Wheat				

Table 3. Matrixes of interest for ELISA methods targeting egg and milk

Egg	Milk
Chicken	Cookies, baked goods
Ice cream	Dark chocolate
Pasta	Drink mixes (ex. alcoholic beverage premix)
Salad dressing	Orange juice
Soy milk	Infant formula
Wine	Wine

all priority allergens, but specific items may be included on some lists, depending on particular concerns, e.g., genetic homology (crustaceans and dust mites) or matrixes of likely exposure. Table 3 lists matrixes of interest for ELISA methods that target egg and milk.

Information on calibrators.—The calibrators provided in the kit must be clearly defined. Information should address the following questions:

What is the calibrator that is supplied with the kit and used to generate the calibration curve? How was the calibrator prepared and assayed? Is the calibrator made from raw or processed material? Was the calibrator extracted or purified and if so how? Is the calibrator in extraction or dilution buffer?

It is very important to identify how the concentration of the calibrator is being expressed, what the units are, and whether it refers to the whole commodity or to a level of protein. If the calibrator is expressed as a level of protein, it should be clarified whether it refers to total protein or soluble protein and how the level of protein was determined, e.g., bicinchoninic acid assay with bovine serum albumin as the standard. Information on whether the calibrator is commercially available should also be provided.

Information on matrixes.—ELISA methods can be susceptible to matrix effects or perform differently in different matrixes. The method developer should clearly identify which matrixes the method is applicable for, on the basis of their in-house data, recognizing the variability of specific formulations. The developer should also identify any matrixes that the method is known to have difficulty with, and identify clearly which states of the food allergen (raw, cooked, or both) the method is capable of detecting.

LOQ, LOD, and lower limit of application (LLA).—LOD is defined as the lowest concentration or mass of analyte in a test sample that can be distinguished from a true blank sample at a specified probability level. LOQ is the lowest level of analyte in a test sample that can be reasonably quantified at a specified level of precision.

Manufacturers or method developers are free to define an LLA at whatever level of confidence they choose. This value may be higher than the LOQ and represents a level below which the method developer does not support or recommend use of the method.

Before conducting an interlaboratory study (precollaborative), a single-laboratory validation study of the ELISA-based allergen detection method should be carried out in-house by the method developer. Guidelines for single-laboratory validation of methods of analysis are readily available (2). The LOD should be estimated by a statistical analysis of the calibration data according to the ISO standard ISO 11843-2 (6) for linear data, or ISO 11843-5 (7) for linear and nonlinear data, using as default probabilities $\alpha = \beta = 0.05$, where α and β represent the probability of a false positive and false negative, respectively. When doing this estimation, care should be taken to include as many sources of variation as possible within a single laboratory. Calibration data from at least three analysts over a minimum of three different runs should be included, preferably using different instruments, if possible.

Ruggedness and lot-to-lot variability of method performance.—Ruggedness refers to the ability of a method to resist changes in the final results when minor deviations are made in the experimental conditions described in the procedure. The ruggedness of the method should be investigated by performing experiments in which specific parameters are changed to determine the impact on the experimental result. In particular, the effect of deviations in incubation times, reagent volumes, extraction conditions (time and temperature) should be investigated. It is recommended that deviations for time and volume be investigated at ±5% or more, and incubation temperatures tried at ±3°C or more. If any of these experimental conditions are particularly important in achieving consistent results, this should be mentioned in the kit insert information.

The shelf life should include the stability of all the reagents provided with the test kit, ideally through real-time testing of reagents under normal storage conditions. Accelerated stability testing at higher than normal storage temperatures can also be used to estimate stability. An expiration date for each test kit should be clearly indicated, along with appropriate conditions for storage before use.

A small number of test kits from each lot should be set aside for comparison with previous or future lots. When a new lot of test kits is produced, it should be tested against the previous lot. New lots should have characteristics similar to those of the previous lots. For example, a positive control sample, such as an incurred test sample or spiked sample, should be analyzed with each new lot to be sure that consistent results are achieved. Information on lot-to-lot variability should be provided by the kit manufacturer as part of the data submission package.

