The Scientific Association Dedicated to Analytical Excellence®

AOAC INTERNATIONAL
Stakeholder Panel on
Infant Formula and Adult Nutritionals

EXPERT REVIEW PANEL
Final Action Review of Methods

Thursday, June 8, 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.

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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.

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

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

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

Do's and Don'ts

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

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

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

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

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

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

Procedures

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

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

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

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

ASUREQUALITY LIMITED, NEW ZEALAND

AOAC INTERNATIONAL
STAKEHOLDER PANEL ON INFANT FORMULA AND ADULT NUTRITIONALS

MULTI LABORATORY TESTING REPORT

DETERMINATION OF BIOTIN BY LIQUID CHROMATOGRAPHY COUPLED WITH IMMUNOAFFINITY COLUMN CLEAN-UP EXTRACTION

AOAC FIRST ACTION OFFICIAL METHOD 2016.02

George Joseph
AOAC MLT Study Director
AsureQuality New Zealand
January 2017

The method was approved by the SPIFAN ERP in March 2016 as a First Action Official Method 2016.02 and published in Journal of AOAC Volume 99, Number 4, 2016, pages 1110 to 1112.

TABLE OF CONTENTS

1. INTRODUCTION

1.1. Study Title

Determination of biotin by liquid chromatography coupled with immunoaffinity column clean-up extraction, AOAC International First Action Official Method 2016.02, Multi Laboratory Testing.

1.2. Study Objective

The objective of the MLT study is to establish the precision and accuracy of the method in various laboratories to demonstrate the suitability of the method as an international reference method with endorsement as AOAC Final Action, and possible adoption by ISO and Codex.

1.3. Study Director

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1.4. Study Monitor

The study is monitored by AOAC International (SPIFAN) Official Method Board or by its delegated personnel.

1.5. Abbreviations

AOAC: Association of Official Analytical Chemists
SPIFAN: Stakeholder Panel on Infant Formula and Adult Nutritionals
ERP: Expert Review Panel
MLT: Multi Laboratory Testing
HPLC: High Performance Liquid Chromatography
UV: Ultraviolet
SMPR: Standard Method Performance Requirements
NIST SRM: National Institute of Standards and Technology, Standard Reference Material

2. THE MLT OVERVIEW

The study is divided in two parts: method set up and qualification of participants (Part 1) and multi laboratory testing by the qualified participants (Part 2).

2.1. Method set up and qualification of participants

The participating laboratories were requested to analyse two practice samples in duplicate using the method provided and report the results to the study director. It was communicated to the laboratories that any deviation, such as necessity to substitute reagents, columns, apparatus or instruments, must be duly recorded and reported. Electronic templates were provided to the participants for data reporting. Moreover raw data was requested wherever necessary. After review, the study director identified the laboratories which have the capability to run the analysis successfully. The study director also had discussion with those laboratories produced unacceptable data to see any technical reasons which can be resolved within reasonable time frame to include them in the second part of the study.

2.2. Multi laboratory testing by qualified participants

The qualified laboratories were then asked to analyse the MLT samples on two different days following a carefully designed protocol provided. The results were submitted to the study director for evaluation.

Unless otherwise specified in the protocol, all powdered samples were analysed on a reconstituted basis, using 25 grams of sample into 225 grams with water, as stated in the attached method. An electronic template was provided for data reporting including system suitability, linearity, peak areas of the standard curve as well as of the sample extracts. Furthermore, detailed information on the different weights and volumes used during sample preparation as indicated in the method, as well as raw data (chromatograms of standards and samples) were requested.

Laboratories are asked to report final biotin and biocytin results in µg/100 g to two decimal places.

2.3. Statistical evaluation

After data collection, outliers were detected using Cochran and Grubbs tests. The number and coded identity of statistical outlier laboratories is included in the final report. Average biotin concentrations, standard deviations of repeatability (S_r) and relative standard deviations of repeatability (RSD_r) were estimated from blind duplicates in MLT samples. The blind coded duplicates were analysed on the same day. Standard deviations of reproducibility (S_R), relative standard deviations of reproducibility (RSD_R), and HorRat values (RSD_R/predicted RSD_R) were also determined.

3. MATERIALS AND METHODS

3.1. AOAC SPIFAN Matrices

The study was carried out using SPIFAN matrices, which represent most of the products in the scope of the project (Infant Formula and Adult Nutritionals made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with and without intact protein). Fourteen different product types were provided to each laboratory which includes two practice samples and 12 samples for multi laboratory testing over two separate days. Two split samples of each matrix were included in the package as blind coded duplicates to analyse on the same day. Please refer to Table 1 for sample details.

Homogeneity assessment of AOAC SPIFAN product matrices were performed by analysing several active nutritional ingredients. The testing was performed by Covance Laboratories Inc. in one of their facilities. The homogeneity report was provided to the study director before commencing the MLT programme.

Table 1: AOAC SPIFAN Matrices

Samples	Product Description	Batch / Lot #	Blind Duplicate Codes	
Practice 1	Infant Formula Powder Partially Hydrolysed Soy Based	410457651Z	SWUO667	SWUO667
Practice 2	Infant Formula Powder FOS/GOS Based	50350017W1	URTF231	URTF231
MLT - 1	Infant Formula Powder Partially Hydrolysed Milk Based	410057652Z	KDOX966	ATAN351
MLT - 2	Infant Elemental Powder	00795RF	ECHL425	UOPM297
MLT - 3	Infant Formula RTF Milk Based	EV4H2R	XKIP216	HYJU890
MLT - 4	Adult Nutritional RTF High Fat	00729RF00	DYLB360	ZMQM883
MLT - 5	Infant Formula Powder Milk Based	4044755861	NSRB999	JSDT587
MLT - 6	Infant Formula Powder Soy Based	E10NWZC	TJHR217	OACN211
MLT - 7	NIST SRM 1849a	CLC10-b	KGSZ273	LTCT316
MLT - 8	Adult Nutritional Powder Low Fat	00859RF00	LYNY751	PZGP859
MLT - 9	Child Formula Powder	00866RF00	RQXQ518	GVPE615
MLT - 10	Toddler Formula Powder Milk-Based	4052755861	EFXN778	BFA0941
MLT - 11	Infant Formula Powder Milk Based	K16NTAV	CULF358	GBZC169
MLT - 12	Adult Nutritional RTF High Protein	00730RF00	FPTE312	DOMY545

3.2. MLT Participants

Extensive campaign was carried out through AOAC and ISO/IDF meetings / contacts to recruit potential laboratories for the multi lab testing. Formal invitation has been sent to more than 30 laboratories around the world by the study director and 15 laboratories agreed to participate. Delivery of SPIFAN matrices to a laboratory in Thailand was failed / cancelled due to issues with the recipient's / country's import clearance requirements. Two laboratories could not progress at all with the MLT on time due to inadequate resources or alternative problems. The details of the remaining 12 laboratories from 10 different countries participated in the MLT study is given below in Table 2.

Table 2: MLT Participants

Lab #	Name of the laboratories	Physical Address	Country
1	Abbott Laboratories	3300 Stelzer Road, Columbus, Ohio 43219	United States
2	Aquanal Laboratoire Aquitaine Analyses	151 bis, Avenue Jean Jaures, Pessac 33600	France
3	AsureQuality New Zealand Limited	131 Boundary Road, Auckland 0600	New Zealand
4	AsureQuality Singapore Pte.	29 Tai Seng Avenue 534119	Singapore
5	DTS Food Laboratories	71 Boundary Road, Melbourne VIC 3051	Australia
6	FirstSource Laboratory Solutions LLP	IDA, Nacharam Cross Rd, Hyderabad-500076	India
7	Fonterra Co-operative Group Limited	Cnr No.1 Rd & SH26, Waitoa 3341	New Zealand
8	Merieux NutriSciences	3600 Eagle Nest Dr, Crete IL 60417	United States
9	R-Biopharm Rhône Ltd	45 Acre Road, Glasgow G20 0XA	Scotland
10	Mead Johnson, China	Xia Yuan Rd, Dongji ID, Guangzhou, 510730	P.R. China
11	Mead Johnson, Netherlands	Middenkampweg2, Nijmegen, 6545CJ	Netherlands
12	Nestle Research Centre, Switzerland	Case Postale 44, Lausanne 26, CH-1000	Switzerland

3.3. Analytical Method

The analytical method provided to the laboratories for the MLT is the same as it is published in Journal of AOAC Volume 99, Number 4, 2016, pages 1110 to 1112 which is codified as AOAC 2016.02. Extensive details of the sample preparation, chromatography, calculation, reporting criteria were specified in the method and the protocol provided to the laboratories. None of the laboratories that participated in the MLT recorded any modifications or deviations from the documented procedure. This information was requested to assess the suitability of the method for further approval as Final Action AOAC method.

3.3.1. Title

DETERMINATION OF BIOTIN BY LIQUID CHROMATOGRAPHY COUPLED WITH IMMUNOAFFINITY COLUMN CLEAN-UP EXTRACTION

3.3.2. Introduction

AsureQuality Auckland Laboratory has initiated a method to facilitate a specific, precise, accurate and robust procedure for the analysis of biotin from Infant Formula and Adult / Pediatric Nutritional Formulas. The method also has an assured limit of quantification of 0.1µg/100g (1ppb) based on a simple mathematical relationship between lowest standard and dilution. The method involves immunoaffinity column (R-Biopharm Rhone, EASI-EXTRACT biotin column or equivalent) clean-up and extraction followed by liquid chromatography with UV detection at 200nm.

3.3.3. Principle / Methodology

The sample is dispersed in phosphate buffered saline and autoclaved at 121±2^{0}C for 25 minutes. The sample is cooled to room temperature and then diluted to 100mL in a volumetric flask. The extract is centrifuged and filtered using a Whatman glass microfiber filter paper. Clear filtrate is collected for clean-up and extraction. Biotin immunoaffinity column is mounted onto a SPE manifold. A disposable syringe barrel is connected to the immunoaffinity column as a reservoir. The buffer in the affinity column is drained and the sample filtrate is loaded through the reservoir and allowed to flow through by gravity. The column is

washed with phosphate buffered saline followed by water. Air is passed through the column to remove residual liquid.

Biotin from the column is eluted with methanol and collected in a reacti-vial. The eluent is evaporated to dryness using a heating block set at 85±5°C under a gentle stream of nitrogen and the sample is re-constituted in 1mL of water. The biotin in the reconstituted sample is quantified by HPLC using a UV detector set at 200nm. Identification of biotin peak is based on absolute retention time. Spectrum scan can be used for peak purity confirmation if required.

3.3.4. Chemicals

1. Laboratory Reagent Grade Water
2. Sodium Dihydrogen Phosphate Dihydrate
3. Disodium Hydrogen Phosphate Dihydrate
4. Sodium Hydroxide
5. Methanol, HPLC grade
6. Acetonitrile, HPLC grade
7. Ortho-phosphoric acid, 85%
8. Phosphate buffered saline (PBS) pH 7.4 (Life Technologies 100100.31 or equivalent)
9. Biotin, purity ≥ 99% (Sigma B4501 or equivalent)
10. Biocytin, purity ≥ 98% (Sigma B4261 or equivalent)

3.3.5. Reagents

1. **2 M Sodium Hydroxide**: Weigh 80g of sodium hydroxide into a 1L volumetric flask, dissolve in water and make up to mark.
2. **0.15 M Sodium Phosphate Buffer**: Weigh 9.15g of sodium dihydrogen phosphate dihydrate and 16.31g of disodium hydrogen phosphate dihydrate into a 1L volumetric flask. Dissolve in water and make up to mark. Adjust pH to 7 with 2M sodium hydroxide.
3. **0.1% Phosphoric acid:** In a 1L volumetric flask, add 500 mL water. Add 1.2 mL of ortho-phosphoric acid. Mix and make up to mark with water.

3.3.6. Apparatus

1. Whatman Glass Microfiber Filters, CAT No. 1820-125
2. R-Biopharm Rhone Easy Extract Biotin Immunoaffinity Column P82/P82B or equivalent
3. SPE Manifold with accessories
4. Autoclave set at 121±3°C
5. Centrifuge (variable speed)
6. Analytical Balance 4 dp
7. Amber glass screw cap bottle, 250mL and 100mL
8. Horizontal shaker
9. Volumetric flasks, 1L, 250mL, 100mL, 10mL
10. Pipettors, calibrated, 10.0mL, 5.0mL, 1.0mL, 200µL, 100µL and 50µL.
11. Measuring cylinder, 100mL, 50mL
12. Reacti-vials
13. Reacti-therm heating block with nitrogen blow down (Thermo Scientific)
14. Ultrasonic bath set at 50ºC
15. Centrifuge tubes, 50mL
16. Vortex mixer
17. Syringe filter, PTFE 0.45µm (Advantec Cat No HP045AN)
18. Disposable syringes, 10mL and 1mL
19. HPLC vials, 2mL with glass inserts (200µL)

3.3.7. Sample Preparation

Reconstitution of powder samples: Accurately weigh and transfer 25g sample (W1) to a 250mL amber glass screw cap bottle. Dissolve using warm water (~50ºC) and make up to a total weight of 225g (W2) using water. Mix well until the suspension is homogeneous or use a laboratory homogeniser (Polytron®) if necessary. Weigh an aliquot of homogeneous reconstituted sample (W3) as specified in Appendix 1 of the protocol into 100mL amber glass screw cap bottle and continue with IAC Clean-up extraction. Some homogenous powder samples are excluded from reconstitution, please refer to Appendix 1.

Liquid samples: Mix well to ensure homogeneity of the sample portion and weigh the specified quantity as in Appendix 1 (W3) into 100mL amber glass screw cap bottle and continue with IAC Clean-up extraction (3.3.8).

3.3.8. IAC Clean-up Extraction

1. Add 0.15 M sodium phosphate buffer to an approximate volume of 50mL.
2. Swirl gently to mix well
3. Autoclave the sample preparation at 121ºC for 25 minutes.
4. Cool the sample to room temperature. Quantitatively transfer the extracts into a 100mL volumetric flask and make up to volume with 0.15 M sodium phosphate buffer, mix well.
5. Transfer extracts into centrifuge tubes and centrifuge the samples at 4000rpm for 15min.
6. Filter the samples using the Whatman glass microfiber filter paper, collect the filtrate.
7. Set up the SPE manifold. Attach the immunoaffinity column (IAC) connected to a 10mL reservoir. Drain off the buffer just above the gel.
8. Load the specified volume of sample filtrate onto the IAC as per Appendix 1 and initialise the flow with the gentle suction of a vacuum pump.
9. Let the solution pass through the column by gravity at the rate of one drop per second.
10. Wash the column by passing 10mL of PBS through the column followed by 10mL of water (initialise the flow with the help of vacuum at every step and leave it for gravity).
11. Remove any residual liquid from the column by introducing gentle vacuum.
12. Introduce a reacti-vial and elute the analyte under gravity with 2mL methanol. Elute further with additional 1mL of methanol. Back flush at least 3 times when eluting and this can be achieved by gentle up and down motion of the syringe plunger to maximize the elution.
13. Evaporate the eluent to dryness using a heating block set at 85±50C, under a gentle stream of nitrogen.
14. Cool down to room temperature by keeping it outside for about 15 minutes.
15. Re-dissolve with 1mL water; cap the reacti-vials and vortex for 30 seconds.
16. Filter using syringe filter into a clean glass insert for HPLC analysis.

3.3.9. Standard Preparation

Stock Standard Biotin (100µg/mL)

Weigh 25mg of biotin reference standard into a 250mL amber volumetric flask. Add 150mL of water and sonicate at room temperature for 90 minutes with occasional shaking. Make up to volume with water.

Stock Standard Biocytin (100µg/mL)

Weigh 10mg of biocytin reference standard into a 100mL amber volumetric flask. Add 60mL of water and sonicate at room temperature for 90 minutes with occasional shaking. Make up to volume with water.

Mixed Intermediate Standard (100µg/100mL)

Pipette 1mL of the biotin and biocytin stock standards to a 100mL volumetric flask and dilute to volume with water.

Calibration Standards

Standard 1 (1.0µg/100mL): Dilute 0.10mL of mixed intermediate standard to 10mL with water.

Standard 2 (2.5µg/100mL): Dilute 0.25mL of mixed intermediate standard to 10mL with water.
Standard 3 (5.0µg/100mL): Dilute 0.50mL of mixed intermediate standard to 10mL with water.
Standard 4 (7.5µg/100mL): Dilute 0.75mL of mixed intermediate standard to 10mL with water.
Standard 5 (10µg/100mL): Dilute 1.0mL of mixed intermediate standard to 10mL with water.
Standard 6 (20µg/100mL): Dilute 2.0mL of mixed intermediate standard to 10mL with water.

Note: The concentrations given above are indicative only; calculate the actual concentrations of biotin and biocytin in each calibration standards using the following formula.

Biotin / Biocytin (µg/100mL) = (W1 x P x 10 x Vis) ÷ (V x 10)

W1= Weight of biotin or biocytin (mg)
P = Percentage purity from the certificate of analysis
Vis = Volume of mixed intermediate standard used for the calibration standard (mL)
V = Volume of stock standard (250mL for biotin and 100mL for biocytin)

3.3.10. Chromatographic Conditions

1. Mobile Phase A : 0.1% Phosphoric acid
2. Mobile Phase B : 100% Acetonitrile
3. Mobile Phase C : 80% Acetonitrile
4. Column: Phenomenex Kinetex Phenyl-Hexyl, 00F-4495-E0 (150 x 4.6mm x 2.6µm x 100Å)
5. Column Temperature: 25±2ºC
6. Retention times: Biocytin 4.5 to 5.5 minutes and biotin 16 to 17 minutes
7. Run time: 27 minutes
8. Detector: PDA operating at 200nm (PDA scan 200 to 350nm)
9. Injection volume: 100µL

Table 3: HPLC Gradient Program

Time (min)	Flow Rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)	Mobile Phase C (%)
0.0	0.6	90	10	0
18.0	0.6	90	10	0
18.5	0.8	0	0	100
24.0	0.8	0	0	100
24.5	0.6	90	10	0
27.0	0.6	90	10	0

3.3.11. System Suitability

1. Inject the calibration **standard 4** six times at the beginning of the analytical sequence
2. Run the full set of calibration standards at the start and the end of the sequence
3. The six point calibration should give a correlation coefficient NLT 0.997.
4. Inject one of the calibration standards after every 5 sample injections.

3.3.12. Calculation and Reporting

The chromatography software will automatically calculate the concentration of the sample (as is basis) in µg/100g, provided the concentration of the standards in (µg/100mL), sample weight (g) and dilution are entered correctly.

Manual calculation can also be performed by using the following equation:

$$\text{Biotin or Biocytin (µg/100g)} = \frac{\text{(Sample Area x Dilution)}}{\text{(Slope x Sample weight)}}$$

Dilution = 10 (100 x 1 ÷ 10) Sample made up to 100mL, 10mL used for IAC clean-up to a final volume of 1mL with water for HPLC analysis. The dilution will be 20 if 5mL is used for IAC clean-up.

Slope = Valid slope calculation based on concentration on X-axis and peak area on Y-axis

Sample weight: Calculate powder equivalent in grams using the following equation for reconstituted powder samples.

Sample weight (powder equivalent) in grams = (W1 x W3) / W2

For ready to feed liquid samples, the sample weight used for extraction is used for the calculation.
Report results to two decimal places as biotin or biocytin in µg/100g

3.3.13. Chromatograms

Chromatograms of calibration standard and a SPIFAN MLT sample (00859RF00 / PZGP859) are given below in Figure 1 and 2 respectively.