Key Elements of Interlaboratory Validation

Number of Laboratories Required

The required number of participating laboratories will be based on AOAC *Appendix D* guidelines (1), currently set at a minimum of eight laboratories contributing usable data at the end of the study.

In order to encourage participation from as diverse a group of laboratories as possible, the AOAC Presidential Task Force on Food Allergens and the Allergen Working Group of the MoniQA network require that, to minimize bias, no more than one-fourth of the total number of laboratories contributing data which is used in the final analysis of the study may be from the same organization. For the purposes of this requirement, the term organization refers to a particular company, such as the method developer or kit manufacturer, or to any other body, such as a regulatory body or other government agency.

Recruiting enough qualified laboratories to conduct a proper validation study for food allergens is difficult. However, the purpose of an interlaboratory validation study is to document the performance of the method in the hands of other laboratories, and this could not be accomplished if many of the laboratories participating in the study were from the same organization. If method developers use laboratories from their own organization as part of the validation study, the results generated by these laboratories shall have the same dispersion of results as those generated by other participating laboratories.

Table 4. Example of raw data

	0 ppm		0.5 ppm		1.0 ppm		2.5 ppm		5 ppm	
Lab	A	B	A	B	A	B	A	B	A	B
1	0.61	0.46	1.10	1.13	1.24	1.97	3.08	2.80	3.65	3.61
2	–0.27	–0.41	0.41	0.29	0.57	0.71	2.80	2.07	4.51	4.84
3	0.37	0.21	0.62	0.11	0.45	0.70	2.82	2.93	4.24	3.93
4	0.13	0.13	1.06	0.62	0.79	0.41	1.95	2.37	5.22	4.96
5	0.24	–0.10	0.29	0.29	1.60	1.56	3.24	3.54	5.59	5.82
6	–0.23	–0.30	0.89	0.72	1.11	1.07	2.32	2.36	4.67	5.22
7	0.15	0.07	0.04	0.25	0.35	0.01	2.09	2.01	5.37	5.55
8	0.02	0.10	0.67	0.47	0.46	0.19	1.52	1.58	6.35	5.53
9	–0.02	–0.18	1.19	0.64	1.40	1.42	2.37	1.56	4.28	3.75
10	–0.10	–0.09	0.68	0.79	0.87	0.77	1.98	2.52	3.04	3.74

The AOAC Presidential Task Force on Food Allergens and the MoniQA food allergen community will attempt to develop a list of external laboratories from around the world that method developers could enlist to participate in validation studies. This will mitigate issues associated with the quality of results generated by the laboratories, or shipping of study samples across borders.

Number of Matrixes, Concentration Levels, and Replicates Required

The food allergen working group recommends that minimum requirements for any validation study include two matrixes, four concentration levels per matrix, and two replicate samples of each concentration per matrix in each laboratory. This is in compliance with AOAC *Appendix D* requirements for a minimum of five materials. For the concentration levels, one of the levels must be the zero level or blank. As an example, for a study using the minimum four concentration levels, two replicates and two matrixes, each participating laboratory would receive 16 samples for analysis.

In addition to a blank or zero level, one of the remaining concentration levels must be less than or equal to two times the LLA stated for the kit so that at least one of the concentration levels is at the lower end of the calibration curve. The remaining non-zero levels should be evenly distributed throughout the range of the calibration curve.

In general, more replicates per laboratory will result in greater certainty in the estimates of both repeatability and reproducibility. As with most estimates of variation, there is a law of diminishing returns with respect to increasing the sample size: the greatest advantage is made in the first few increases in sample size (replicates), but not much afterwards. These decisions are eventually made based on the tradeoffs between improved statistical estimates and resources needed to manage and perform the study. For allergen ELISA methods, the food allergen working group has concluded that a minimum of two replicates per laboratory will optimize the statistical confidence while not imposing undue burden on study participants.

Acceptance Criteria

Acceptance criteria are defined as numerical limits, ranges, or other suitable measures for acceptance of the analytical results to which a food allergen method should conform to be considered acceptable for its intended use. Acceptability of method performance is generally based on a number of factors, including percent recovery for spiked or incurred samples.