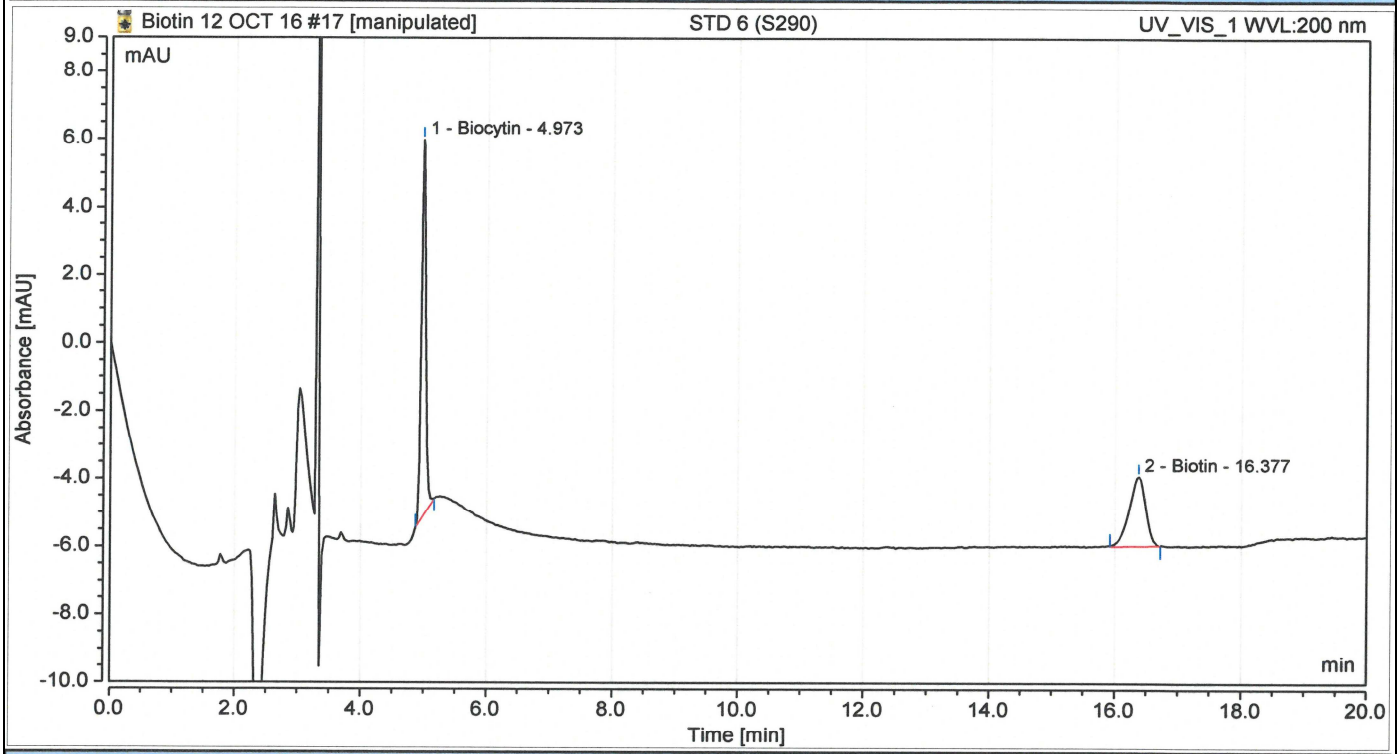
Figure 1: Calibration standard

Chromatogram and Results

Injection Details

Injection Name:	**STD 6 (S290)**	*Run Time (min):*	**27.00**
Vial Number:	**RA6**	*Injection Volume:*	**100.00**
Injection Type:	**Calibration Standard**	*Channel:*	**UV_VIS_1**
Calibration Level:	**06**	*Wavelength:*	**200.0**
Instrument Method:	**Biotin 11 Aug 15 ver 8**	*Bandwidth:*	**4**
Processing Method:	**Biotin**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**13-Oct-16 00:25**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount ug/100mL
1	Biocytin	4.973	0.892	11.102	59.09	84.42	19.8967
2	Biotin	16.377	0.618	2.049	40.91	15.58	19.6813
Total:			**1.510**	**13.151**	**100.00**	**100.00**	

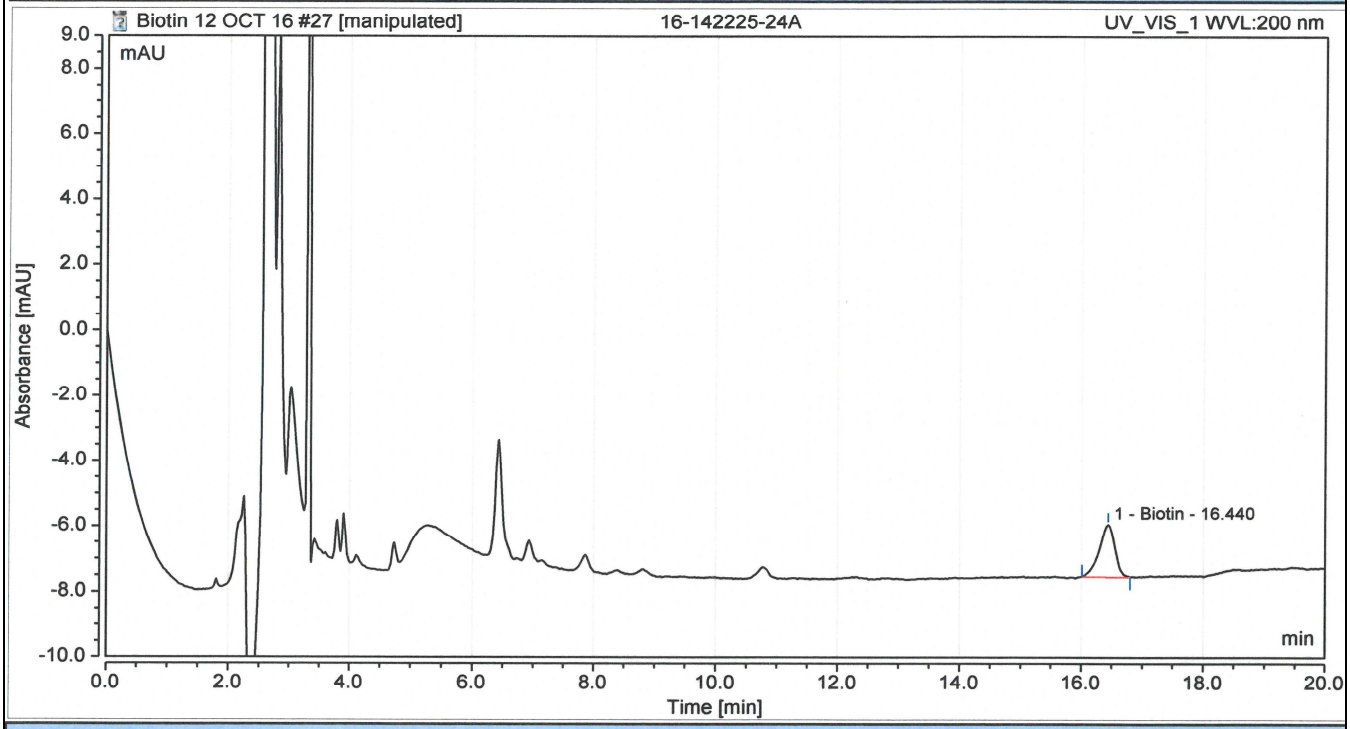
Figure 2: MLT Sample 8B (00859RF00 / PZGP859)

Chromatogram and Results

Injection Details

Injection Name:	**16-142225-24A**	*Run Time (min):*	**27.00**
Vial Number:	**RB7**	*Injection Volume:*	**100.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**200.0**
Instrument Method:	**Biotin 11 Aug 15 ver 8**	*Bandwidth:*	**4**
Processing Method:	**Biotin**	*Dilution Factor:*	**20.0000**
Injection Date/Time:	**13-Oct-16 05:12**	*Sample Weight:*	**1.1154**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount ug/100mL
n.a.	Biocytin	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	Biotin	16.440	0.477	1.599	100.00	100.00	272.4113
Total:			**0.477**	**1.599**	**100.00**	**100.00**	

4. RESULTS AND DISCUSSION

4.1. The overview

The details of the 12 laboratories from 10 different countries participated in the MLT study is given in Table 2. The laboratories were requested to analyse biotin and biocytin in the samples, however none of the samples were found to have biocytin and only biotin results were reported and evaluated in the study. All the biotin results presented in the report are expressed as *µg/100g* of sample as received basis from AOAC International.

Three of the eleven laboratories (Labs 10, 11 and 12) could only report the results of practice samples on the due date of the study, 27 Jan 2017. In general, the results from Lab 7 is showing low bias which was investigated further. The possible reasons for the low bias could be the practice followed in sample preparation especially the steps related to loading of immunoaffinity column and the elution technique where triple backflush is specified. The investigation from Lab 7 reported that the analysts did not follow the backflush technique effectively and that may have caused the incomplete elution of biotin from the cartridges. The laboratory also reported that they had deviated from the protocol in reconstitution of powder samples by taking 10g of powder in 80mL. The results from Labs 7, 10, 11 and 12 are included in the MLT report and for statistical evaluation of the results to calculate precision of the analytical method.

4.2. Linearity and Range

The retention time of biocytin ranges from 3.3 minutes to 6.1 minutes and that of biotin from 13.8 min to 22.5 minutes. The retention times reported from Lab 9 stands out from other laboratories though the chromatographic conditions were followed as documented. The variation is likely due to difference in the hardware configuration of the liquid chromatographs used for the analysis. The information was not available from Lab 10 at the time of the data processing.

All the participants used more or less the same calibration range 1 to 20 µg/100mL for biocytin and biotin as specified in the method. The range showed excellent correlation coefficient among the participants of not less than 0.999, confirming the linearity of the method over the calibration range (Table 4).

Table 4: Retention times and correlation coefficients

Lab #	Retention Time (Min)		Correlation Coefficient (r^2)	
	Biocytin	Biotin	Biocytin	Biotin
1	4.6	16.1	0.9981	0.9995
2	3.8	15.9	0.9996	0.9999
3	4.9	16.2	0.9987	0.9993
4	5.9	18.1	0.9983	0.9997
5	4.4	15.5	0.9997	0.9993
6	4.9	16.5	0.9995	0.9995
7	3.3	14.5	0.9990	0.9990
8	4.1	13.8	0.9991	0.9973
9	6.1	22.5	0.9998	0.9995
11	4.9	15.1	0.9995	0.9988
12	4.1	12.9	0.9990	0.9993

4.3. MLT Results and Statistical Evaluation

The biotin results from practice and MLT samples (Day 1 and 2) are given in Table 5, 6 and 7 respectively. Fourteen different product types were analysed in duplicate by nine laboratories which includes two practice samples and 12 samples for multi laboratory testing over two separate days. The practice samples were analysed in duplicates and the blind coded duplicates were analysed during MLT on the same day by the participants. Laboratories 10, 11 and 12 submitted results for practice samples only and the results are presented in Table 5.

The results are closely comparable between the duplicates and among all the participants and all the data have been used for statistical evaluation to calculate repeatability, reproducibility and HorRat. The statistical analysis of the data was carried out using *International Study Workbook Version 2.1 for Blind (Unpaired) Replicates* from AOAC International. The data as presented in the report as *µg/100g* were used as such for statistical calculation with a Factor for Units of Measurement of 1.00E-08. The summary of relative standard deviation of repeatability (RSD_r) and reproducibility (RSD_R) along with HorRat performance ratios is tabulated in Table 8.

4.4. Relative Standard Deviation of Repeatability (RSD_r)

Repeatability expresses the precision under the same operating conditions (intra-assay) over a short interval of time. The AOAC SPIFAN SMPR 2014.005 for the biotin analysis specifies a maximum repeatability standard deviation of not more than 6% for biotin levels greater than 1 µg/100g. The mean RSD_r of the matrices analysed in the study is 4.6% and is well within the limit of the SMPR. However MLT samples 5 and 9 recorded slightly higher RSD_r of 6.79% and 7.03% respectively. The duplicate results from Lab 8 are causing the outlier for MLT sample 5 and removing the data from the lab bring the RSD_r down to 5.68%. Similarly, the removal of duplicate data from Lab 9 will bring the RSD_r of MLT sample 9 down to 5.81%. The removal of these values still leaves statistically significant duplicate sets (8 sets) for a valid RSD_r calculation. However the values are kept in the report as the overall repeatability meets the SMPR criteria and the SLV demonstrated the repeatability precision before the present study.

4.5. Relative Standard Deviation of Reproducibility (RSD_R)

Reproducibility expresses the precision among the laboratories. The relative standard deviation of reproducibility (RSD_R) exceeds the SMPR criteria of not more than 12% for all the 14 matrices analysed in the study confirming the precision of the analytical method. The maximum RSD_R noticed was 9.5% in the MLT sample 1, which is partially hydrolysed milk based infant formula matrix. The reproducibility precision confirms the suitability of the SPIFAN method AOAC 2016.02 as a strong candidate as a reference method for global dispute resolution hence the inter-laboratory variation is minimal and exceeds the expectation of the SMPR limits.

4.6. Horwitz Ratio (HorRat)

The Horwitz ratio (HorRat) is a normalized performance parameter indicating the acceptability of methods of analysis with respect to among-laboratory precision (reproducibility). It is the ratio of the observed relative standard deviation among laboratories calculated from the actual performance data, RSD_R, to the corresponding predicted relative standard deviation ($PRSD_R$) calculated from the Horwitz equation. The formula for the Horwitz ratio as presented in the *International Study Workbook* of AOAC International is applicable only when the concentration is in the unit/unit form (e.g., µg/µg or g/g, etc.). When the analyte concentration is a mass fraction amount as µg/100g, the appropriate factor for unit of measurement should be selected to generate correct HorRat values. The factor selected in the study for the calculation is 1.00E-08.

Under reproducibility conditions, the acceptable HorRat value range is 0.5 to 2 as per AOAC International. The HorRat values obtained in this study were within 0.3 to 0.5 in all SPIFAN matrices except the NIST SRM 1849a. The performance requirements of SPIFAN methods are generally tighter than routine analytical methods and therefore $PRSD_R$ used in the formula may be slightly higher pushing the HorRat values down. Nevertheless, the lower Horwitz ratios confirm the enhanced performance of the method and homogeneity of the matrices used for the study. The NIST SRM 1849a recorded the lowest HorRat value of 0.21 and undoubtedly the most homogeneous certified reference material used in the MLT.

4.7. Accuracy (NIST SRM 1849a)

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The established procedure for accuracy assessment is by analysing samples with known concentrations such as certified reference materials. The SPIFAN SMPR 2014.005 recommends NIST SRM 1849a for accuracy evaluation and the SRM is included in the SPIFAN matrices as blind coded duplicates.

The NIST SRM 1849a results were all within the certified limits of biotin confirming the accuracy of the method. It has been noticed that one of the duplicate results of NIST reference sample was slightly on the low bias (185.41µg/100g) for Lab 7 but this was investigated as explained before. The NIST SRM 1849a has comparatively higher biotin content and homogeneous therefore unlike other powder samples it was not reconstituted with water and 1g powder was directly weighed for the analysis.

Matrix recoveries were carried out during SLV at different levels of calibration range and the recoveries were within 95 to 105%.

4.8. Selectivity (Comparison with LCMS/MS)

Selectivity / specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Selectivity of the method is confirmed by analysing the SPIFAN matrices by LCMS/MS method by one of the laboratories participated in the MLT study (Lab 5). The method is a modified version of AOAC Official Method 2012.16 for pantothenic acid and uses a UPLC platform with triple quadrupole mass spectrometer. The biotin results by LCMS/MS method were closely comparable to the MLT results for the range of SPIFAN matrices used in the study. Please refer to Table 9 for comparison data.

Table 5: Biotin (µg/100g) Practice Samples

Lab #	PRACTICE Sample 1		PRACTICE Sample 2	
	A	B	A	B
1	39.28	39.28	15.50	15.50
2	37.76	38.74	14.69	15.68
3	40.21	40.21	15.11	15.11
4	39.40	39.40	15.17	15.17
5	42.90	37.30	13.20	15.90
6	39.33	39.92	14.73	15.79
7	31.73	32.26	12.38	11.50
8	40.49	40.38	15.98	16.48
9	40.83	40.61	14.41	15.53
10	38.58	38.58	14.73	14.73
11	39.80	39.10	15.10	15.30
12	34.29	35.89	16.00	14.87
Mean	**38.72**	**38.47**	**14.75**	**15.13**
%RSD	**7.75**	**6.17**	**7.14**	**8.19**

Table 6: Biotin (µg/100g) MLT samples (Day 1)

Lab #	MLT Sample 1		MLT Sample 2		MLT Sample 3		MLT Sample 4		MLT Sample 5		MLT Sample 6	
	A	B	A	B	A	B	A	B	A	B	A	B
1	32.96	31.42	68.94	72.48	3.45	3.88	70.84	73.68	24.42	24.78	46.79	43.44
2	39.47	38.48	81.71	83.53	4.33	4.49	78.33	68.43	27.42	28.91	51.02	48.20
3	33.38	33.21	80.37	81.62	4.03	4.22	69.58	72.92	26.25	27.38	46.18	45.42
4	35.41	34.62	81.88	80.43	3.79	3.74	71.65	70.80	28.39	24.24	44.15	44.03
5	37.49	34.70	84.88	79.87	3.84	4.37	77.67	74.07	26.12	25.93	47.15	43.55
6	29.09	35.06	77.44	81.29	4.43	4.05	74.44	74.98	29.29	32.95	41.88	47.62
7	33.45	32.33	76.96	75.88	4.29	3.96	57.51	59.17	24.82	24.66	39.25	41.15
8	27.85	30.34	75.70	79.96	3.66	4.00	72.86	70.76	25.36	30.22	39.91	45.41
9	34.96	37.95	81.26	81.41	4.33	4.43	74.23	74.41	27.94	26.37	42.89	47.41
Mean	**33.78**	**34.23**	**78.79**	**79.61**	**4.02**	**4.13**	**71.90**	**71.02**	**26.67**	**27.27**	**44.36**	**45.14**
%RSD	**10.89**	**8.04**	**5.95**	**4.24**	**8.68**	**6.37**	**8.53**	**6.94**	**6.31**	**10.72**	**8.57**	**5.16**

Table 7: Biotin (µg/100g) MLT Samples (Day 2)

Lab #	MLT Sample 7		MLT Sample 8		MLT Sample 9		MLT Sample 10		MLT Sample 11		MLT Sample 12	
	A	B	A	B	A	B	A	B	A	B	A	B
1	195.27	200.37	270.20	265.83	171.08	155.73	10.69	9.80	44.26	44.68	48.66	56.39
2	200.63	191.30	271.16	266.46	180.38	159.53	11.11	10.68	46.01	43.08	52.02	53.47
3	196.99	196.48	265.19	272.41	179.63	168.93	10.40	10.24	42.19	43.96	54.56	54.37
4	191.31	205.32	251.11	265.49	171.89	162.31	11.19	9.20	44.02	44.66	54.54	54.92
5	196.80	196.60	260.67	247.84	170.46	161.75	9.76	9.37	40.82	40.13	52.48	49.79
6	193.53	186.75	263.54	248.41	184.86	161.43	9.88	9.89	43.31	42.48	53.84	56.11
7	185.41	190.38	219.30	213.21	153.16	140.19	10.05	9.15	38.43	39.03	50.09	50.14
8	201.99	199.82	268.69	254.35	175.94	163.60	9.36	9.59	40.44	42.14	52.00	44.45
9	202.58	205.62	278.04	274.41	183.98	158.33	11.02	10.72	46.05	46.44	55.47	54.89
Mean	**196.06**	**196.96**	**260.88**	**256.49**	**174.60**	**159.09**	**10.38**	**9.85**	**42.84**	**42.96**	**52.63**	**52.73**
%RSD	**2.81**	**3.33**	**6.64**	**7.37**	**5.55**	**5.02**	**6.35**	**6.02**	**6.05**	**5.42**	**4.24**	**7.39**

Table 8: Statistical evaluation biotin results from SPIFAN matrices

Samples	Product Description	p	Mean	%RSD_r	%RSD_R	HorRat
Practice 1	Infant Formula Powder Partially Hydrolysed Soy Based	12	38.59	3.18	6.98	0.38
Practice 2	Infant Formula Powder FOS/GOS Based	12	14.94	4.92	7.74	0.36
MLT - 1	Infant Formula Powder Partially Hydrolysed Milk Based	9	34.01	5.53	9.48	0.50
MLT - 2	Infant Elemental Powder	9	79.20	2.64	5.14	0.31
MLT - 3	Infant Formula RTF Milk Based	9	4.07	5.52	7.59	0.29
MLT - 4	Adult Nutritional RTF High Fat	9	71.46	3.88	7.75	0.46
MLT - 5	Infant Formula Powder Milk Based	9	26.97	6.79	8.76	0.45
MLT - 6	Infant Formula Powder Soy Based	9	44.75	5.77	6.96	0.39
MLT - 7	NIST SRM 1849a	9	196.5	2.38	3.04	0.21
MLT - 8	Adult Nutritional Powder Low Fat	9	258.7	2.81	7.03	0.51
MLT - 9	Child Formula Powder	9	166.8	7.03	7.03	0.47
MLT - 10	Toddler Formula Powder Milk-Based	9	10.12	5.74	6.65	0.29
MLT - 11	Infant Formula Powder Milk Based	9	42.90	2.26	5.72	0.31
MLT - 12	Adult Nutritional RTF High Protein	9	52.68	5.14	5.89	0.33

p = total number of laboratories
RSD_r = relative standard deviation of repeatability
RSD_R = relative standard deviation of reproducibility
HorRat = Horwitz Ratio (RSD_R / $PRSD_R$)

Table 9: Comparison with LCMS/MS technique

Sample #	Product Description	Blind Duplicates	Biotin (µg/100g)		
			AOAC 2016.02	LCMS/MS	Difference
MLT 1	Infant Formula Powder Partially Hydrolysed Milk Based	A	33.78	35.55	-1.77
		B	34.23	39.10	-4.87
MLT 2	Infant Elemental Powder	A	78.79	82.45	-3.66
		B	79.61	81.15	-1.54
MLT 3	Infant Formula RTF Milk Based	A	4.02	3.73	0.29
		B	4.13	3.84	0.29
MLT 4	Adult Nutritional RTF High Fat	A	71.90	66.06	5.84
		B	71.02	77.49	-6.47
MLT 5	Infant Formula Powder Milk Based	A	26.67	26.99	-0.32
		B	27.27	25.25	2.02
MLT 6	Infant Formula Powder Soy Based	A	44.36	43.00	1.36
		B	45.14	42.27	2.87
MLT 7	NIST SRM 1849a	A	196.06	186.58	9.48
		B	196.96	181.50	15.46
MLT 8	Adult Nutritional Powder Low Fat	A	260.88	263.90	-3.02
		B	256.49	246.17	10.32
MLT 9	Child Formula Powder	A	174.60	171.69	2.91
		B	159.09	162.77	-3.68
MLT 10	Toddler Formula Powder Milk-Based	A	10.38	9.87	0.51
		B	9.85	10.30	-0.45
MLT 11	Infant Formula Powder Milk Based	A	42.84	41.66	1.18
		B	42.96	39.77	3.19
MLT 12	Adult Nutritional RTF High Protein	A	52.63	54.05	-1.42
		B	52.73	51.57	1.16

5. SUMMARY AND CONCLUSION

The SLV and the MLT data provide systematic scientific evidence for a simple, selective, accurate and precise method as a potential candidate reference method for dispute resolution for the determination of total biotin in all forms of infant, adult, and/or pediatric formula. The method fully meets the intended purpose and applicability statement by complying with standard method performance requirement outlined in AOAC SPIFAN SMPR 2014.005.

The method was applied to a cross section of matrix types and established acceptable precision and accuracy. The analytical platform is inexpensive and the method can be used in almost any labs worldwide with basic facilities. The immunoaffinity column clean-up extraction is the key step to successful analysis. Although R-Biopharm IAC was used for the MLT, alternative IAC was compared during SLV with comparable results. The performance parameters of the method are compared with AOAC SPIFAN SMPR 2014.005 and the key points are summarised in the Table 10.

Table 10: Comparison of method performance with SMPR 2014.005

Parameters	SMPR 2014.005	AOAC 2016.02	Comments
Analytical Range	0.1 - 150 µg/100g	0.1 - 300 µg/100g	Analytical range of the proposed method is wider based on the sample weight and loading volume for IAC extraction.
Limit of Quantitation	≤0.1 µg/100g	≤0.1 µg/100g	The proposed method meets the LOQ of 0.1 µg/100g as required by SMPR 2014.005.
Repeatability (RSD_r)	>1 µg/100g: ≤6%	>1 µg/100g: ≤5%	Repeatability of the proposed method is better than the SMPR 2014.005.
Reproducibility (RSD_R)	>1 µg/100g: ≤12%	>1 µg/100g: ≤10%	Reproducibility of the proposed method is better than the SMPR 2014.005.
Horwitz Ratio (HorRat)	0.5 to 2.0	0.3 to 0.5	Excellent precision performance rating.
Recovery (>1µg/100g)	90 to 110%	95 to 105%	The spiked recoveries by the proposed method are better than the recovery range specified by SMPR 2014.005.
NIST SRM 1849a	199 ± 13	197 ± 10	NIST 1949a results are within the certified limits.

6. RECOMMENDATIONS

A complete AOAC International MLT study workbook along with the statistical report and draft copy of the study report summarising the outcomes of this collaborative study were submitted with the recommendation that the AOAC First Action Official Method 2016.02 be accepted as a SPIFAN endorsed AOAC Official Final Action Method.

7. NOTES FROM THE STUDY DIRECTOR

I would like to place on record my learnings and experience with the collaborators during the course of the study. I believe that ideas for improvement, feedback and challenges are valuable information for future enhancement to the method in some way or other.

Filtrate for IAC Clean-up: One of the participants commented that the filtrate for IAC clean-up is not clear, suggested that if the same extraction would be also efficient if performed at pH 4, which would precipitate many of the proteins, yielding a clear filtrate. My laboratory tested few SPIFAN matrices using sodium acetate solution 0.4M, pH 4.0 (In to a 2000mL volumetric flask, weigh 108.8 g sodium acetate trihydrate. Add about 1800mL water. Dissolve. Add 50mL acetic acid, and adjust pH to 4.0 with acetic acid, dilute to volume with water). The filtrate was clear, IAC clean-up was faster and the biotin results were comparable to the current method. However this change was not advised to any of the collaborators and the participant was advised to proceed with current method as published as First Action Official AOAC 2016.02.

IAC loading speed: Two laboratories obtained lower biotin results for practice samples initially were advised to follow IAC loading by gravity as documented in the method and the results came back higher and comparable to other labs.

Elution of biotin from IAC: While assisting a participant investigating low biotin results, it became apparent that the "back flush" technique was inexactly followed, mainly it seems because the exact description of this technique was not given. So, rather than performing a true "back flush", the 3mL methanol eluate was recycled through the IA cartridge three times. It is possible that this may partly explain slightly low recovery for some samples, given that the IA cartridge may well have retained a small portion of this eluent fraction / biotin. The details of the back flush using a syringe plunger / reverse pressure is now detailed in the method.

Syringe Filter: At least two laboratories experienced interference with chromatography. The investigation isolated the issue with syringe filters with extractable chromophores. It was advised to test the filters as part of the reagent blank preparation

Quality Water: Quality of water**,** especially the water used for the final extraction is very important to get clean chromatogram. Type 1 water is recommended and this information will be updated in the method.

UPLC Platform: The availability of stationary phase (solid-core, kinetex phenyl hexyl) in smaller particle size for UPLC platforms is encouraging. This would significantly reduce analysis time and improve throughput, my laboratory is doing further work to validate the change.

Calculation errors: The calculation error is a common problem which was noticed. It was advised to participants to use reference samples to prompt any issues with calculation before reporting the results.

8. REFERENCES

1. AOAC SPIFAN SMPR 2014.005 (2014)
2. AOAC Official First Action Method 2016.02
3. AOAC International, International Study Workbook Version 2.1 for Blind (Unpaired) Replicates
4. Appendix F: Guidelines for SMPR, AOAC International (2012)
5. Appendix D: Guidelines for collaborative study procedures to validate characteristics of a method of analysis, AOAC International (2012).
6. Biotin (Bio-02) SLV Report 14/05V (2014)

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13. *David Wollard,* Eurofins, New Zealand
14. *Adrienne McMahon,* Wyeth Nutrition Askeaton, Ireland

Determination of total biotin by liquid chromatography coupled with immunoaffinity column clean-up extraction: Multi Laboratory Testing, Final Action 2016.02

First Action 2016
Final Action 2017

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A collaborative study was carried out on AOAC First Action Official Method 2016.02: Determination of total biotin by liquid chromatography coupled with immunoaffinity column clean-up extraction. Subsequent to the successful method optimisation and analysis practice samples by 12 laboratories covering 10 different countries, 9 laboratories completed the analysis 12 pairs of blind duplicates before the due date of the study. Carefully selected SPIFAN matrices were used for the multi laboratory testing.

The sample is dispersed in phosphate buffered saline (PBS) and autoclaved at 121±2^{0}C for 25 minutes. The sample is cooled to room temperature and then diluted to 100mL in a volumetric flask. The extract is centrifuged and filtered using a Whatman glass microfiber filter paper (GE Healthcare Life Sciences, Buckinghamshire, UK). Clear filtrate is collected for clean-up and extraction. Biotin immunoaffinity column is mounted onto a SPE manifold. A disposable syringe barrel is connected to the immunoaffinity column as a reservoir. The buffer in the affinity column is drained and the sample filtrate is loaded through the reservoir and allowed to flow through by gravity. The column is washed with PBS followed by water. Air is passed through the column to remove residual liquid.

Biotin and Biocytin from the column is eluted with methanol and collected in a reacti-vial (Cat. No. 13223, Thermo Scientific). The eluent is evaporated to dryness using a heating block set at 85±5°C under a gentle stream of nitrogen and the sample is re-constituted in 1mL of water. The biotin / biocytin in the reconstituted sample are analysed simultaneously by HPLC using a PDA set at 200nm. Identification of peaks is based on absolute retention time. Quantification was by multipoint external calibration using peak area responses of the analytes. Spectrum scan (200 nm to 350 nm) can be used for the purity and identity confirmation as required.

Biotin / Vitamin H / Vitamin B_7 is a B group vitamin involved in the production of energy and functions as a coenzyme in bicarbonate-dependent carboxylation reactions. Biotin exists as free biotin and in protein-bound forms in foods. Signs of biotin deficiency are quite evident in humans who consume raw egg white over long periods. Raw egg white contains avidin, a protein that has shown to bind biotin in the small intestine and prevent its absorption. Clinical findings of biotin deficiency include dermatitis, conjunctivitis, alopecia and central nervous system abnormalities.

Biotin (Hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-4-pentanoic acid; CAS 58-85-5) is the fusion of an imidazolidone ring with a tetrahydrothiophen group linked to a valeric acid side chain. The chemical formula of biotin is $C_{10}H_{16}N_2O_3S$ with a molecular weight of 244.31.

Biocytin (N6-[5-[(3aS,4S,6aR)-Hexahydro-2-oxo¬-1H-thieno[3,4-d]imidazol-4-yl]-1-oxopentyl]-L-lysine; CAS 576-19-2) is a bound form of biotin (the linked molecule is lysine) is also biologically active in human system. The chemical formula of biocytin is $C_{16}H_{28}N_4O_4S$ with a molecular weight of 372.48.

Naturally active forms of biotin in foodstuffs are d-biotin and d-biocytin though the fortified form is exclusively d-biotin. These two forms should therefore be determined in order to estimate the total biotin nutritional value.

In view of the absence of a standard analytical procedure for total biotin, the biotin working group of SPIFAN activity mooted by AOAC International has developed a Standard Method Performance Requirements (SMPR 2014.005) in search for a candidate method to be considered as a global dispute resolution standard.

The AsureQuality Auckland Laboratory has developed a method to facilitate a specific, precise, accurate and robust procedure for the analysis of biotin and biocytin from Infant Formula and Adult / Pediatric Nutritional Formulas (1-8). The method meets the SMPR and also has an assured limit of quantification of 0.1µg/100g (1ppb) based on a simple mathematical relationship between lowest standard and dilution. The method involves immunoaffinity column (R-Biopharm Rhone, EASI-EXTRACT biotin column or equivalent) clean-up and extraction followed by LC - UV set at 200nm. The laboratory has carried out extensive Single Laboratory Validation (SLV) study following SPIFAN SLV guidelines (Appendix L: AOAC International) using SPIFAN kit and NIST SRM 1849a. The SLV along with the method was reviewed by the AOAC SPIFAN expert review panel (ERP) in 2016 and approved for First Action Official Method of Analysis (OMA) 2016.02.

Collaborative Study (Multi Laboratory Testing)

The objective of the Multi Laboratory Testing (MLT) study was to establish the precision and accuracy of the method in various laboratories to demonstrate the suitability of the method as an international reference method with endorsement as AOAC Final Action, and possible adoption by ISO and Codex.

The study was chaired by Dr George Joseph from AsureQuality New Zealand and is monitored by AOAC International (SPIFAN) Official Method Board through its delegated personnel. The study is divided in two parts: method set up and qualification of participants (Part 1) and multi laboratory testing by the qualified participants (Part 2).

Extensive campaign was carried out through AOAC and ISO/IDF meetings / contacts to recruit potential laboratories for the MLT. Formal invitation has been sent to more than 30 laboratories around the world by the study director and 15 laboratories agreed to participate. Delivery of SPIFAN matrices to a laboratory in Thailand was failed / cancelled due to issues with the recipient's / country's import clearance requirements. Two laboratories could not progress at all with the MLT within the time frame due to inadequate resources or alternative problems. The details of the remaining 12 laboratories from 10 different countries participated in the MLT study (Table 2016.02A)

Table 2016.02A: MLT Participants

Lab #	Name of the laboratories	Physical Address	Country
1	Abbott Laboratories	3300 Stelzer Road, Columbus, Ohio 43219	United States
2	Aquanal Laboratoire Aquitaine Analyses	151 bis, Avenue Jean Jaures, Pessac 33600	France
3	AsureQuality New Zealand Limited	131 Boundary Road, Auckland 0600	New Zealand
4	AsureQuality Singapore Pte.	29 Tai Seng Avenue 534119	Singapore
5	DTS Food Laboratories	71 Boundary Road, Melbourne VIC 3051	Australia
6	FirstSource Laboratory Solutions LLP	IDA, Nacharam Cross Rd, Hyderabad-500076	India
7	Fonterra Co-operative Group Limited	Cnr No.1 Rd & SH26, Waitoa 3341	New Zealand
8	Merieux NutriSciences	3600 Eagle Nest Dr, Crete IL 60417	United States
9	R-Biopharm Rhône Ltd	45 Acre Road, Glasgow G20 0XA	Scotland
10	Mead Johnson, China	Xia Yuan Rd, Dongji ID, Guangzhou, 510730	P.R. China
11	Mead Johnson, Netherlands	Middenkampweg2, Nijmegen, 6545CJ	Netherlands
12	Nestle Research Centre, Switzerland	Case Postale 44, Lausanne 26, CH-1000	Switzerland

The study was carried out using SPIFAN II matrices, which represent most of the products in the scope of the project (Infant Formula and Adult Nutritionals made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with and without intact protein). Fourteen different product types were provided to each laboratory which includes two practice samples and 12 samples for multi laboratory testing over two separate days. Two split samples of each matrix were included in the package as blind coded duplicates to analyse on the same day. The sample details are summarised in Table 2016.02B

Table 2016.02B: AOAC SPIFAN Matrices

Samples	Product Description	Batch / Lot #	Blind Duplicate Codes	
Practice 1	Infant Formula Powder Partially Hydrolysed Soy Based	410457651Z	SWUO667	SWUO667
Practice 2	Infant Formula Powder FOS/GOS Based	50350017W1	URTF231	URTF231
MLT - 1	Infant Formula Powder Partially Hydrolysed Milk Based	410057652Z	KDOX966	ATAN351
MLT - 2	Infant Elemental Powder	00795RF	ECHL425	UOPM297
MLT - 3	Infant Formula RTF Milk Based	EV4H2R	XKIP216	HYJU890
MLT - 4	Adult Nutritional RTF High Fat	00729RF00	DYLB360	ZMQM883
MLT - 5	Infant Formula Powder Milk Based	4044755861	NSRB999	JSDT587
MLT - 6	Infant Formula Powder Soy Based	E10NWZC	TJHR217	OACN211
MLT - 7	NIST SRM 1849a	CLC10-b	KGSZ273	LTCT316
MLT - 8	Adult Nutritional Powder Low Fat	00859RF00	LYNY751	PZGP859
MLT - 9	Child Formula Powder	00866RF00	RQXQ518	GVPE615
MLT - 10	Toddler Formula Powder Milk-Based	4052755861	EFXN778	BFA0941
MLT - 11	Infant Formula Powder Milk Based	K16NTAV	CULF358	GBZC169
MLT - 12	Adult Nutritional RTF High Protein	00730RF00	FPTE312	DOMY545

Homogeneity assessment of AOAC SPIFAN product matrices were performed by analysing several active nutritional ingredients. The testing was performed by Covance Laboratories Inc. in one of their facilities. The homogeneity report was provided to the study director which is carefully evaluated before commencing the MLT programme.

The participating laboratories were requested to analyse two practice samples in duplicate using the first action method provided and report the results to the study director. It was communicated to the laboratories that any deviation, such as necessity to substitute reagents, columns, apparatus or instruments, must be duly recorded and reported. Electronic templates were provided to the participants for data reporting. Moreover raw data was requested wherever necessary. After review, the study director identified the laboratories which have the capability to run the analysis successfully. The study director also had discussion with those laboratories produced unacceptable data to see any technical reasons which can be resolved within reasonable time frame to include them in the second part of the study.

The qualified laboratories were then asked to analyse the MLT samples on two different days following a carefully designed protocol provided. The results were submitted to the study director for evaluation.

Unless otherwise specified in the protocol, all powdered samples were analysed on a reconstituted basis, using 25 grams of sample into 225 grams with water, as stated in the method. An electronic template was provided for data reporting including system suitability, linearity, peak areas of the standard curve as well as of the sample extracts. Furthermore, detailed information on the different weights and volumes used during sample preparation as indicated in the method, as well as raw data (chromatograms of standards and samples) were requested. Laboratories are asked to report final biotin and biocytin results in µg/100 g to two decimal places.

After data collection, outliers were detected using Cochran and Grubbs tests. The number and coded identity of statistical outlier laboratories is included in the final report. Average biotin concentrations, standard deviations of repeatability (Sr) and relative standard deviations of repeatability (RSDr) were estimated from blind duplicates in MLT samples. The blind coded duplicates were analysed on the same day. Standard deviations of reproducibility (SR), relative standard deviations of reproducibility (RSDR), and HorRat values (RSDR/predicted RSDR) were also determined.

The analytical method provided to the laboratories for the MLT is the same as it is published in Journal of AOAC Volume 99, Number 4, 2016, pages 1110 to 1112 which is codified as First Action AOAC 2016.02. Extensive details of the sample preparation, chromatography, calculation, reporting criteria were specified in the method and the protocol provided to the laboratories. None of the laboratories that participated in the MLT recorded any modifications or deviations from the documented procedure. This information was requested to assess the suitability of the method for further approval as Final Action AOAC method.

AOAC Official Method 2016.02

Determination of total biotin by liquid chromatography coupled with immunoaffinity column clean-up extraction

First Action 2016
Final Action 2017

Applicable to the determination of total biotin in all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids and liquid concentrates)

Caution: Refer safety data sheets for all chemicals prior to use. Ensure all appropriate personal protective equipment and follow good laboratory practices.

A. Principle / Methodology

The sample is dispersed in phosphate buffered saline (PBS) and autoclaved at 121±2^{0}C for 25 minutes. The sample is cooled to room temperature and then diluted to 100mL in a volumetric flask. The extract is centrifuged and filtered using a Whatman glass microfiber filter paper (GE Healthcare Life Sciences, Buckinghamshire, UK). Clear filtrate is collected for clean-up and extraction. Biotin immunoaffinity column is mounted onto a SPE manifold. A disposable syringe barrel is connected to the immunoaffinity column as a reservoir. The buffer in the affinity column is drained and the sample filtrate is loaded through the reservoir and allowed to flow through by gravity. The column is washed with PBS followed by water. Air is passed through the column to remove residual liquid.

Biotin / Biocytin from the column is eluted with methanol and collected in a reacti-vial (Cat. No. 13223, Thermo Scientific). The eluent is evaporated to dryness using a heating block set at 85±5°C under a gentle stream of nitrogen and the sample is re-constituted in 1mL of water. The biotin / biocytin in the reconstituted sample are analysed simultaneously by HPLC using a PDA set at 200nm. Identification of peaks is based on absolute retention time. Quantification was by multipoint external calibration using peak area responses of the analytes. Spectrum scan (200 nm to 350 nm) can be used for the purity and identity confirmation as required.

B. Chemicals

1. Laboratory Reagent Grade Water
2. Sodium Dihydrogen Phosphate Dihydrate
3. Disodium Hydrogen Phosphate Dihydrate
4. Sodium Hydroxide
5. Methanol, HPLC grade
6. Acetonitrile, HPLC grade
7. Ortho-phosphoric acid, 85%
8. PBS - pH 7.4 (Cat. No. 10010031 Life Technologies / Thermo Scientific or equivalent)
9. Biotin - Purity ≥ 99% (Cat. No. B4501 Sigma Chemical Co., St. Louis, MO, USA, or equivalent)
10. Biocytin - Purity ≥ 98% (Cat. No. B4261 Sigma Chemical Co., St. Louis, MO, USA,or equivalent)

C. Reagents

(a) ***Sodium Hydroxide*****, 2M. -** Weigh 80g of sodium hydroxide in a 1L volumetric flask, then dissolve in water and make up to the mark.

(b) ***Sodium Phosphate Buffer*****, 0.15 M. -** Weigh 9.15g of sodium dihydrogen phosphate dihydrate and 16.31g of disodium hydrogen phosphate dihydrate in a 1L volumetric flask, then dissolve in water and make up to the mark. Adjust the pH to 7 with 2M sodium hydroxide.

(c) ***Phosphoric acid*****, 0.1%. -** In a 1L volumetric flask, add 500 mL water. Add 1.2 mL of ortho-phosphoric acid. Mix and make up to the mark with water.

D. Apparatus

(a) Whatman Glass Microfiber Filters. - CAT No. 1820-125.
(b) R-Biopharm Rhone Easy Extract Biotin Immunoaffinity Column Pack. - P82/P82B or equivalent.
(c) SPE Manifold. - With accessories.
(d) Autoclave. - Set at 121°C.
(e) Centrifuge. - Variable speed.
(f) Analytical Balance. - 4 dp.
(g) Amber glass screw-cap bottle. - 100mL.
(h) Horizontal shaker.
(i) Volumetric flasks. - 1L and 250mL, 100mL and 10mL.
(j) Pipettors. - Calibrated, 10.0mL, 5.0mL, 1.0mL and 200µL, 100µL and 50µL.
(k) Measuring cylinder. - 100mL, 50mL.
(l) Reacti-Vials
(m) Reacti-therm heating block. - With nitrogen blow down (Thermo Scientific).
(n) Ultrasonic bath. - Set at 50ºC.
(o) Centrifuge tubes. - 50mL.
(p) Vortex mixer.
(q) Syringe filter. - PTFE 0.45µm (Cat. No. 13HP045AN; Advatec Syringe Filters, Cole Parmer, Vernon Hills, IL, USA).
(r) Disposable syringes. - 10mL and 1mL.
(s) HPLC vials. - 2mL with 200µL glass inserts.

E. Sample Preparation

Note: For weight and loading volumes for the different ranges of product, see Table 2016.02A. Slurry may be used wherever product heterogeneity is expected.

For the slurry, reconstitute the 25 g powder with warm water (~50°C) to a total weight of 200 g. Mix thoroughly on a horizontal shaker for 15 min and then sonicate at 50°C for 10 min. Cool to room temperature. For liquid samples, mix well to ensure homogeneity of the sample portion and weigh the specified quantity.

(a) Weigh sample/slurry into a 100 mL amber glass screw-cap bottle. See Table 2016.02A.
(b) Add 0.15 M sodium phosphate buffer to a volume of 50 mL.
(c) Swirl gently to mix.
(d) Autoclave the sample preparation at 121°C for 25 min.
(e) Cool the sample to room temperature. Quantitatively transfer the extracts into a 100 mL volumetric flask and make up to the mark with 0.15 M sodium phosphate buffer, mixing well.
(f) Transfer extracts into centrifuge tubes and centrifuge the samples at 4000 rpm for 15 min.
(g) Filter the samples using Whatman glass microfiber filter paper and collect the filtrate.
(h) Set up the SPE manifold. Attach the immunoaffinity column connected to a 10 mL reservoir. Drain off buffer just above the gel.
(i) Load the sample filtrate onto the column as per Table 2016.02A and initialize the flow with the help of a vacuum pump.
(j) Let the solution pass through the column by gravity at a rate of one drop per second.
(k) Wash the column by passing 10 mL PBS through the column, followed by 10 mL water (initialize the flow with the help of vacuum at every step and leave it for gravity).
(l) Remove any residual liquid from the column by introducing gentle vacuum.
(m) Introduce a Reacti-Vial and elute the analyte under gravity with 2 mL methanol. Elute further with an additional 1 mL methanol. Backflush at least three times when eluting and this can be achieved by gentle up and down motion of the syringe plunger to maximize the elution.