Ideal percent recovery levels would range from 80 to 120%. Recovery levels are affected by both the efficiency of the extraction step and the ELISA procedure. With ELISA methods for food allergens, this level of recovery is not always possible, particularly when certain difficult matrixes are analyzed. In addition, the recovery from incurred samples can be substantially different from those obtained using spiked samples. For this reason, recoveries between 50 and 150% will be considered acceptable so long as they can be shown to be consistent.

Data Analysis for Interlaboratory Studies

The ISO standard for method validation, ISO 5725-2 (8), and the AOAC *Official Methods of Analysis* (9) are the standards that outline how to analyze data stemming from interlaboratory trials in the context of analytical method validation. Each matrix/level combination should be treated as a separate experiment. For each matrix/level combination, the following analyses should be performed: Outliers should be tested sequentially by Cochran's and Grubbs' tests, as indicated in AOAC *Official Methods of Analysis*, *Appendix D* (1). Mean, accuracy (if applicable), repeatability (S_r), reproducibility (S_R), RSD of repeatability (RSD_r), and RSD of reproducibility (RSD_R) should be calculated and reported.

For each matrix, the LOD and LOQ of the method should be estimated using the sample S_R by the methods described in the IUPAC Nomenclature guidelines for LOD and LOQ (10). These guidelines call for a probabilistic estimation of LOD based on the variance observed at zero or near-zero concentration levels. If all assumptions are met (variance is constant and normally distributed, and the blank distribution is centered on zero), the LOD can be estimated as 3.3 times the SD of the distribution of blank results. This corresponds to false-positive and false-negative risks of 5% each ($\alpha = \beta = 0.05$), which is the recommended level for LOD estimation. LOQ can be set at 10 times the S_R.

Example of LOD Estimation for ELISA Collaborative Study Data

The following example uses data from a hypothetical collaborative study performed with an ELISA allergen test kit and shows the various steps required to calculate the LOD and LOQ for the method in a particular matrix as well as how to construct an operating characteristic (OC) curve for the method at a given concentration, such as the LOQ. Because different matrixes could give different results, data from each matrix in the study should be analyzed separately. The example is for samples spiked at nominal

Table 5. Example of data analysis following AOAC/ISO 5725 Standard

		0 ppm	0.5 ppm	1.0 ppm	2.5 ppm	5 ppm
Total number of laboratories	p	10	10	10	10	10
Total number of replicates	Sum(n(L))	20	20	20	20	20
Overall mean of all data (grand mean)	$\bar{\bar{x}}$	0.040	0.612	0.882	2.395	4.694
Repeatability SD	s_r	0.108	0.211	0.220	0.305	0.325
Reproducibility SD	s_R	0.269	0.350	0.536	0.580	0.913
Repeatability RSD	RSD_r	273.438	34.456	24.888	12.721	6.925
Reproducibility RSD	RSD_R	680.549	57.203	60.711	24.228	19.455
HorRat value	HorRat	26.164	3.322	3.724	1.727	1.535

levels of 0, 0.5, 1.0, 2.5, and 5 ppm. The samples were analyzed in duplicate by 10 laboratories. It should be noted that these values may not reflect the full range of the calibration curve for this ELISA method, which could go much higher than 5 ppm. The results of the collaborative study and an example of how to use the data to calculate LOD are as follows:

Step 1: Collect data (*see* Table 4).

Step 2: Data analysis following AOAC/ISO 5725 standard (*see* Table 5).

Step 3: Model (S_R) by mean as per ISO 5725 (*see* Table 6).

Figure 1 gives an example plot of S_R versus mean. This model uses an ordinary least square estimate. Weighted least square analysis would also be acceptable.

Step 4: Estimate LOD and LOQ. Basic formula:

$$LOD = 3.3 \times s(0) = 1.0 \text{ ppm}$$

$$LOQ = 10 \times s(0) = 3.0 \text{ ppm}$$

Advanced formula to adjust for increase in s_R as mean increases: slope = 0.1285; intercept = 0.3081; xbar(0) = 0.039553; LOD = (xbar(0) + 3.3 × intercept)/(1–1.65 × slope); LOD = 1.3405; LOQ = 3 × LOD = 4.0215. These estimates are likely to be more accurate than those obtained following the simple formula.