(n) Evaporate the eluent to dryness using a heating block set at 85 ± 5°C, under a gentle nitrogen blow down.

(o) Cool down to room temperature by keeping it outside for about 15 min

(p) Redissolve with 1 mL water and then cap the Reacti-Vials and vortex for 30 s. Filter by using a syringe filter in a clean glass insert for the HPLC analysis

Table 2016.02A. Sample Preparation

Product (µg/100g)		Sample Preparation				Conc (µg/100mL)	
Min	Max	Weight (g)	Volume (mL)	Load (mL)	Final	Min	Max
0.1	0.5	20	100	50	1 mL	1	5
0.5	1.0	10	100	20	1 mL	1	2
1.0	5.0	10	100	10	1 mL	1	5
5.0	50.0	2.0 (Slurry 16g)	100	10	1 mL	1	10
50.0	100.0	1.0 (Slurry 8g)	100	10	1 mL	5	10
100.0	400.0	0.5 (Slurry 4g)	100	5	1 mL	2.5	10

F. Standard Preparation

(a) Stock Standard Biotin (100 µg/mL).—Weigh 25 mg biotin reference material in a 250 mL amber volumetric flask. Add 150 mL water and sonicate at room temperature for 90 min with occasional shaking. Make up to volume with water.

(b) Stock Standard Biocytin (100 µg/mL).—Weigh 10 mg biotin reference material in a 100 mL amber volumetric flask. Add 60 mL water and sonicate at room temperature for 90 min with occasional shaking. Make up to volume with water.

(c) Mixed intermediate standard (100 µg/100 mL).—Dilute 1 mL each of stock standards to 100 mL with water.

(1) Standard 1 (1.0 µg/100 mL).—Dilute 100 µL mixed intermediate standard to 10 mL with water.

(2) Standard 2 (2.5 µg/100 mL).—Dilute 250 µL mixed intermediate standard to 10 mL with water.

(3) Standard 3 (5.0 µg/100 mL).—Dilute 500 µL mixed intermediate standard to 10 mL with water.

(4) Standard 4 (7.5 µg/100 mL).—Dilute 750 µL mixed intermediate standard to 10 mL with water.

(5) Standard 5 (10 µg/100 mL).—Dilute 1 mL mixed intermediate standard to 10 mL with water.

(6) Standard 6 (20 µg/100 mL).—Dilute 2 mL mixed intermediate standard to 10 mL with water

Note: The concentrations given above are indicative only; calculate the actual concentrations of biotin and biocytin in each calibration standards using the following formula.

Biotin / Biocytin (µg/100mL) = (W1 x P x 10 x Vis) ÷ (V x 10)

W1= Weight of biotin or biocytin (mg)
P = Percentage purity from the certificate of analysis
Vis = Volume of mixed intermediate standard used for the calibration standard (mL)
V = Volume of stock standard (250mL for biotin and 100mL for biocytin)

G. Chromatographic Conditions

(a) Mobile phase A. - 0.1% Phosphoric acid
(b) Mobile phase B. - 100% Acetonitrile
(c) Mobile phase C. - 80% Acetonitrile
(d) Column: Kinetex Phenyl-Hexyl (Cat. No. 00F-4495-E0; Phenomenex, Torrance, CA, USA), 150 × 4.6 mm × 2.6 µm × 100 Å.
(e) Column temperature. - 25±2ºC
(f) Retention times. - Biocytin 4.5 to 5.5 minutes and biotin 16 to 17 minutes
(g) Run time. - 27 minutes
(h) Detector. – Photodiode array detector operating at 200 nm (spectrum scan 200–350 nm)
(i) Injection volume. - 100µL

For gradient program see Table 2016.02B.

Table 2016.02B. Gradient Program

Time (min)	Flow Rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)	Mobile Phase C (%)
0.0	0.6	90	10	0
18.0	0.6	90	10	0
18.5	0.8	0	0	100
24.0	0.8	0	0	100
24.5	0.6	90	10	0
27.0	0.6	90	10	0

H. Quality Control

(a) Check system suitability by injecting Standard 3 five times. RSD should be ≤2%.
(b) Run the calibration standards at the beginning and end of the sequence (slope drift ≤2%).
(c) The six-point calibration should give a correlation coefficient ≥0.997.
(d) Test one in five samples in duplicate. The duplicates should be within the method repeatability.
(e) Inject one of the calibration standards after every five sample injections.
(f) Analyze a reference sample (e.g., National Institute of Standards and Technology Standard Reference Material 1849a) in duplicate.
(g) Identification of biotin peak is based on absolute retention time. Spectrum scan can be used for peak purity confirmation if required.
(h) Perform three high level recoveries with every batch of immuno affinity columns

I. Calculation and Reporting

The chromatography software will automatically calculate the concentration of the sample in µg/100g, provided the concentration of the standards in (µg/100mL), sample weight (g) and dilution are entered correctly.

Manual calculation can also be performed by using the following equation:

$$\text{Biotin or Biocytin (µg/100g)} = \frac{\text{(Sample Area x Dilution)}}{\text{(Slope x Sample weight in grams)}}$$

Dilution = 10 (100 x 1 ÷ 10) Sample made up to 100mL, 10mL used for IAC clean-up to a final volume of 1mL with water for HPLC analysis. The dilution will be 20 if 5mL is used for IAC clean-up.

Slope = Valid slope calculation based on concentration on X-axis and peak area on Y-axis

Sample weight: Calculate powder equivalent in grams using the following equation for reconstituted powder samples.

Sample weight (powder equivalent) in grams = (W1 x W3) / W2

For ready to feed liquid samples, the sample weight used for extraction is used for the calculation.

Report results to three significant figures, using microgram-per-100-gram units or convert to other units as required.

J. Repeatability

The difference between the results of duplicate portions of the same sample tested at the same sequence should not exceed 6% of the mean result.

K. Reproducibility

The difference between the results of duplicate determinations tested on different days should not exceed 12% of the mean result

L. Uncertainty of Measurement

Uncertainty of the method was calculated as 7%, using appropriate statistical procedure (square root of the sum of squares of the errors expressed as a percentage).

M. Limit of Quantitation

The LOQ was calculated based on the lowest working standard and dilution factor,

Limit of Quantitation (LOQ) was calculated based on the lowest working standard and the dilution factor.

LOQ = (1 x 100) / (20 x 50) = 0.1µg/100g (1ppb)

1 = 1µg/100mL lowest standard
100 = Volume (mL)
20 = 20g sample
50 = Volume (mL) loaded on immunoaffinity column
1= Final volume (mL)

RESULTS AND DISCUSSION

The laboratories were requested to analyse biotin and biocytin in the samples, however none of the samples were found to have biocytin and only biotin results were reported and evaluated in the study. All the biotin results presented in the report are expressed as *µg/100g* of sample as received basis from AOAC International.

Three of the twelve laboratories (Labs 10, 11 and 12) could only report the results of practice samples prior to the due date of the study, 27 Jan 2017. In general, the results from Lab 7 is showing low bias which was investigated further. The possible reasons for the low bias could be the practice followed in sample preparation especially the steps related to loading of immunoaffinity column and the elution technique where triple backflush is specified. The investigation from Lab 7 reported that the analysts did not follow the backflush technique effectively and that may have caused the incomplete elution of biotin from the cartridges. The laboratory also reported that they had deviated from the protocol in reconstitution of powder samples by taking 10g of powder in 80mL. The results from Labs 7, 10, 11 and 12 are included in the MLT report and for statistical evaluation of the results to calculate precision of the analytical method.

Linearity and Range

The retention time of biocytin ranges from 3.3 minutes to 6.1 minutes and that of biotin from 13.8 min to 22.5 minutes. The retention times reported from Lab 9 stands out from other laboratories though the chromatographic conditions were followed as documented. The variation is likely due to difference in the hardware configuration of the liquid chromatographs used for the analysis. The information was not available from Lab 10 at the time of the data processing.

All the participants used more or less the same calibration range 1 to 20 µg/100mL for biocytin and biotin as specified in the method. The range showed excellent correlation coefficient among the participants of not less than 0.999, confirming the linearity of the method over the calibration range (Table 2016.02C).

Table 2016.02C: Retention times and correlation coefficients

Lab #	Retention Time (Min)		Correlation Coefficient (r^2)	
	Biocytin	Biotin	Biocytin	Biotin
1	4.6	16.1	0.9981	0.9995
2	3.8	15.9	0.9996	0.9999
3	4.9	16.2	0.9987	0.9993
4	5.9	18.1	0.9983	0.9997
5	4.4	15.5	0.9997	0.9993
6	4.9	16.5	0.9995	0.9995
7	3.3	14.5	0.9990	0.9990
8	4.1	13.8	0.9991	0.9973
9	6.1	22.5	0.9998	0.9995
11	4.9	15.1	0.9995	0.9988
12	4.1	12.9	0.9990	0.9993

MLT Results and Statistical Evaluation

The biotin results from practice and MLT samples (Day 1 and 2) are given in Tables 2016.02D, E and F respectively. Fourteen different product types were analysed in duplicate by nine laboratories which includes two practice samples and 12 samples for MLT over two separate days. The practice samples were analysed in duplicates and the blind coded duplicates were analysed during MLT on the same day by the participants. Laboratories 10, 11 and 12 submitted results for practice samples only.

The results are closely comparable between the duplicates and among all the participants and all the data have been used for statistical evaluation to calculate repeatability, reproducibility and HorRat. The statistical analysis of the data was carried out using *International Study Workbook Version 2.1 for Blind (Unpaired) Replicates* from AOAC International. The data as presented in the report as *µg/100g* were used as such for statistical calculation with a Factor for Units of Measurement of 1.00E-08. The summary of relative standard deviation of repeatability (RSD_r) and reproducibility (RSD_R) along with HorRat performance ratios is tabulated in Table 2016.02G.

Relative Standard Deviation of Repeatability (RSD_r)

Repeatability expresses the precision under the same operating conditions (intra-assay) over a short interval of time. The AOAC SPIFAN SMPR 2014.005 for the biotin analysis specifies a maximum repeatability standard deviation of not more than 6% for biotin levels greater than 1 µg/100g. The mean RSD_r of the matrices analysed in the study is 4.6% and is well within the limit of the SMPR. However MLT samples 5 and 9 recorded slightly higher RSD_r of 6.79% and 7.03% respectively. The duplicate results from Lab 8 are causing the outlier for MLT sample 5 and removing the data from the lab bring the RSD_r down to 5.68%. Similarly, the removal of duplicate data from Lab 9 will bring the RSD_r of MLT sample 9 down to 5.81%. The removal of these values still leaves statistically significant duplicate sets (8 sets) for a valid RSD_r calculation. However the values are kept in the report as the overall repeatability meets the SMPR criteria and the SLV demonstrated the repeatability precision before the present study.

Relative Standard Deviation of Reproducibility (RSD_R)

Reproducibility expresses the precision among the laboratories. The relative standard deviation of reproducibility (RSD_R) exceeds the SMPR criteria of not more than 12% for all the 14 matrices analysed in the study confirming the precision of the analytical method. The maximum RSD_R noticed was 9.5% in the MLT sample 1, which is partially hydrolysed milk based infant formula matrix. The reproducibility precision confirms the suitability of the SPIFAN method AOAC 2016.02 as a strong candidate as a reference method for global dispute resolution hence the inter-laboratory variation is minimal and exceeds the expectation of the SMPR limits.

Horwitz Ratio (HorRat)

The Horwitz ratio (HorRat) is a normalized performance parameter indicating the acceptability of methods of analysis with respect to among-laboratory precision (reproducibility). It is the ratio of the observed relative standard deviation among laboratories calculated from the actual performance data, RSD_R, to the corresponding predicted relative standard deviation ($PRSD_R$) calculated from the Horwitz equation. The formula for the Horwitz ratio as presented in the *International Study Workbook* of AOAC International is applicable only when the concentration is in the unit/unit form (e.g., µg/µg or g/g, etc.). When the analyte concentration is a mass fraction amount as µg/100g, the appropriate factor for unit of measurement should be selected to generate correct HorRat values. The factor selected in the study for the calculation is 1.00E-08.

Under reproducibility conditions, the acceptable HorRat value range is 0.5 to 2 as per AOAC International. The HorRat values obtained in this study were within 0.3 to 0.5 in all SPIFAN matrices except the NIST SRM 1849a. The performance requirements of SPIFAN methods are generally tighter than routine analytical methods and therefore $PRSD_R$ used in the formula may be slightly higher pushing the HorRat values down. Nevertheless, the lower Horwitz ratios confirm the enhanced performance of the method and homogeneity of the matrices used for the study. The NIST SRM 1849a recorded the lowest HorRat value of 0.21 and undoubtedly the most homogeneous certified standard reference material used in the MLT.

Accuracy (NIST SRM 1849a)

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The established procedure for accuracy assessment is by analysing samples with known concentrations such as certified reference materials. The SPIFAN SMPR 2014.005 recommends NIST SRM 1849a for accuracy evaluation and the SRM is included in the SPIFAN matrices as blind coded duplicates.

The NIST SRM 1849a results were all within the certified limits of biotin confirming the accuracy of the method. It has been noticed that one of the duplicate results of NIST reference sample was slightly on the low bias (185.41µg/100g) for Lab 7 but this was investigated as explained before. The NIST SRM 1849a has comparatively higher biotin content and homogeneous therefore unlike other powder samples it was not reconstituted with water and 1g powder was directly weighed for the analysis.

Matrix recoveries were carried out during SLV at different levels of calibration range and the recoveries were within 95 to 105%.

Selectivity (Comparison with LCMS/MS)

Selectivity / specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Selectivity of the method is confirmed by analysing the SPIFAN matrices by LCMS/MS method by one of the laboratories participated in the MLT study (Lab 5). The method is a modified version of AOAC Official Method 2012.16 for pantothenic acid and uses a UPLC platform with triple quadrupole mass spectrometer. The biotin results by LCMS/MS method were closely comparable to the MLT results for the range of SPIFAN matrices used in the study. Please refer to Table 2016.02H for comparison data.

Table 2016.02D: Biotin (µg/100g) Practice Samples

Lab #	PRACTICE Sample 1		PRACTICE Sample 2	
	A	B	A	B
1	39.28	39.28	15.50	15.50
2	37.76	38.74	14.69	15.68
3	40.21	40.21	15.11	15.11
4	39.40	39.40	15.17	15.17
5	42.90	37.30	13.20	15.90
6	39.33	39.92	14.73	15.79
7	31.73	32.26	12.38	11.50
8	40.49	40.38	15.98	16.48
9	40.83	40.61	14.41	15.53
10	38.58	38.58	14.73	14.73
11	39.80	39.10	15.10	15.30
12	34.29	35.89	16.00	14.87
Mean	**38.72**	**38.47**	**14.75**	**15.13**
%RSD	**7.75**	**6.17**	**7.14**	**8.19**

Table 2016.02E: Biotin (µg/100g) MLT samples (Day 1)

Lab #	MLT Sample 1		MLT Sample 2		MLT Sample 3		MLT Sample 4		MLT Sample 5		MLT Sample 6	
	A	B	A	B	A	B	A	B	A	B	A	B
1	32.96	31.42	68.94	72.48	3.45	3.88	70.84	73.68	24.42	24.78	46.79	43.44
2	39.47	38.48	81.71	83.53	4.33	4.49	78.33	68.43	27.42	28.91	51.02	48.20
3	33.38	33.21	80.37	81.62	4.03	4.22	69.58	72.92	26.25	27.38	46.18	45.42
4	35.41	34.62	81.88	80.43	3.79	3.74	71.65	70.80	28.39	24.24	44.15	44.03
5	37.49	34.70	84.88	79.87	3.84	4.37	77.67	74.07	26.12	25.93	47.15	43.55
6	29.09	35.06	77.44	81.29	4.43	4.05	74.44	74.98	29.29	32.95	41.88	47.62
7	33.45	32.33	76.96	75.88	4.29	3.96	57.51	59.17	24.82	24.66	39.25	41.15
8	27.85	30.34	75.70	79.96	3.66	4.00	72.86	70.76	25.36	30.22	39.91	45.41
9	34.96	37.95	81.26	81.41	4.33	4.43	74.23	74.41	27.94	26.37	42.89	47.41
Mean	**33.78**	**34.23**	**78.79**	**79.61**	**4.02**	**4.13**	**71.90**	**71.02**	**26.67**	**27.27**	**44.36**	**45.14**
%RSD	**10.89**	**8.04**	**5.95**	**4.24**	**8.68**	**6.37**	**8.53**	**6.94**	**6.31**	**10.72**	**8.57**	**5.16**

Table 2016.02F: Biotin (µg/100g) MLT Samples (Day 2)

Lab #	MLT Sample 7		MLT Sample 8		MLT Sample 9		MLT Sample 10		MLT Sample 11		MLT Sample 12	
	A	B	A	B	A	B	A	B	A	B	A	B
1	195.27	200.37	270.20	265.83	171.08	155.73	10.69	9.80	44.26	44.68	48.66	56.39
2	200.63	191.30	271.16	266.46	180.38	159.53	11.11	10.68	46.01	43.08	52.02	53.47
3	196.99	196.48	265.19	272.41	179.63	168.93	10.40	10.24	42.19	43.96	54.56	54.37
4	191.31	205.32	251.11	265.49	171.89	162.31	11.19	9.20	44.02	44.66	54.54	54.92
5	196.80	196.60	260.67	247.84	170.46	161.75	9.76	9.37	40.82	40.13	52.48	49.79
6	193.53	186.75	263.54	248.41	184.86	161.43	9.88	9.89	43.31	42.48	53.84	56.11
7	185.41	190.38	219.30	213.21	153.16	140.19	10.05	9.15	38.43	39.03	50.09	50.14
8	201.99	199.82	268.69	254.35	175.94	163.60	9.36	9.59	40.44	42.14	52.00	44.45
9	202.58	205.62	278.04	274.41	183.98	158.33	11.02	10.72	46.05	46.44	55.47	54.89
Mean	**196.06**	**196.96**	**260.88**	**256.49**	**174.60**	**159.09**	**10.38**	**9.85**	**42.84**	**42.96**	**52.63**	**52.73**
%RSD	**2.81**	**3.33**	**6.64**	**7.37**	**5.55**	**5.02**	**6.35**	**6.02**	**6.05**	**5.42**	**4.24**	**7.39**

Table 2016.02G: Statistical evaluation biotin results from SPIFAN matrices

Samples	Product Description	p	Mean	%RSD_r	%RSD_R	HorRat
Practice 1	Infant Formula Powder Partially Hydrolysed Soy Based	12	38.59	3.18	6.98	0.38
Practice 2	Infant Formula Powder FOS/GOS Based	12	14.94	4.92	7.74	0.36
MLT - 1	Infant Formula Powder Partially Hydrolysed Milk Based	9	34.01	5.53	9.48	0.50
MLT - 2	Infant Elemental Powder	9	79.20	2.64	5.14	0.31
MLT - 3	Infant Formula RTF Milk Based	9	4.07	5.52	7.59	0.29
MLT - 4	Adult Nutritional RTF High Fat	9	71.46	3.88	7.75	0.46
MLT - 5	Infant Formula Powder Milk Based	9	26.97	6.79	8.76	0.45
MLT - 6	Infant Formula Powder Soy Based	9	44.75	5.77	6.96	0.39
MLT - 7	NIST SRM 1849a	9	196.5	2.38	3.04	0.21
MLT - 8	Adult Nutritional Powder Low Fat	9	258.7	2.81	7.03	0.51
MLT - 9	Child Formula Powder	9	166.8	7.03	7.03	0.47
MLT - 10	Toddler Formula Powder Milk-Based	9	10.12	5.74	6.65	0.29
MLT - 11	Infant Formula Powder Milk Based	9	42.90	2.26	5.72	0.31
MLT - 12	Adult Nutritional RTF High Protein	9	52.68	5.14	5.89	0.33

p = total number of laboratories
RSD_r = relative standard deviation of repeatability
RSD_R = relative standard deviation of reproducibility
HorRat = Horwitz Ratio (RSD_R / $PRSD_R$)

Table 2016.02H: Comparison with LCMS/MS technique

Sample #	Product Description	Blind Duplicates	Biotin (µg/100g)		
			AOAC 2016.02	LCMS/MS	Difference
MLT 1	Infant Formula Powder Partially Hydrolysed Milk Based	A	33.78	35.55	-1.77
		B	34.23	39.10	-4.87
MLT 2	Infant Elemental Powder	A	78.79	82.45	-3.66
		B	79.61	81.15	-1.54
MLT 3	Infant Formula RTF Milk Based	A	4.02	3.73	0.29
		B	4.13	3.84	0.29
MLT 4	Adult Nutritional RTF High Fat	A	71.90	66.06	5.84
		B	71.02	77.49	-6.47
MLT 5	Infant Formula Powder Milk Based	A	26.67	26.99	-0.32
		B	27.27	25.25	2.02
MLT 6	Infant Formula Powder Soy Based	A	44.36	43.00	1.36
		B	45.14	42.27	2.87
MLT 7	NIST SRM 1849a	A	196.06	186.58	9.48
		B	196.96	181.50	15.46
MLT 8	Adult Nutritional Powder Low Fat	A	260.88	263.90	-3.02
		B	256.49	246.17	10.32
MLT 9	Child Formula Powder	A	174.60	171.69	2.91
		B	159.09	162.77	-3.68
MLT 10	Toddler Formula Powder Milk-Based	A	10.38	9.87	0.51
		B	9.85	10.30	-0.45
MLT 11	Infant Formula Powder Milk Based	A	42.84	41.66	1.18
		B	42.96	39.77	3.19
MLT 12	Adult Nutritional RTF High Protein	A	52.63	54.05	-1.42
		B	52.73	51.57	1.16

SUMMARY AND CONCLUSION

The SLV and the MLT data provide systematic scientific evidence for a simple, selective, accurate and precise method as a potential candidate reference method for dispute resolution for the determination of total biotin in all forms of infant, adult, and/or pediatric formula. The method fully meets the intended purpose and applicability statement by complying with standard method performance requirement outlined in AOAC SPIFAN SMPR 2014.005.