Step 5: Construct OC curve based on results of Steps 3 and 4. Calculate the SD over a range of concentrations bracketing the LOQ using the formula:

$$SD = 0.1285 \times \text{concentration} + 0.3081$$

where 0.1285 and 0.3081 are the slope and intercept of the curve from Step 3.

Use a normal distribution calculation function to calculate the probability of obtaining a result higher than the LOQ (4.0) for the given concentration using the calculated SD and assuming a normal distribution. The probability thus calculated is plotted against the concentration to obtain the OC curve.

The curve below was calculated in Excel using the following equation to calculate the probability of a result higher than LOQ:

$$= 1 - \text{NORMDIST}(LOQ, \text{mean concentration}, S_R, 1)$$

where the LOQ is set at 4.0 ppm, the mean concentration is on the *x* axis, and the S_R is calculated from the mean concentration using the equation from Step 3.

Figure 2 presents an example of the OC curve. This OC curve shows the probability of obtaining a result above 4 ppm based on the concentration present in a sample. When the concentration in the sample is 4 ppm, there is a 50% chance the result will be above 4 ppm.

It is very important for collaborators to report all results obtained by the method without censoring to a predetermined LOD or LOQ. For nonspiked samples, this may mean half of the responses are negative numbers. It is critical to keep this information in the data set, as censoring will result in biased LOD/LOQ estimates.

For the results of the interlaboratory study, model S_R by concentration mean as detailed in ISO 5725-2. If the slope is significantly greater than zero, it should be taken that variance of the method increases with increased concentration. In this event, LOD estimates will need to be corrected with a general formula, which is shown above. If the general formula for LOD is used, LOQ can be estimated as three times LOD.

Additional guidance on the handling and analysis of data generated during interlaboratory studies will be provided through implementation studies conducted following this validation protocol.

Allergen-Specific Criteria

Certain criteria are dependent upon the specific target food allergen. For example, reference materials, spiking methods and food matrixes will vary from one food allergen to the next. General guidance on allergen-specific criteria and specific guidance for milk and egg allergens are as follows:

Reference materials.—Choosing a reference material for use in an allergen method validation can be extremely challenging. A perfect representative material rarely exists. Different species of the same food commodity may have different protein profiles. Processing methods can also drastically affect protein content, conformation, solubility, and reactivity. In general, a reference material is representative of the allergenic food commodity, is well-characterized, can be produced or supplied with robust reproducible

Table 6. Example of (S_R) modeling

Level	Mean	s_R
0	0.039553	0.26918
0.5	0.612395	0.350308
1.0	0.882414	0.535725
2.5	2.395355	0.580356
5.0	4.693936	0.913203

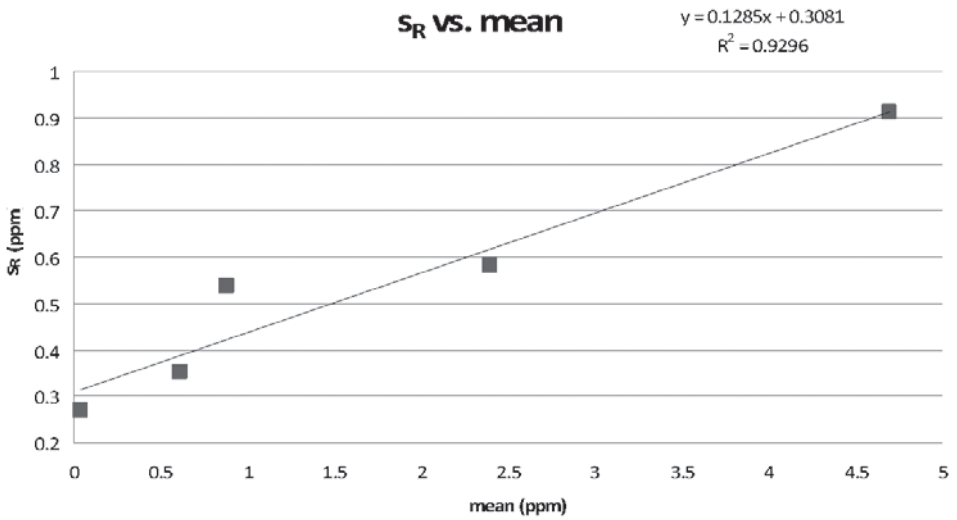


Figure 1. Example curve of S_R versus mean.

characteristics, and can be used as a calibration standard, control, or spiking material. Food allergens can be present in many different forms, processed or unprocessed, depending on the food matrix in which they are found, and with very divergent characteristics and functions in a food. It is unlikely a single material can represent many different possibilities at once. However, a widely available reference material will provide a common reference point for data comparison purposes between kits designed for the same food allergen.