The method was applied to a cross section of matrix types and established acceptable precision and accuracy. The analytical platform is inexpensive and the method can be used in almost any labs worldwide with basic facilities. The immunoaffinity column (IAC) clean-up extraction is the key step to successful analysis. Although R-Biopharm IAC was used for the MLT, alternative IAC was compared during SLV with comparable results. The performance parameters of the method are compared with AOAC SPIFAN SMPR 2014.005 and the key points are summarised in the Table 2016.02I.

Table 2016.02I: Comparison of method performance with SMPR 2014.005

Parameters	SMPR 2014.005	AOAC 2016.02	Comments
Analytical Range	0.1 - 150 µg/100g	0.1 - 300 µg/100g	Analytical range of the proposed method is wider based on the sample weight and loading volume for IAC extraction.
Limit of Quantitation	≤0.1 µg/100g	≤0.1 µg/100g	The proposed method meets the LOQ of 0.1 µg/100g as required by SMPR 2014.005.
Repeatability (RSD_r)	>1 µg/100g: ≤6%	>1 µg/100g: ≤5%	Repeatability of the proposed method is better than the SMPR 2014.005.
Reproducibility (RSD_R)	>1 µg/100g: ≤12%	>1 µg/100g: ≤10%	Reproducibility of the proposed method is better than the SMPR 2014.005.
Horwitz Ratio (HorRat)	0.5 to 2.0	0.3 to 0.5	Excellent precision performance rating.
Recovery (>1µg/100g)	90 to 110%	95 to 105%	The spiked recoveries by the proposed method are better than the recovery range specified by SMPR 2014.005.
NIST SRM 1849a	199 ± 13	197 ± 10	NIST 1949a results are within the certified limits.

RECOMMENDATIONS

A complete AOAC International MLT study workbook along with the statistical report and draft copy of the study report summarising the outcomes of this collaborative study were submitted with the recommendation that the AOAC First Action Official Method 2016.02 be accepted as a SPIFAN endorsed AOAC Official Final Action Method.

NOTES FROM THE STUDY DIRECTOR

I would like to place on record my learnings and experience with the collaborators during the course of the study. I believe that ideas for improvement, feedback and challenges are valuable information for future enhancement to the method in some way or other.

Filtrate for IAC Clean-up: One of the participants commented that the filtrate for IAC clean-up is not clear, suggested that if the same extraction would be also efficient if performed at pH 4, which would precipitate many of the proteins, yielding a clear filtrate. My laboratory tested few SPIFAN matrices using sodium acetate solution 0.4M, pH 4.0 (In to a 2000mL volumetric flask, weigh 108.8 g sodium acetate trihydrate. Add about 1800mL water. Dissolve. Add 50mL acetic acid, and adjust pH to 4.0 with acetic acid, dilute to volume with water). The filtrate was clear, IAC clean-up was faster and the biotin results were comparable to the current method. However this change was not advised to any of the collaborators and the participant was advised to proceed with current method as published as First Action Official AOAC 2016.02.

IAC loading speed: Two laboratories obtained lower biotin results for practice samples initially were advised to follow IAC loading by gravity as documented in the method and the results came back higher and comparable to other labs.

Elution of biotin from IAC: While assisting a participant investigating low biotin results, it became apparent that the "back flush" technique was inexactly followed, mainly it seems because the exact description of this technique was not given. So, rather than performing a true "back flush", the 3mL methanol eluate was recycled through the IA cartridge three times. It is possible that this may partly explain slightly low recovery for some samples, given that the IA cartridge may well have retained a small portion of this eluent fraction / biotin. The details of the back flush using a syringe plunger / reverse pressure is now detailed in the method.

Syringe Filter: At least two laboratories experienced interference with chromatography. The investigation isolated the issue with syringe filters with extractable chromophores. It was advised to test the filters as part of the reagent blank preparation

Quality Water: Quality of water**,** especially the water used for the final extraction is very important to get clean chromatogram. Type 1 water is recommended and this information will be updated in the method.

UPLC Platform: The availability of stationary phase (solid-core, kinetex phenyl hexyl) in smaller particle size for UPLC platforms is encouraging. This would significantly reduce analysis time and improve throughput, my laboratory is doing further work to validate the change.

Calculation errors: The calculation error is a common problem which was noticed. It was advised to participants to use reference samples to prompt any issues with calculation before reporting the results.

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(9) AOAC SPIFAN SMPR 2014.005 (2014)
(10) AOAC Official First Action Method 2016.02
(11) AOAC International, International Study Workbook Version 2.1 for Blind (Unpaired) Replicates
(12) Appendix F: Guidelines for SMPR, AOAC International (2012)
(13) Appendix D: Guidelines for collaborative study procedures to validate characteristics of a method of analysis, AOAC International (2012).
(14) Biotin (Bio-02) SLV Report 14/05V

ACKNOWLEDGEMENTS

I would like to thank the following individuals for their participation in this study and support during the course of method set up and the MLT study. Special thanks to Simon Bevis and Elaine Marley of R-Biopharm Rhône Ltd for supporting the program by providing immunoaffinity columns. Thanks to Neil Choi, DTS Food Laboratories for analysing the SPIFAN matrices using LCMS/MS method and Shellie Stassie, Covance Laboratories for organising the MLT samples.

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8. *Sneh Bhandari and Tiffany Gallegos-Peretz*, Merieux NutriSciences, United States
9. *Elaine Marley and Dave Leeman*, R-Biopharm Rhône Ltd, Scotland
10. *Hua Chen and Yan Green,* Mead Johnson, China
11. *Maurice Seegers and Nico Dekker*, Mead Johnson, Netherlands
12. *Esther Campos Gimenez,* Nestle Research Centre, Switzerland
13. *David Wollard,* Eurofins, New Zealand
14. *Adrienne McMahon,* Wyeth Nutrition Askeaton, Ireland

Chromatograms

Chromatograms of calibration standard and a SPIFAN MLT sample (00859RF00 / PZGP859) are given below in Figure 1 and 2 respectively.

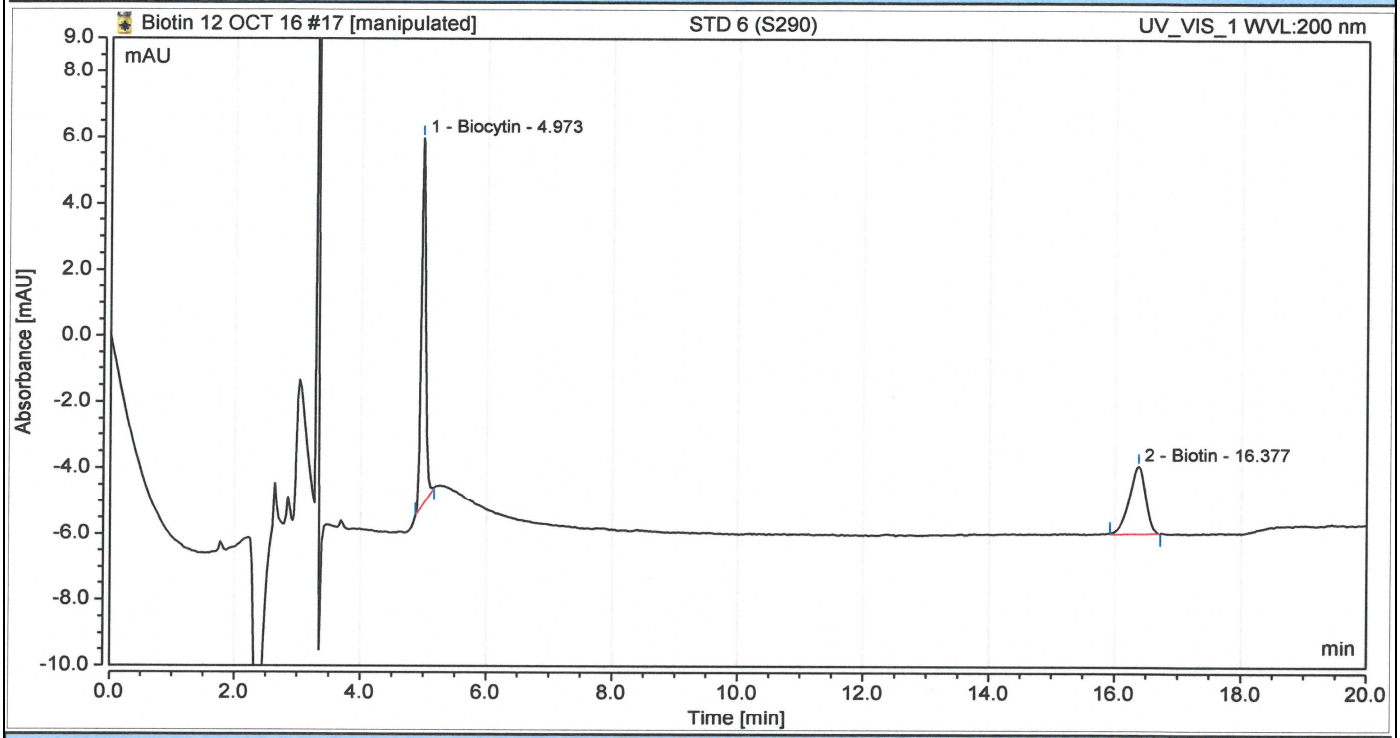
Figure 1: Calibration standard

Chromatogram and Results

Injection Details

Injection Name:	**STD 6 (S290)**	*Run Time (min):*	**27.00**
Vial Number:	**RA6**	*Injection Volume:*	**100.00**
Injection Type:	**Calibration Standard**	*Channel:*	**UV_VIS_1**
Calibration Level:	**06**	*Wavelength:*	**200.0**
Instrument Method:	**Biotin 11 Aug 15 ver 8**	*Bandwidth:*	**4**
Processing Method:	**Biotin**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**13-Oct-16 00:25**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount ug/100mL
1	Biocytin	4.973	0.892	11.102	59.09	84.42	19.8967
2	Biotin	16.377	0.618	2.049	40.91	15.58	19.6813
Total:			**1.510**	**13.151**	**100.00**	**100.00**	

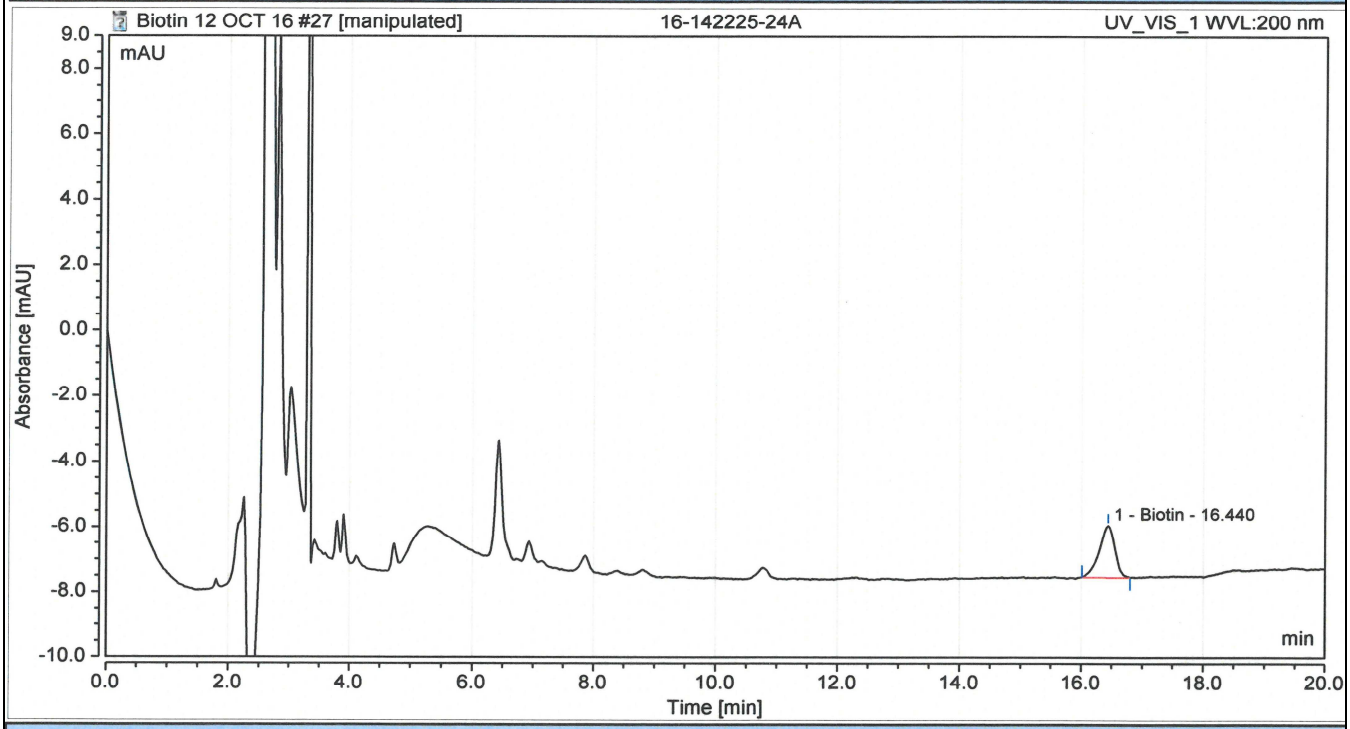
Figure 2: MLT Sample 8B (00859RF00 / PZGP859)

Chromatogram and Results

Injection Details

Injection Name:	**16-142225-24A**	*Run Time (min):*	**27.00**
Vial Number:	**RB7**	*Injection Volume:*	**100.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**200.0**
Instrument Method:	**Biotin 11 Aug 15 ver 8**	*Bandwidth:*	**4**
Processing Method:	**Biotin**	*Dilution Factor:*	**20.0000**
Injection Date/Time:	**13-Oct-16 05:12**	*Sample Weight:*	**1.1154**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount ug/100mL
n.a.	Biocytin	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	Biotin	16.440	0.477	1.599	100.00	100.00	272.4113
Total:			**0.477**	**1.599**	**100.00**	**100.00**	

AOAC INTERNATIONAL

Stakeholder Panel on
Infant Formula and Adult Nutritionals

(2016.02 - Biotin Comments)

Method	Method Title	Comments
OMA# 2016.02	*AOAC 2016.02: Determination of biotin by liquid chromatography coupled with immunoaffinity column clean-up extraction*	○ See reviewer form(s) ○ .12 in the sample with back to back flushing; information not clear in the original, but changed in the current version ○ Method was easily applied ○ In the cartridge (address in final version) ○ Method contains unknown content ○ Clarity around the column usage ○ Stability of the column ○ System suitability ○ Ability to use other IA columns ➢ Any immunoaffinity column could be used (data can be found in the SLV report)

Multi-Laboratory Testing Report

AOAC 2016.05: Vitamin D in Nutritional Products by LC-MS/MS

27 January 2017

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Summary

A multi-laboratory collaborative study of AOAC First Action 2016.05 (1), an LC-MS/MS method for the analysis of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) in infant formula and nutritional products was undertaken. This study consisted of analyses of blind duplicates of 5 different unfortified nutritional products containing endogenous levels <1 µg hg^{-1}, blind duplicates of 13 vitamin D_3 supplemented powders and ready-to-feed nutritional products containing ~1–12 µg hg^{-1} and two reference materials, NIST 1849a and NIST 1869 containing ~11 µg hg^{-1} vitamin D_3 and ~14 µg hg^{-1} vitamin D_2 respectively by 9 different laboratories. For all supplemented nutritional products, precision as repeatability ranged from 1.9–5.8% RSD_r and reproducibility values ranged from 6.4–12.7% RSD_R, within limits set in the SPIFAN Vitamin D SMPR (2). Acceptable repeatability and reproducibility was also demonstrated with HorRat values for the method ranging from 0.2–0.6. On the basis of the results of this study, it is recommended that AOAC First Action 2016.05 be endorsed as Final Action for the determination of vitamin D in infant formula and nutritional products.

Table of Contents

Study Title

Analysis of Vitamin D_2 and Vitamin D_3 by LC MS/MS in Infant Formulas and Nutritionals Products, First Action 2016.05: Multi-Laboratory Testing.

Method Principle

Samples are saponified at high temperature then lipid soluble components are extracted into isooctane. A portion of the isooctane layer is transferred, washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is added to derivatise vitamin D to form a high molecular mass, easily ionisable adduct. The vitamin D-adduct is then back-extracted into a small volume of acetonitrile and analysed by reversed-phase liquid chromatography. Detection is by mass spectrometry using multiple reaction monitoring (MRM). Stable isotope labelled *d6*-vitamin D_2 and *d6*-vitamin D_3 internal standards are used for quantitation to correct for losses in extraction and any variation in derivatisation and ionisation efficiencies.

Study Author

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Objective

The objective of this study is to complete the validation of AOAC 2016.05 by determination of inter-laboratory precision (reproducibility) with a multi-laboratory testing study as fulfilment of the requirements as a SPIFAN Final Action method.

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The assistance of Melissa Phillips (NIST, USA) for the supply of NIST1869 included as part of this study and Shellie Stassi (Covance, USA) for arranging the shipping of the SPIFAN kits to participating labs is greatly appreciated.

Study Design

A total of 12 laboratories agreed to participate as part of this study, however, only 9 laboratories were able to submit data for evaluation prior to submission deadline, with 2 laboratories not reporting any data and 1 laboratory unable to achieve acceptable results for the practice sample. The participating laboratories represent a wide range of food testing laboratories including governmental agencies, infant formula manufacturers, and contract analytical services. Prior to commencement of the study, each collaborator received a detailed study protocol to allow familiarisation with the technique and an opportunity to communicate any difficulties.

The SPIFAN Kit was used for this study and included an extra sample, NIST1869, which was fortified with vitamin D_2. The protocol consisted of testing SPIFAN kit samples (Appendix I) over two separate days as blind-coded duplicates pairs. A practice sample was run by participants and if acceptable data was obtained, approval to proceed to full analysis was given.

Reporting

Upon completion of the full sample analysis, each participating laboratory reported results as well as raw data such as sample identification, weights, volumes, UV absorbance, and peak areas. Participants were also asked to document any deviation from the method and any other pertinent comments based on their experiences in adapting the method into their laboratory. Results received from participants were tabulated and summarised in Table 1 and Figures 1–20. All 9 collaborating laboratories returned acceptable standard calibration parameters based on linear regression correlation coefficients (r^2: ≥ 0.998).