For egg detection methods, based on a preliminary multilaboratory study, a suggested material is the National Institute of Standards and Technology (NIST) egg powder (NIST RM-8445). This is the first NIST reference material specifically intended for use in food allergen testing. The kit manufacturer is expected to provide a conversion factor relative to the NIST egg powder if a different material is used.

For milk detection methods, a suggested material is the NIST nonfat milk powder (NIST RM-1549). Although this reference material was not specifically intended for use in food allergen testing, it has been used in the past for method validations and has performed well as a reference material for milk ELISAs. The kit manufacturer is expected to provide a conversion factor relative to the NIST milk powder if a different material is used.

Spiking methods.—The best source of information on method performance for allergen detection methods is an incurred sample, which is defined as one in which a known amount of the food allergen has been incorporated during processing, mimicking as closely as possible the actual conditions under which the sample matrix would normally be manufactured. This kind of real-life sample would give the most accurate representation of the recovery and response of a particular method for that particular matrix. Whenever possible, validation studies for allergen detection tests should be run using incurred samples. Unfortunately, incurred samples can be difficult and costly to obtain, particularly in larger quantities required for a validation study.

Because of these limitations, validation studies using samples with food allergens added to them after manufacturing (spiked

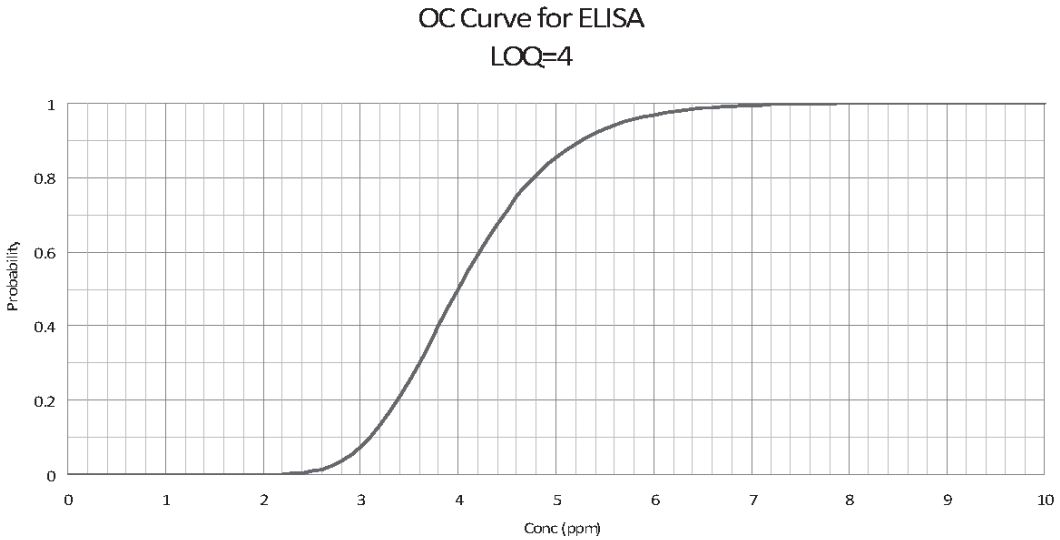


Figure 2. Example of OC curve.

samples) are still considered an acceptable way to generate information about the performance of a method in specific matrixes. However, spiked samples may result in an artificially higher recovery than would be obtained from incurred samples; hence, some regulatory bodies may be unwilling to consider approval of validation data without the inclusion of data generated with incurred samples prepared with known and controlled amounts of the reference material for the allergen being targeted.