Table 1: Tabulated raw data for replicate analyses of SPIFAN kit

Lab #	NIST1849a CRM Vitamin D_3 (µg hg^{-1}) KGSZ273/LTCT316		KGSZ273/LTCT316		IF powder, p/h soy-based Vitamin D_3 (µg hg^{-1}) SWUO667/MYHK654		Infant elemental powder Vitamin D_3 (µg hg^{-1}) ECHL425/UOPM297		Adult nutritional RTF – high protein Vitamin D_3 (µg hg^{-1}) FPTE312/DOMY545		Soy-based IF Vitamin D_3 (µg hg^{-1}) TJHR217/OACN211	
1	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—
3	10.78	10.19	10.52	10.05	7.89	8.93	9.21	8.96	0.97	0.98	11.54	10.36
4	9.92	10.55	10.61	10.51	8.69	9.11	8.02	7.95	0.97	0.94	10.28	10.45
5	—	—	—	—	—	—	—	—	—	—	—	—
6	10.69	10.94	10.48	10.85	9.13	9.53	8.61	8.29	1.10	1.08	10.62	10.33
7	10.22	10.58	10.13	10.28	9.07	8.83	8.33	8.53	0.98	0.92	9.81	10.02
8	10.04	10.13	10.09	9.87	8.11	8.26	8.04	8.14	0.93	0.92	9.76	9.64
9	11.11	11.73	12.18	11.34	9.22	9.74	9.07	9.00	1.21	1.11	11.43	10.77
10	9.83	9.39	9.44	9.46	8.26	8.38	7.50	7.52	0.85	0.94	9.38	9.45
11	9.58	9.12	9.27	9.61	8.49	8.21	8.03	7.64	1.02	0.89	8.78	9.77
12	9.60	8.92	10.20	10.14	7.34	6.90	7.40	6.87	0.77	0.74	7.82	7.54

Lab #	Adult nutritional RTF – high protein placebo Vitamin D_3 (µg hg^{-1}) YMZB323/BYJK962		Child elemental powder Vitamin D_3 (µg hg^{-1}) NSRB999/JSDT587		Adult nutritional RTF – high fat placebo Vitamin D_3 (µg hg^{-1}) IQVG111/VVOL664		Infant formula RTF – milk-based Vitamin D_3 (µg hg^{-1}) XKIP216/HYJU890		Adult nutritional RTF – high fat Vitamin D_3 (µg hg^{-1}) DYLB360/ZMQM883		Infant formula RTF – milk-based Vitamin D_3 (µg hg^{-1}) TJMN542/XJDD334	
1	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—
3	0.00	0.00	9.10	9.00	0.00	0.00	0.70	0.72	1.30	1.34	0.00	0.00
4	0.02	0.01	8.61	8.86	0.10	0.16	0.68	0.69	1.38	1.24	0.02	0.01
5	—	—	—	—	—	—	—	—	—	—	—	—
6	0.00	0.00	8.70	9.00	0.00	0.00	0.72	0.71	1.39	1.46	0.00	0.00
7	0.01	0.00	8.59	8.71	0.01	0.02	0.67	0.66	1.26	1.28	0.00	0.00
8	0.00	0.00	7.82	7.93	0.00	0.00	0.67	0.65	1.19	1.16	0.00	0.00
9	0.00	0.00	8.44	8.87	0.08	0.05	0.81	0.73	1.30	1.24	0.01	0.01
10	0.00	0.00	7.95	7.92	0.02	0.02	0.59	0.59	1.23	1.25	0.00	0.00
11	0.00	0.00	8.57	7.44	0.00	0.00	0.63	0.68	1.23	1.27	0.00	0.00
12	0.00	0.00	7.66	6.84	0.00	0.00	0.51	0.52	1.18	1.13	0.00	0.00

— Data not submitted prior to MLT submission deadline

Table 1 (continued): Raw data for replicate analyses of SPIFAN kit

Lab #	Child formula powder – milk-based Vitamin D_3 (µg hg^{-1}) RQXQ518/GVPE615		Infant formula powder, p/h milk-based Vitamin D_3 (µg hg^{-1}) KDOX966/ATAN351		Child elemental powder Vitamin D_3 (µg hg^{-1}) EFXN778/BFAO941		Milk-based infant formula Vitamin D_3 (µg hg^{-1}) CULF358/GBZC169		IF powder – FOS/GOS based Vitamin D_3 (µg hg^{-1}) URTF231/WKHN288		Infant elemental powder placebo Vitamin D_3 (µg hg^{-1}) ARJT349/SAEQ748	
1	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—
3	7.93	7.75	9.05	8.86	9.60	9.36	10.35	10.63	7.16	7.44	0.00	0.00
4	8.32	8.09	9.29	9.27	8.94	9.28	10.36	10.25	7.17	7.00	0.50	0.30
5	—	—	—	—	—	—	—	—	—	—	—	—
6	8.21	8.65	9.25	9.35	9.36	9.15	10.58	10.67	7.01	6.83	0.00	0.00
7	7.36	7.37	9.04	8.76	9.06	9.24	10.11	10.23	6.95	6.66	0.01	0.04
8	7.78	7.78	8.32	8.41	8.72	8.44	9.77	10.03	6.70	6.77	0.00	0.00
9	7.26	7.52	9.90	9.63	9.87	9.67	11.86	10.78	8.09	7.93	0.03	0.03
10	7.50	7.50	8.47	8.39	8.40	8.32	9.48	9.61	6.57	6.49	0.01	0.00
11	6.82	6.77	8.85	7.75	7.02	7.45	8.78	9.79	5.75	6.23	0.00	0.00
12	7.43	7.64	8.32	8.29	8.09	7.41	8.10	9.66	7.04	5.80	0.00	0.00

Lab #	Child formula powder – milk-based placebo Vitamin D_3 (µg hg^{-1}) DRWO880/YXBH789		Low-Fat adult nutritional powder Vitamin D_3 (µg hg^{-1}) LYNY751/PZGP859		NIST1869 CRM Vitamin D_2 (µg hg^{-1}) GBAN911/PTLO721		GBAN911/PTLO721	
1	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—
3	0.00	0.00	3.20	3.02	15.07	14.76	13.14	14.05
4	0.06	0.04	3.74	3.55	13.62	14.49	13.75	14.44
5	—	—	—	—	—	—	—	—
6	0.00	0.00	3.28	3.56	*21.09	*18.01	14.24	14.10
7	0.01	0.02	3.35	3.45	12.79	12.80	13.19	12.72
8	0.00	0.00	2.64	2.83	13.20	13.34	12.60	12.64
9	0.03	0.04	4.06	3.81	16.75	17.54	17.61	17.34
10	0.00	0.01	3.15	3.13	14.30	13.30	13.30	13.40
11	0.00	0.00	2.98	2.83	15.44	15.49	13.06	11.93
12	0.00	0.00	2.89	3.25	10.49	11.14	12.21	11.99

— Data not submitted prior to MLT submission deadline; * Results removed as Cochran outlier prior to precision calculation

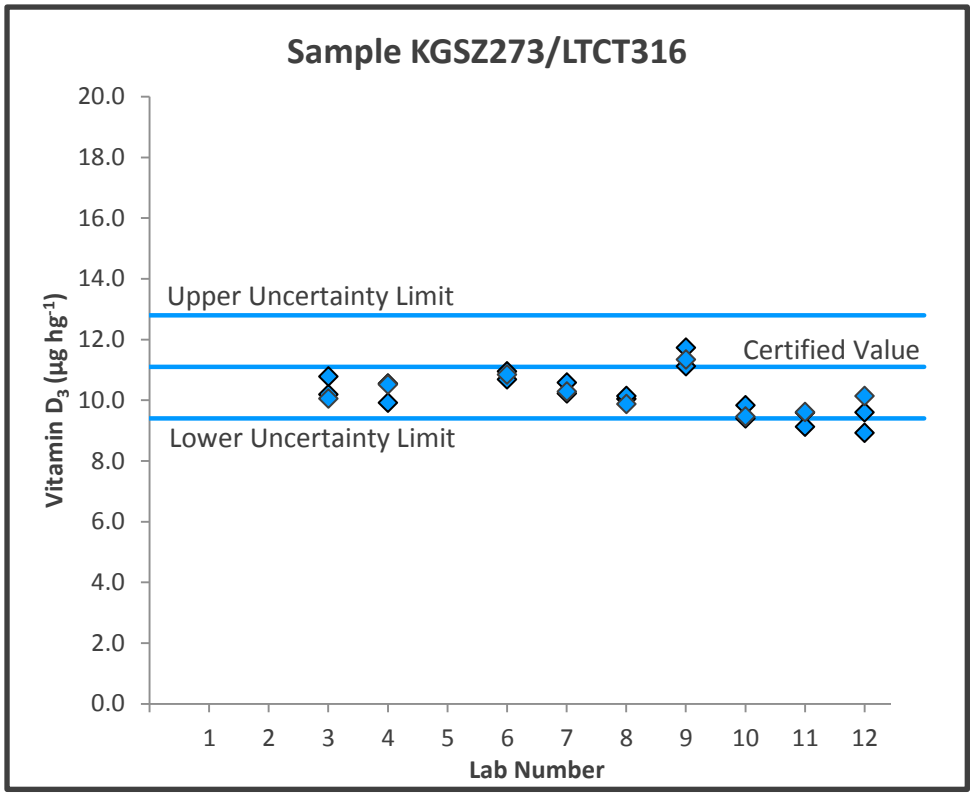

Figure 1: Plotted raw data for NIST 1849a CRM powder (fortified)

Vitamin D_3 (µg hg^{-1})

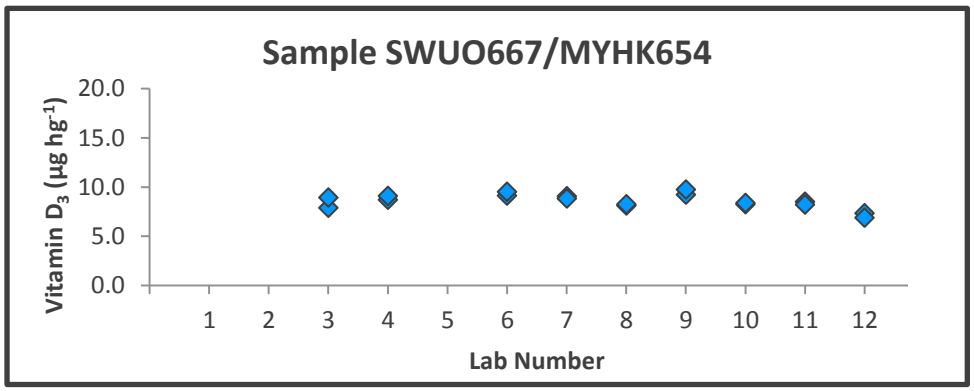

Figure 2: Plotted raw data for partially-hydrolysed soy-based infant formula powder (fortified)

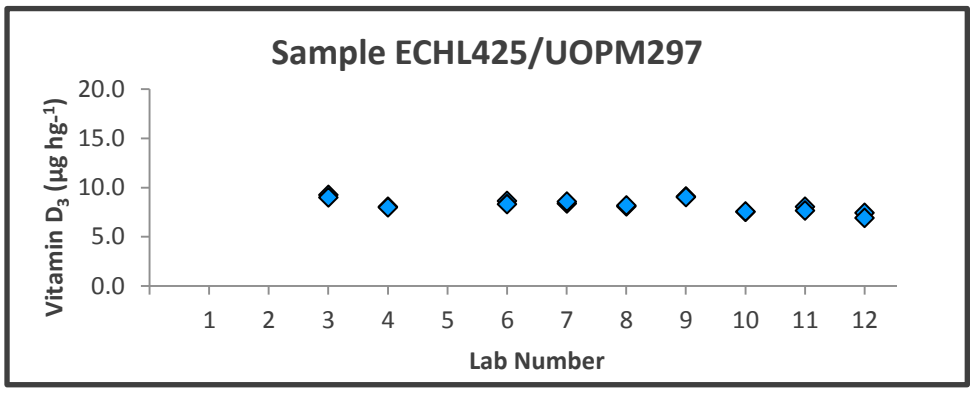

Figure 3: Plotted raw data for infant elemental powder (fortified)

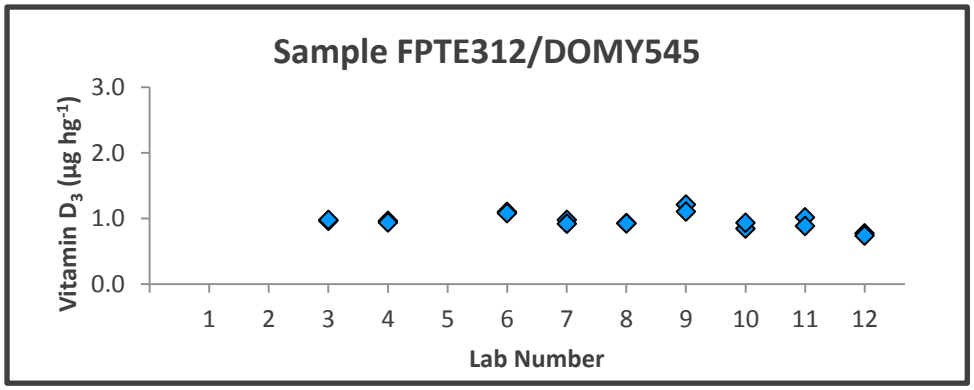

Figure 4: Plotted raw data for high protein ready-to-feed adult nutritional (fortified)

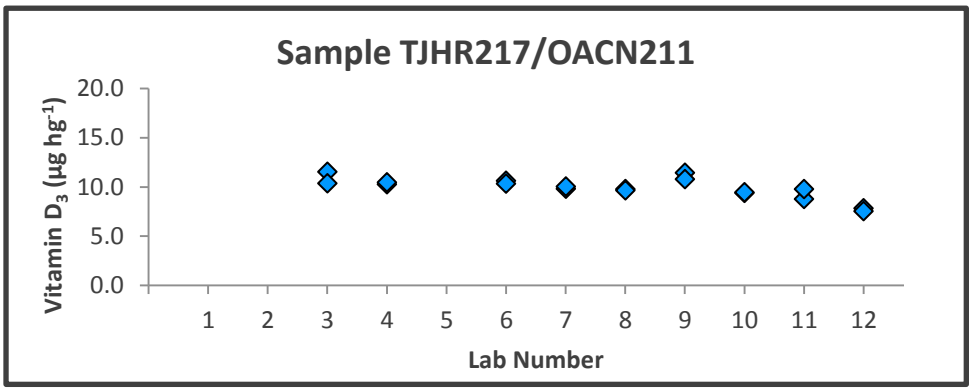

Figure 5: Plotted raw data for soy-based infant formula powder (fortified)

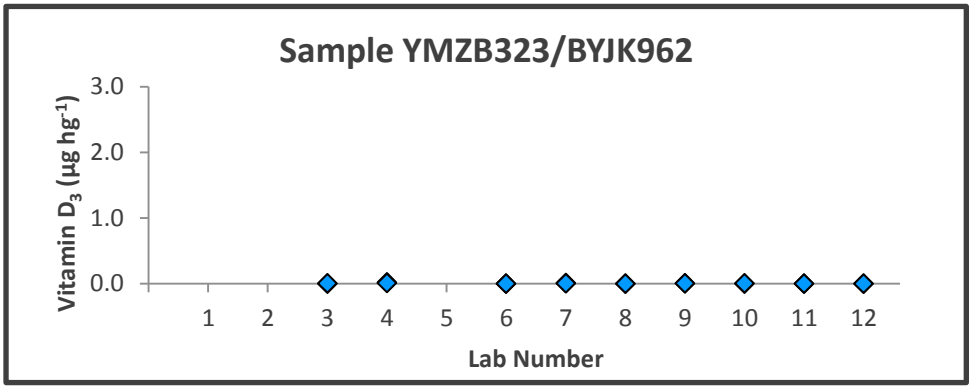

Figure 6: Plotted raw data for high protein ready-to-feed adult nutritional (placebo)

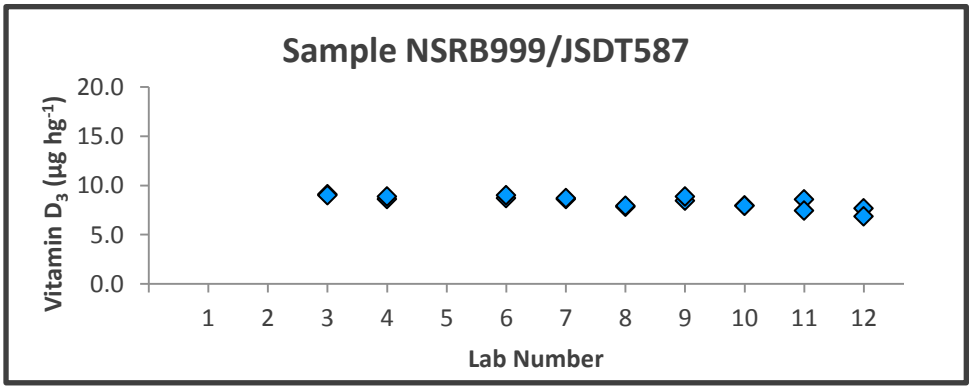

Figure 7: Plotted raw data for child elemental powder (fortified)

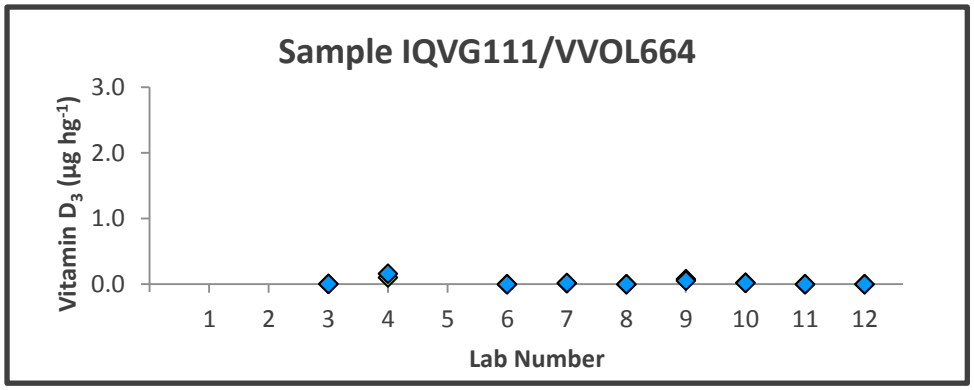

Figure 8: Plotted raw data for high fat adult nutritional powder (placebo)

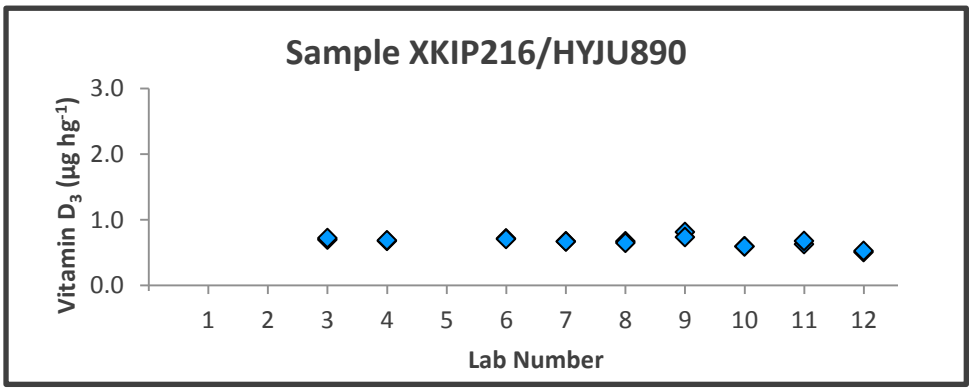

Figure 9: Plotted raw data for milk-based ready-to-feed infant formula (fortified)

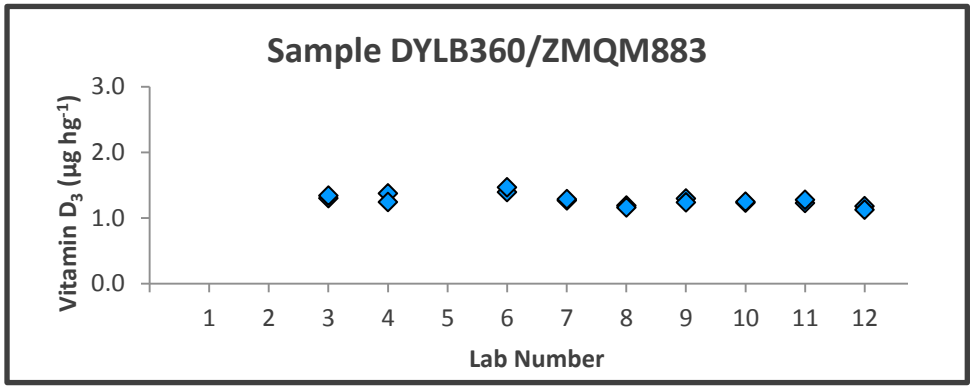

Figure 10: Plotted raw data for high fat ready-to-feed adult nutritional (fortified)

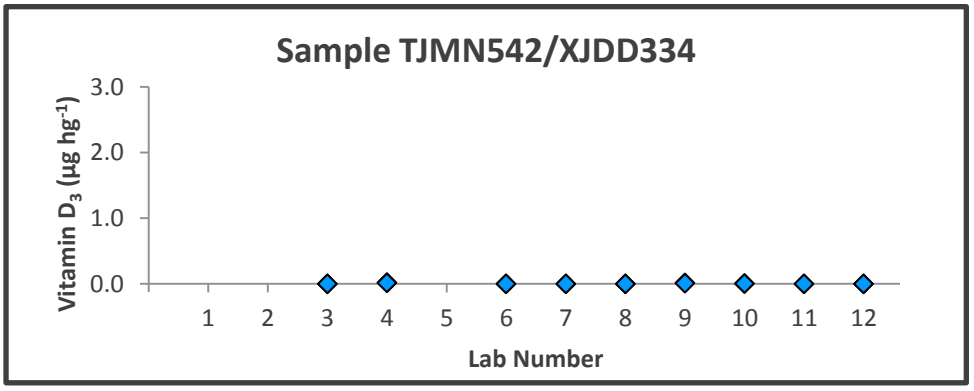

Figure 11: Plotted raw data for milk-based ready-to-feed infant formula (placebo)

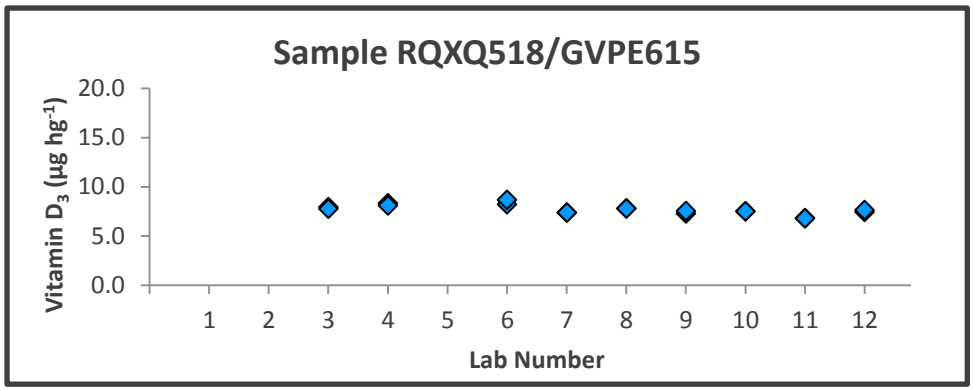

Figure 12: Plotted raw data for milk-based child formula powder (fortified)

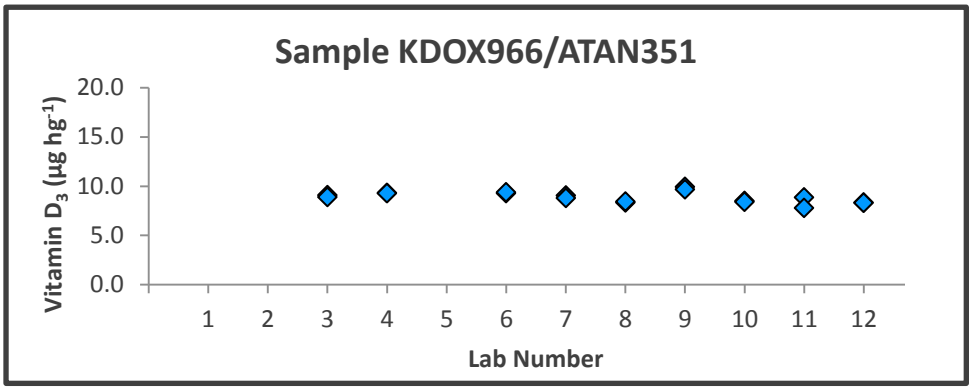

Figure 13: Plotted raw data for partially-hydrolysed milk-based infant formula powder (fortified)