There are several ways to prepare spiked samples. One way involves the preparation of a large batch of a food sample that contains a specific food allergen, then gradual dilution of the allergen by mixing with more of the food sample that does not contain the allergen. This kind of sample preparation works best for samples that can be mixed well in an attempt to reach homogeneity, such as liquids or fine powders. An example would be the use of pasta containing a known amount of egg that had been ground to a fine powder and was then mixed with non-egg-containing pasta (also ground to a fine powder) stepwise until the desired concentration of egg was reached. Considerable effort is required to ensure sufficient mixing and to verify the homogeneity of the final batch of material, but this method of sample preparation has the advantage of being relatively similar to an incurred sample.

Because it can be difficult to mix a large batch of samples at a low spiking level to make a homogeneous mixture, the most precise way to spike samples is to add a known amount of a food allergen to each individual sample or test portion. This method results in each sample receiving an accurate amount of analyte, and addresses the issue of homogeneity of the spiked samples. Such a spiking method has been successfully used in the AOAC peanut *Performance Tested Method*℠ study (11). In that study, individual test portions were weighed out and spiked before being sent out for analysis. This method of spiking results in a small part of the actual procedure (weighing of samples) being completed before the samples are distributed to study participants, and eliminates any weighing errors that may be introduced if study participants have to weigh the samples. Although this procedure is not ideal, the AOAC and MoniQA food allergens communities believe it is acceptable in order to overcome problems with production of large batches of food samples homogeneously spiked at a low level with a particular allergen. This type of sample preparation is the most artificial method and least representative of real-life samples.

When spiking samples, unaltered reference material should be used instead of a protein extract of the reference material. If the reference material is completely soluble in the buffer used for spiking, a solution of the reference material can be prepared and diluted to the appropriate level. The spike should be delivered in the same volume for each of the spiking levels.

The stability of the spiking material in the matrix of interest should be investigated by spiking several samples, and then extracting and analyzing them over the same period of time that will be required to complete the entire study. If the response changes significantly over time, this must be accounted for in the study design. Samples will have to be prepared, shipped, and analyzed within a defined time frame to avoid any decrease in response.

The suggested reference materials (NIST RM-1549 for milk and NIST RM-8445 for egg) are both powders that could be used with either of the spiking methods mentioned earlier (spiking a large batch of the matrix followed by serial dilution in a blank matrix, or spiking individual test portions using a spiking solution). Although the NIST nonfat milk powder (NIST RM-1549) is soluble in water or phosphate-buffered saline, the NIST egg powder (NIST RM-8445) is not. However, use of a tissue grinder, such as the Potter-Elvehjem type, will facilitate dispersion of the egg powder to form a homogeneous suspension. Thus, for both egg and milk, a stock solution of the reference material can be made, followed by dilution to the appropriate spiking levels. A recommended starting concentration for the stock solution is 1 mg/mL. In all cases, the method chosen for preparation of the spike and the spiking method should be documented in the validation report.

Food matrixes.—The matrix being analyzed can have a large impact on the performance of an ELISA method. Ideally, methods would be able to analyze all matrixes with equally reliable results. In reality, methods may work better for some matrixes than for others. The choice of matrixes included in a validation study is left to the method developer to meet customer demands. Although no matrixes are mandatory, some are of particular interest for each food allergen and are based on which food products are most likely to be contaminated with a particular allergen. Table 3 lists matrixes of interest for egg and milk. Method developers are encouraged to include as many of these matrixes as possible in their validation studies. However, good performance in one or even several matrixes does not guarantee good performance in others.

Conclusions

The food allergen analytical community is challenged to develop detection methods for multiple allergens in various food products to protect allergic consumers and promote consumer confidence. This protocol reflects the consensus reached through input from various food allergen analytical experts and contains recommendations based on the current knowledge of ELISA methods. Specific recommendations have only been included for two priority allergens, egg and milk. The general considerations of the protocol will be applied to other priority allergens in the future. Meeting the challenges of developing reliable food allergen detection methods requires conscientious and continuous support from the allergen community. Future work is planned for the implementation of this guidance document for egg and milk ELISA methods and for the development of similar guidance pertaining to other priority food allergens.

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