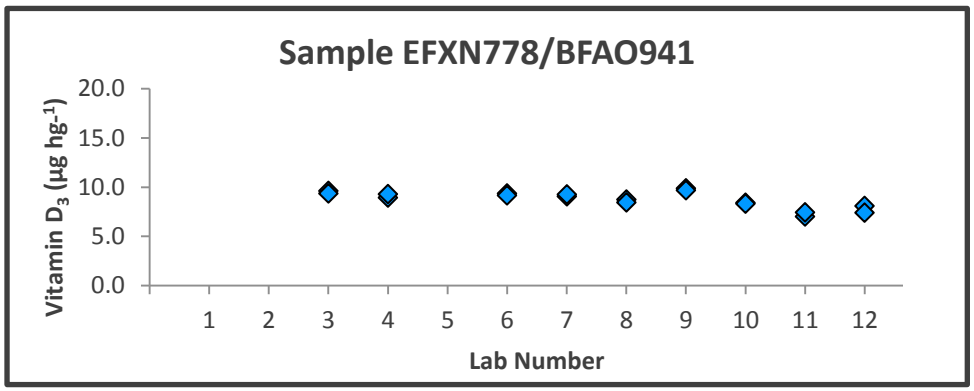

Figure 14: Plotted raw data for child elemental powder (fortified)

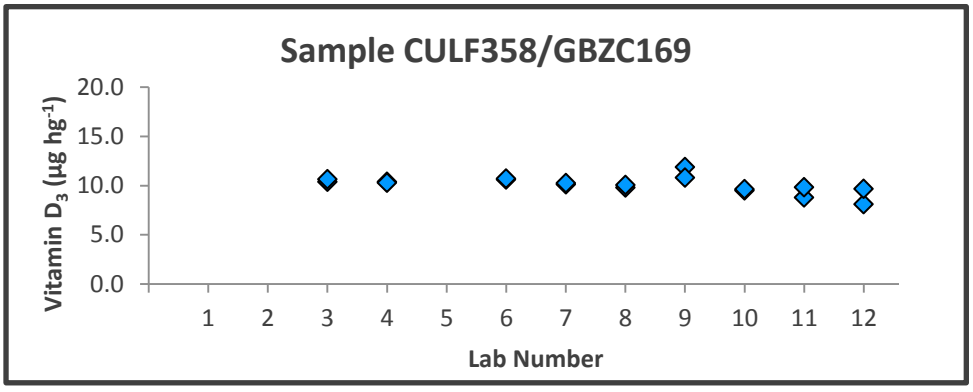

Figure 15: Plotted raw data for milk-based infant formula powder (fortified)

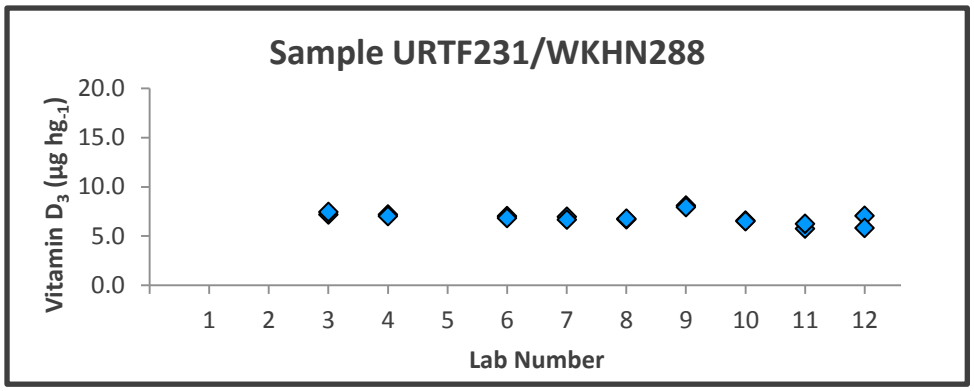

Figure 16: Plotted raw data for FOS/GOS-based infant formula powder (fortified)

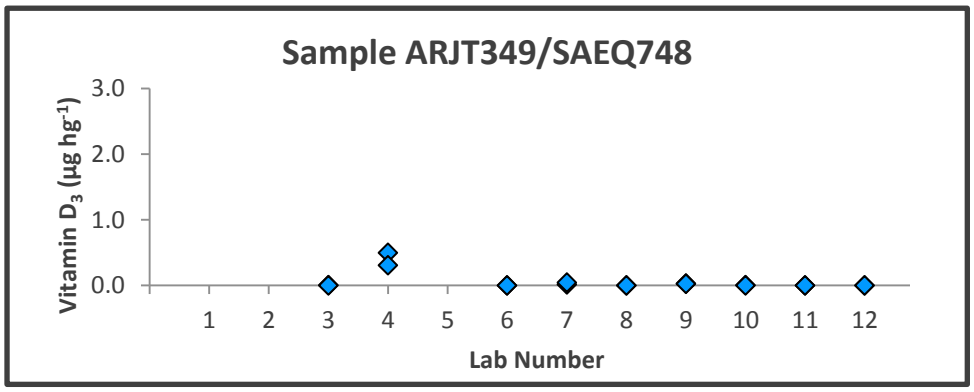

Figure 17: Plotted raw data for infant elemental powder (placebo)

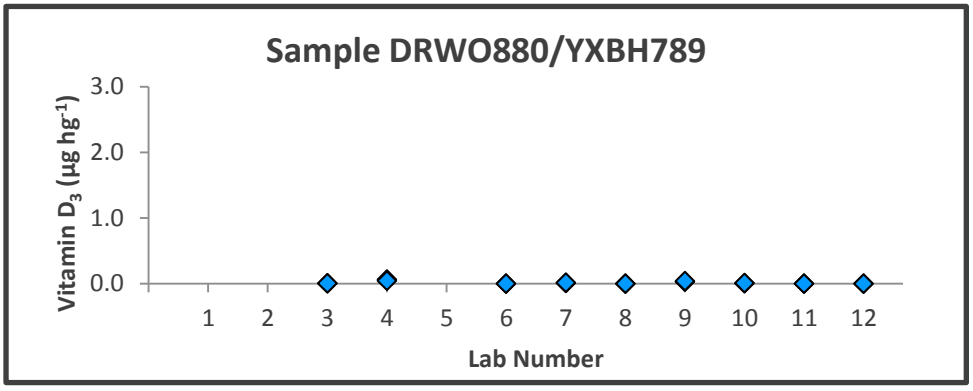

Figure 18: Plotted raw data for milk-based child formula powder (placebo)

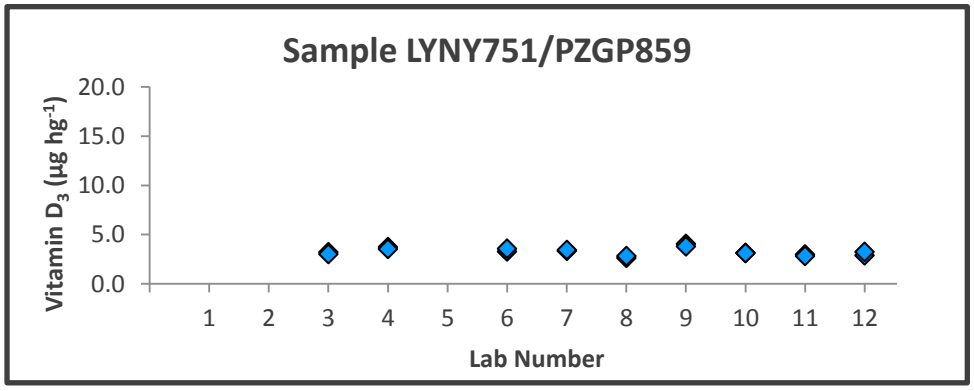

Figure 19: Plotted raw data for low-fat adult nutritional powder (fortified)

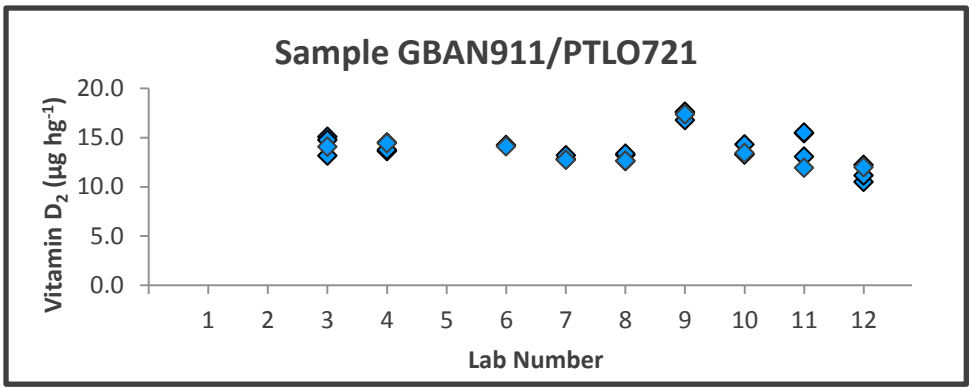

Figure 20: Plotted raw data for NIST 1869 CRM powder (fortified)

Method Precision

The submitted data was statistically analysed using the AOAC protocol for overall mean, intra-laboratory repeatability (S_r), repeatability relative standard deviation (RSD_r), inter-laboratory reproducibility (S_R), reproducibility relative standard deviation (RSD_R), and HorRat (2). Cochran (p = 0.025, 1-tail) and Grubbs (single and double, p = 0.025, 2-tail) tests were utilized to determine outliers (3,4). The statistical analysis of results submitted by participants is given in Table 2 with pertinent values for method illustrated in Figures 21–23. Only a single pair of results for vitamin D_2 (samples GBAN911/PTLO721) from Lab 6 were excluded as Cochran outliers, no other outliers were identified and all other results were used in the generation of precision values.

Precision as repeatability ranged from 1.9–5.8% RSD_r and reproducibility values ranged from 6.4–12.7% RSD_R. For all vitamin D fortified products, the repeatability and reproducibility are within limits set in the Vitamin D SMPR (2). Acceptable reproducibility was also demonstrated with better than expected $HorRat_R$ values for the method ranging from 0.2–0.6 (expected range 0.5–2.0) (4).

Table 2: Precision results for vitamin D fortified samples

Seq.	Precision	Symbol	NIST1849a CRM Vitamin D_3 KGSZ273/LTCT316	IF powder, p/h soy-based Vitamin D_3 SWUO667/MYHK654	Infant elemental powder Vitamin D_3 ECHL425/UOPM297	Adult nutritional RTF – high protein Vitamin D_3 FPTE312/DOMY545
1	Total number of laboratories	p	9	9	9	9
2	Total number of replicates	Sum(n(L))	36	18	18	18
3	Overall mean of all data (grand mean)	$\bar{\bar{X}}$	10.2 µg hg^{-1}	8.6 µg hg^{-1}	8.2 µg hg^{-1}	1.0 µg hg^{-1}
4	Repeatability standard deviation	SD_r	0.3 µg hg^{-1}	0.3 µg hg^{-1}	0.2 µg hg^{-1}	0.0 µg hg^{-1}
5	Reproducibility standard deviation	SD_R	0.7 µg hg^{-1}	0.8 µg hg^{-1}	0.7 µg hg^{-1}	0.1 µg hg^{-1}
6	SMPR Repeatability limit		11.0%	11.0%	11.0%	11.0%
7	Repeatability RSD	RSD_r%	3.2%	3.9%	2.3%	5.1%
8	SMPR Reproducibility limit		15.0%	15.0%	15.0%	15.0%
9	Reproducibility RSD	RSD_R%	7.2%	8.8%	8.2%	12.4%
10	Horwitz Ratio	$HorRat_R$	0.3	0.4	0.3	0.4
Seq.	**Precision**	**Symbol**	**Soy-based IF Vitamin D_3 TJHR217/OACN211**	**Child elemental powder Vitamin D_3 NSRB999/JSDT587**	**Infant formula RTF – milk-based Vitamin D_3 XKIP216/HYJU890**	**Adult nutritional RTF – high fat Vitamin D_3 DYLB360/ZMQM883**
1	Total number of laboratories	p	9	9	9	9
2	Total number of replicates	Sum(n(L))	18	18	18	18
3	Overall mean of all data (grand mean)	$\bar{\bar{X}}$	9.9 µg hg^{-1}	8.3 µg hg^{-1}	0.7 µg hg^{-1}	1.3 µg hg^{-1}
4	Repeatability standard deviation	SD_r	0.4 µg hg^{-1}	0.4 µg hg^{-1}	0.0 µg hg^{-1}	0.0 µg hg^{-1}
5	Reproducibility standard deviation	SD_R	1.1 µg hg^{-1}	0.6 µg hg^{-1}	0.1 µg hg^{-1}	0.1 µg hg^{-1}
6	SMPR Repeatability limit		11.0%	11.0%	11.0%	11.0%
7	Repeatability RSD	RSD_r%	4.2%	4.3%	3.6%	3.4%
8	SMPR Reproducibility limit		15.0%	15.0%	15.0%	15.0%
9	Reproducibility RSD	RSD_R%	10.9%	7.7%	11.4%	6.9%
10	Horwitz Ratio	$HorRat_R$	0.5	0.3	0.3	0.2

Table 2 (continued): Precision results for vitamin D fortified samples

Seq.	Precision	Symbol	Child formula powder – milk-based Vitamin D_3 RQXQ518/GVPE615	Infant formula powder, p/h milk-based Vitamin D_3 KDOX966/ATAN351	Child elemental powder Vitamin D_3 EFXN778/BFAO941	Milk-based infant formula Vitamin D_3 CULF358/GBZC169
1	Total number of laboratories	p	9	9	9	9
2	Total number of replicates	Sum(n(L))	18	18	18	18
3	Overall mean of all data (grand mean)	$\bar{\bar{x}}$	7.6 µg hg^{-1}	8.8 µg hg^{-1}	8.7 µg hg^{-1}	10.1 µg hg^{-1}
4	Repeatability standard deviation	SD_r	0.1 µg hg^{-1}	0.3 µg hg^{-1}	0.2 µg hg^{-1}	0.5 µg hg^{-1}
5	Reproducibility standard deviation	SD_R	0.5 µg hg^{-1}	0.6 µg hg^{-1}	0.9 µg hg^{-1}	0.8 µg hg^{-1}
6	SMPR Repeatability limit		11.0%	11.0%	11.0%	11.0%
7	Repeatability RSD	RSD_r%	1.9%	3.2%	2.7%	5.1%
8	SMPR Reproducibility limit		15.0%	15.0%	15.0%	15.0%
9	Reproducibility RSD	RSD_R%	6.5%	6.4%	9.8%	8.3%
10	Horwitz Ratio	$HorRat_R$	0.3	0.3	0.4	0.4

Seq.	Precision	Symbol	IF powder – FOS/GOS based Vitamin D_3 URTF231/WKHN288	Low-Fat adult nutritional powder Vitamin D_3 LYNY751/PZGP859	NIST1869 CRM Vitamin D_2 GBAN911/PTLO721
1	Total number of laboratories	p	9	9	9
2	Total number of replicates	Sum(n(L))	18	18	34
3	Overall mean of all data (grand mean)	$\bar{\bar{x}}$	6.9 µg hg^{-1}	3.3 µg hg^{-1}	13.8 µg hg^{-1}
4	Repeatability standard deviation	SD_r	0.3 µg hg^{-1}	0.2 µg hg^{-1}	0.8 µg hg^{-1}
5	Reproducibility standard deviation	SD_R	0.6 µg hg^{-1}	0.4 µg hg^{-1}	1.8 µg hg^{-1}
6	SMPR Repeatability limit		11.0%	11.0%	11.0%
7	Repeatability RSD	RSD_r%	4.9%	4.6%	5.8%
8	SMPR Reproducibility limit		15.0%	15.0%	15.0%
9	Reproducibility RSD	RSD_R%	9.1%	12.0%	12.7%
10	Horwitz Ratio	$HorRat_R$	0.4	0.4	0.6

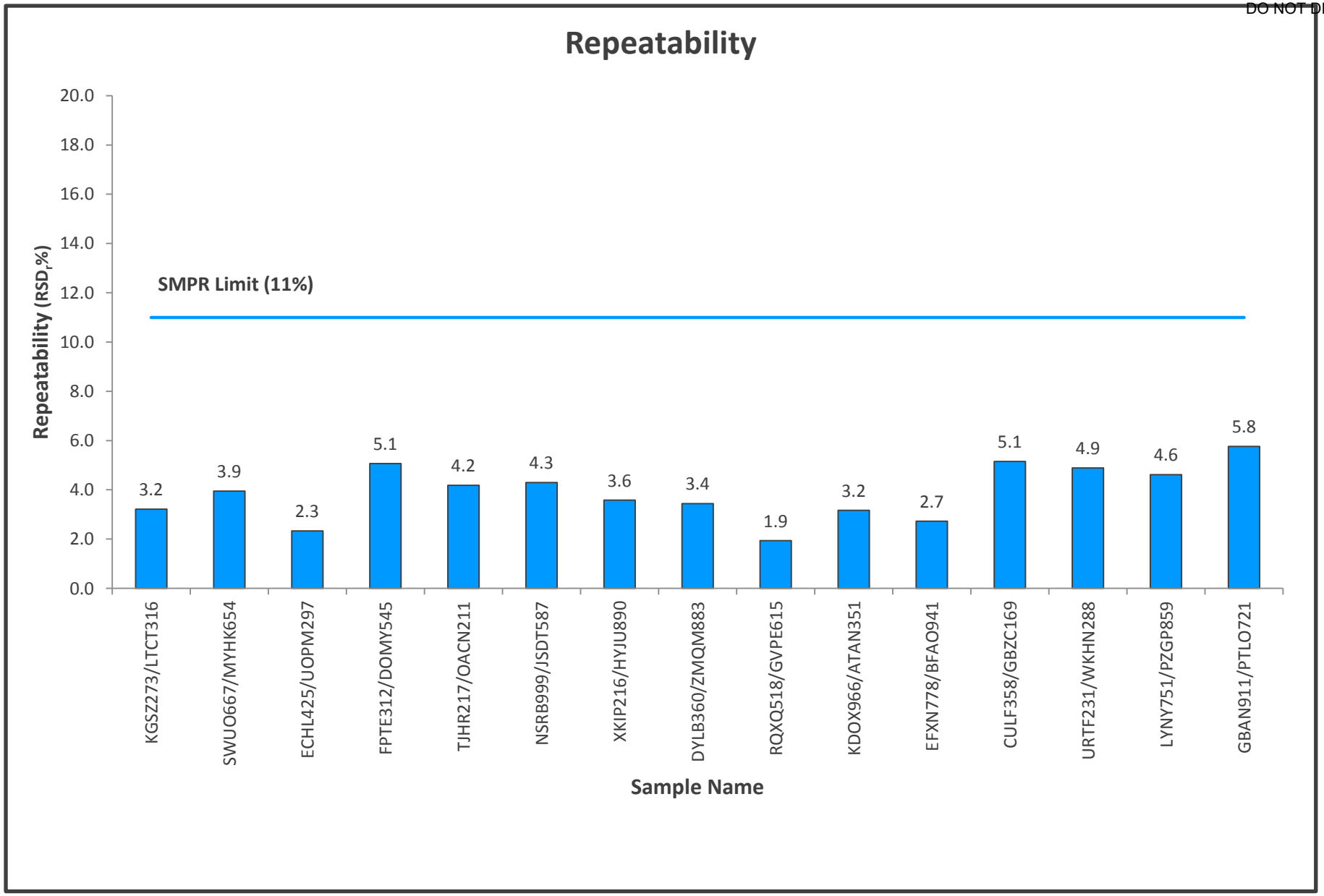

Figure 21: Repeatability results for vitamin D fortified samples

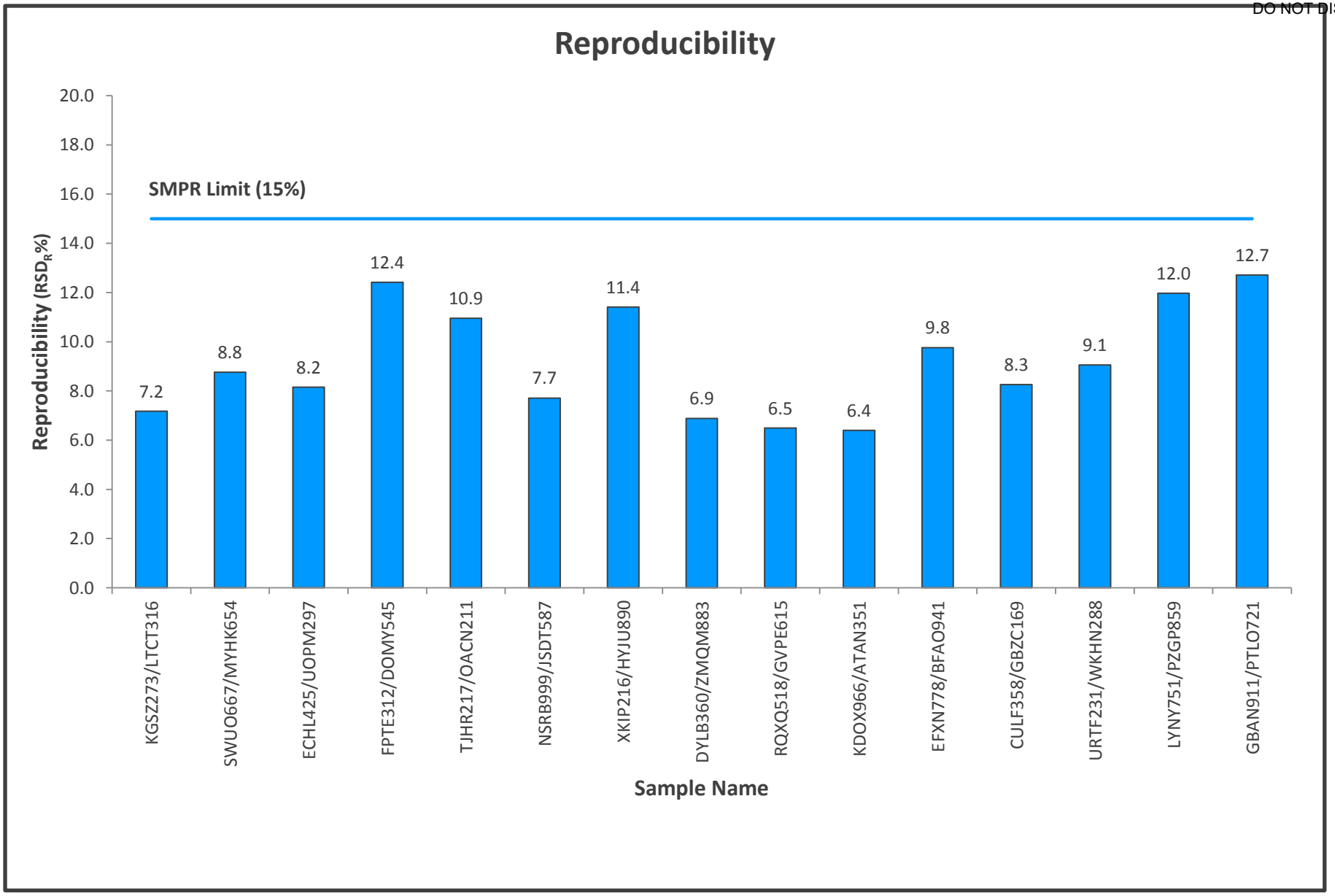

Figure 22: Reproducibility results for vitamin D fortified samples

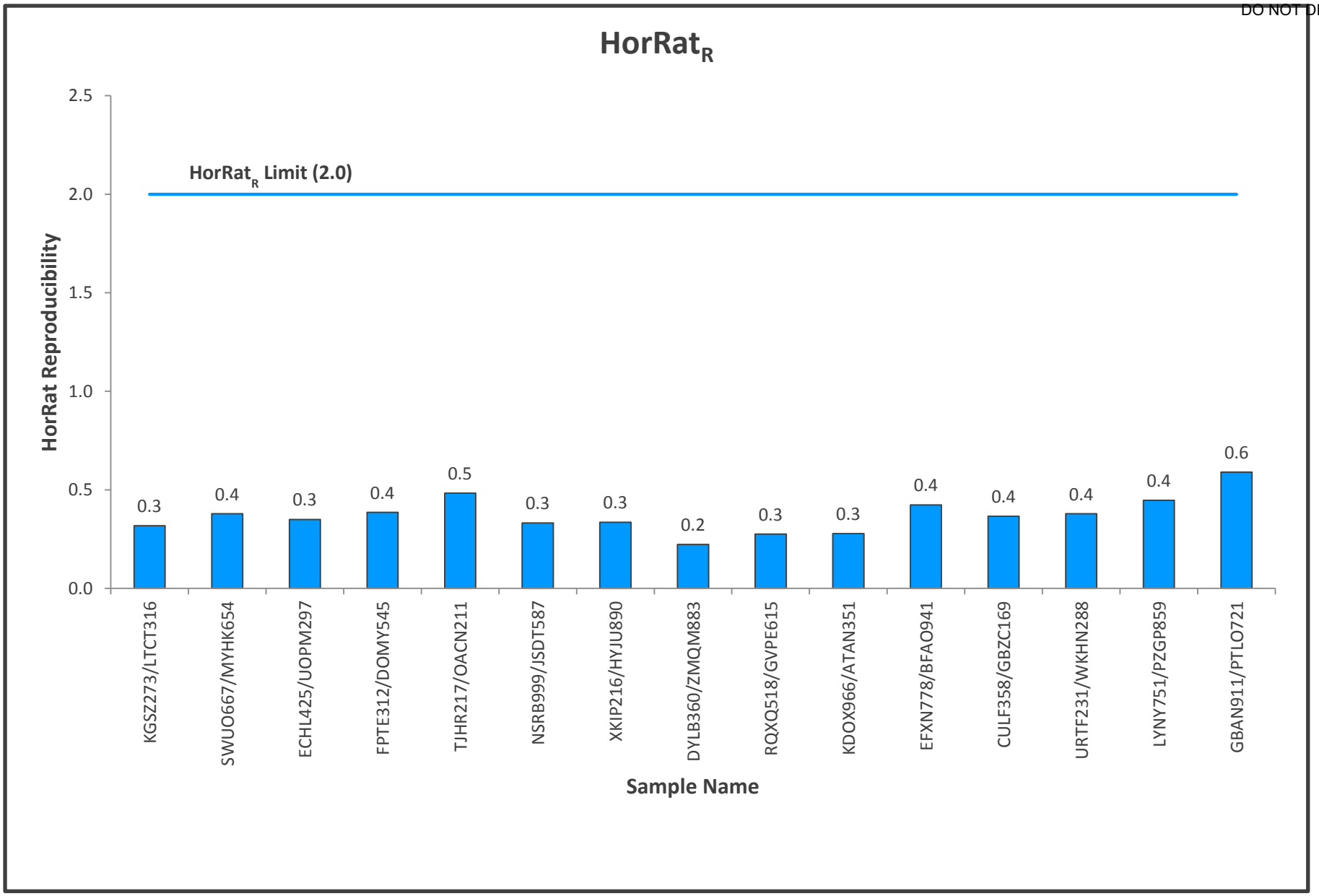

Figure 23: Horwitz ratio for vitamin D fortified samples

Method Accuracy

Method accuracy was assessed in accordance with SPIFAN procedures based on results of the NIST1869a samples tested as part of the final study sample sets (Table 3). The *p*-value of 0.32 indicates no bias was found against the certified value (5). Placebo samples not fortified with vitamin D returned negligible results for vitamin D as expected.

Table 3: Accuracy results for vitamin D in NIST1849a CRM

FORTIFIED: NIST1849a CRM KGSZ273/LTCT316			
Seq.	**Bias**	**Analyte Symbol**	**Vitamin D3 Value**
1	Overall mean of all data (grand mean)	$\bar{\bar{x}}$	10.2 µg hg^{-1}
2	Total number of replicates	Sum(n(L))	36
2	Certified value (ug/hg)	µ	11.1 µg hg^{-1}
3	Uncertainty	U_{CRV}	1.7 µg hg^{-1}
4	Coverage factor	*k*	2.0
5	Students's test-statistic	t_{STAT}	1.0
6	Degrees of freedom	DF	63.0
7	p-value		0.32

Method Applicability

The Vitamin D SMPR applicability statement:
"*Determination of total vitamin D_2 and vitamin D_3 in all forms (powders, ready-to-feed liquids, and liquid concentrates) of infant, adult, and pediatric nutritional formulas. For the purpose of this SMPR, vitamin D2 is defined as ergocalciferol (CAS 8017-28-5) and its previtamin isomer; and vitamin D3 is defined as cholecalciferol (CAS 67-97-0) and its previtamin isomer* (3)

The method has been demonstrated to be suitable for the analysis of vitamin D in a wide range of infant formulas and nutritional products, as illustrated with the range of different matrices used in this collaborative study.

Safety Considerations

There are no major hazards beyond those typically found in chemistry laboratories. Use appropriate safety equipment when handling acids, bases, and solvents. Refer to MSD sheets for detailed safety instructions for each chemical used.

Comments from Collaborators

A summary of each laboratory's performance was sent to participants along with an invitation to make comments on the performance of the method in their laboratory. The following comments were received:

Lab 3: Sample prep centrifugation of the samples at 250 x g did not give a good separation between the two layers. Samples were centrifuged at 2500 x g for 15 minutes.

> Study Author: *Depending on the nature of the tubes used in this step higher centrifuge speed is possible and desirable. Method modification to recommend "*≥ 250 × g"

Lab 4: The use of dry acetone (to dissolve PTAD) was not optimal. I am not convinced by magnesium chloride as drying agent. When the PTAD/acetone solution was added in isooctane, I observed droplets like PTAD/acetone solution was not miscible with isooctane but the derivatization seems to have worked (totally?). The solution PTAD/acetone is fully miscible when I use regular acetone. We use this regular acetone for the derivatization of the Vitamin D in our method and we do not have any problem in the derivatization step. Is it really necessary to dry acetone when we use high quality acetone?

> Study Author: *Agreed, this step was added in response to a bad batch of acetone and seems unnecessary. This step was removed from method for other participants of MLT.*

Lab 6: Sample prep completed steps in a hot block vessel instead of boiling tube, after adding water to hot block vessel and inverting 10 times, total volume was transferred to 50 mL falcon tube for centrifugation… preparation of PTAD solution was done in non-dried acetone,…final steps were completed in amber auto-sampler vials.

> Study Author: *Changes agreed to in advance due to limitations of equipment. Results do not seem to have been compromised*.

Lab 9: Sample 4 (FPTE312) and sample 20 (DOMY545) are slurry and reconstitution was performed. The sample weight input in this table was adjusted by dilution factor of reconstitution. During "step f" in the procedure, isooctane layer of sample 4, 9, 11, 20 can't be separated at 250 g. The centrifuge speed was adjusted to 3000 g to achieve separation.

> Study Author: *As with Lab 3, a higher centrifuge speed is possible and desirable. Method modification to recommend "*≥ 250 × g*".*

Conclusions

A multi-laboratory collaborative study of AOAC First Action 2016.05, an LC-MS/MS method for the analysis of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) in infant formula and nutritional products was undertaken. The method was applied to the SPIAN kit and demonstrated acceptable precision..

Recommendations

On the basis of the results of this study, it is recommended that AOAC First Action 2016.05 be endorsed as Final Action for the determination of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) an LC-MS/MS in fortified milk powders, infant formula, and nutritional products.

References

(1) Gill, B.D.; Abernethy, G.A.; Green, R.J.; Indyk, H.E. (2016) *J AOAC Int*. **99**, 1321–1330.

(2) AOAC SMPR 2011.004 (2012) *J AOAC Int*. **95**, 287–297.

(3) Official Methods of Analysis (2016) 20th Ed., AOAC International, Gaithersburg, MD, Appendix D.

(4) Horwitz, W (1988) Pure Appl. Chem. **60** 855–864.

(5) Gill, B.D.; Indyk, H.E.; Blake, C.J.; Konings, E.J.M.; Jacobs, W.A.; Sullivan, D. (2015) *J AOAC Int*. **98**, 112–115.

Appendix 1: Samples

Table A1: Full sample set used in the MLT study

Day	Sample	Code	Description	Type	Fortification
Practice Sample	1	KGSZ273	NIST1849a	Powder	Cholecalciferol
	2	LTCT316	NIST1849a	Powder	Cholecalciferol
Day1 MLT	1	OACN211	Soy-based IF	Powder	Cholecalciferol
	2	KGSZ273	NIST1849a CRM	Powder	Cholecalciferol
	3	ECHL425	Infant elemental powder	Powder	Cholecalciferol
	4	FPTE312	Adult nutritional RTF high protein	Liquid	Cholecalciferol
	5	UOPM297	Infant elemental powder	Powder	Cholecalciferol
	6	VVOL664	Adult nutritional RTF high fat	Liquid	Placebo
	7	NSRB999	Child elemental	Powder	Cholecalciferol
	8	IQVG111	Adult nutritional RTF high fat	Liquid	Placebo
	9	XKIP216	Infant formula RTF milk-based	Liquid	Cholecalciferol
	10	JSDT587	Child elemental	Powder	Cholecalciferol
	11	HYJU890	Infant formula RTF milk-based	Liquid	Cholecalciferol
	12	ZMQM883	Adult nutritional RTF high fat	Liquid	Cholecalciferol
	13	LTCT316	NIST1849a	Powder	Cholecalciferol
	14	YMZB323	Adult nutritional RTF high protein	Liquid	Placebo
	15	MYHK654	IF powder, p/h soy-based	Powder	Cholecalciferol
	16	BYJK962	Adult nutritional RTF high protein	Liquid	Placebo
	17	TJHR217	Soy-based IF	Powder	Cholecalciferol
	18	DYLB360	Adult nutritional RTF high fat	Liquid	Cholecalciferol
	19	SWUO667	IF powder, p/h soy-based	Powder	Cholecalciferol
	20	DOMY545	Adult nutritional RTF high protein	Liquid	Cholecalciferol
	21	PTLO721	NIST1869	Powder	Ergocalciferol
	22	GBAN911	NIST1869	Powder	Ergocalciferol
Day2 MLT	1	KDOX966	Infant formula, p/h milk-based	Powder	Cholecalciferol
	2	WKHN288	IF powder FOS/GOS based	Powder	Cholecalciferol
	3	ATAN351	Infant formula, p/h milk-based	Powder	Cholecalciferol
	4	LYNY751	Low-fat adult nutritional	Powder	Cholecalciferol
	5	TJMN542	Infant formula RTF milk-based	Liquid	Placebo
	6	URTF231	IF powder FOS/GOS based	Powder	Cholecalciferol
	7	ARJT349	Infant elemental	Powder	Placebo
	8	LTCT316	NIST1849a	Powder	Cholecalciferol
	9	CULF358	Milk-based infant formula	Powder	Cholecalciferol
	10	KGSZ273	NIST1849a	Powder	Cholecalciferol
	11	EFXN778	Child elemental powder	Powder	Cholecalciferol
	12	DRWO880	Child formula powder milk-based	Powder	Placebo
	13	SAEQ748	Infant elemental	Powder	Placebo
	14	BFAO941	Child elemental powder	Powder	Cholecalciferol
	15	YXBH789	Child formula milk-based	Powder	Placebo
	16	GVPE615	Child formula milk-based	Powder	Cholecalciferol
	17	PZGP859	Low-fat adult nutritional	Powder	Cholecalciferol
	18	XJDD334	Infant formula RTF milk-based	Liquid	Placebo
	19	GBZC169	Milk-based infant formula	Powder	Cholecalciferol
	20	RQXQ518	Child formula milk-based	Powder	Cholecalciferol
	21	PTLO721	NIST1869	Powder	Ergocalciferol
	22	GBAN911	NIST1869	Powder	Ergocalciferol

Appendix 2: Analytical Method

Analysis of Vitamin D_2 and Vitamin D_3 by LC MS/MS in Milk Powders, Infant Formulas, and Adult Nutritionals

First Action 2016.05

(Applicable to the determination of vitamin D_2 and vitamin D_3 in supplemented milk powders, infant formula and adult/pediatric nutritional formula).

Caution: Refer to the material safety data sheets for all chemicals prior to use. Use all appropriate personal protective equipment and follow good laboratory practices.

A. Principle

Samples are saponified at high temperature then lipid soluble components are extracted into isooctane. A portion of the isooctane layer is transferred, washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione is added to derivatize vitamin D to form a high molecular mass, easily ionisable adduct. The vitamin D-adduct is then re extracted into a small volume of acetonitrile and analysed by reverse-phase liquid chromatography. Detection is by triple quadrupole mass spectrometer using multiple reaction monitoring (MRM). Stable isotope labelled (SIL) vitamin D_2 and vitamin D_3 internal standards are used for quantitation to correct for losses in extraction and any variation in derivatisation and ionisation efficiencies.

B. Apparatus

(a) UHPLC system, consisting of dual pump system, a sample injector unit, a degasser unit, and a column oven (Shimadzu Nexura or equivalent).
(b) Triple quadrupole mass spectrometer (Sciex 6500 QTrap or equivalent)
(b) Column, Kinetex C_{18} core-shell, 2.6 µm, 2.1 mm × 50 mm, (Phenomenex or equivalent)
(c) Spectrophotometer.—Capable of digital readout to 3 decimal places.
(d) Centrifuge tubes, polypropylene, 15 mL.
(e) Boiling tubes, glass, 60 mL.
(f) Water baths, cold 20 °C, hot 70 °C.
(g) Disposable syringes, capacity 1 mL.
(h) Syringe filters, PTFE, 0.2 µm, 13 mm.
(i) Centrifuges, suitable for 60 mL boiling tubes and 15 mL centrifuge tubes.
(j) Pasteur pipettes, glass, ~140 mm.
(k) Horizontal shaker.
(l) Eppendorf vials, 2 mL.
(m) Filter membranes, 0.2 µm × 47 mm, nylon.
(n) Cryogenic vials, 2 mL.
(o) Schott bottles, 1 L, 100 mL.

C. Reagents

(a) Standards.—Should be ≥99% pure.
  (1) Vitamin D_2, ergocalciferol.
  (2) Vitamin D_3, cholecalciferol.
  (3) *d6*-Vitamin D_2, 26,26,26,27,27,27-*d6* ergocalciferol.
  (4) *d6*-Vitamin D_3, 26,26,26,27,27,27-*d6* cholecalciferol.
(b) PTAD (4-phenyl-1,2,4-triazoline-3,5-dione).
(c) Formic acid (HCO_2H), LC-MS grade.
(d) Potassium hydroxide (KOH).
(e) Pyrogallol ($C_6H_3(OH)_3$).
(f) Ethanol (C_2H_5OH).
(g) Methanol (CH_3OH), LC-MS grade
(h) Isooctane ($(CH_3)_3CCH_2CH(CH_3)_2$).
(i) Acetone (CH_3COCH_3).
(j) Acetonitrile (CH_3CN). LC-MS grade
(k) Water.—Purified with resistivity ≥18 MΩ.

D. Reagent Preparation

(a) PTAD Solution (4-phenyl-1,2,4-triazoline-3,5-dione, 10 mg/mL). Dissolve 50 mg PTAD in 5.0 mL acetone.

(b) Potassium Hydroxide Solution (KOH, 50% w/v). Dissolve 100 g potassium hydroxide in 200 mL water.

(c) Ethanolic Pyrogallol Solution ($C_6H_3(OH)_3$, 1% w/v). Dissolve 5 g pyrogallol in 500 mL of ethanol.

(d) Mobile Phase A (HCO_2H, 0.1% v/v). To 500 mL of water, add 0.5 mL formic acid

(e) Mobile Phase B (CH_3OH, 100% v/v). Methanol, 500 mL

E. Standard Preparation

Vitamin D is sensitive to light; perform all steps under low-level incandescent lighting. If exclusively vitamin D_3 is required for analysis, then standards pertaining to vitamin D_2 need not be used and vice versa. Calibration standards should be bracketed at the beginning and at the end of an analytical run.

(a) Vitamin D_2 Stable Isotope Labelled Stock Standard Solution (~10 µg/mL)—Dispense the contents of a 1 mg vial of *d6*-vitamin D_2 into a 100 mL volumetric flask. Dissolve in 90 mL of ethanol; to promote dissolution, sonicate if necessary. Mix thoroughly, make up to volume with ethanol. Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record concentration. Immediately dispense aliquots (~1.3 mL) into cryogenic vials and freeze at < –15 °C for up to 6 months.

(b) Vitamin D_3 Stable Isotope Labelled Stock Standard Solution (~10 µg/mL)—Dispense the contents of a 1 mg vial of *d6*-vitamin D_3 into a 100 mL volumetric flask. Dissolve in 90 mL of ethanol; to promote dissolution, sonicate if necessary. Mix thoroughly, make up to volume with ethanol. Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record concentration. Immediately dispense aliquots (~1.3 mL) into cryogenic vials and freeze at < –15 °C for up to 6 months.

(c) Stable Isotope Labelled Internal Standard Solution (1 µg/mL)—Depending on the number of samples that need to be analyzed in a run, more or less Stable Isotope Labelled Internal Standard Solution needs to be made up. For every 15 samples (or part thereof) in an analytical run, remove 1 vial of Vitamin D_2 Stable Isotope Labelled Stock Standard Solution and/or 1 vial of Vitamin D_3 Stable Isotope Labelled Stock Standard Solution from the freezer and allow to warm to room temperature. Pipette 1.0 mL of Vitamin D_2 Stable Isotope Labelled Stock Standard Solution and/or 1.0 mL of Vitamin D_3 Stable Isotope Labelled Stock Standard into a 10 mL volumetric flask (use a separate 10 mL volumetric flask for each set of 15 samples). Make each 10 mL volumetric flask to volume with acetonitrile, pool together and mix thoroughly. Make fresh daily.

(d) Vitamin D_2 Non-Labelled Stock Standard Solution (~1 mg/mL)—Weigh accurately, approximately 50 mg of vitamin D_2 into a 50 mL volumetric flask. Dissolve in 40 mL of ethanol; to promote dissolution sonicate if necessary. Mix thoroughly, make up to volume with ethanol. Store in freezer at <–15 °C for up to 1 month.

(e) Vitamin D_3 Non-Labelled Stock Standard Solution (~1 mg/mL)—Weigh accurately, approximately 50 mg of vitamin D_3 into a 50 mL volumetric flask. Dissolve in 40 mL of ethanol; to promote dissolution sonicate if necessary. Mix thoroughly, make up to volume with ethanol. Store in freezer at <–15 °C for up to 1 month.

(f) Vitamin D_2 Non-Labelled Purity Standard Solution (~10 µg/mL) —Pipette 1.0 mL of Vitamin D_2 Non-Labelled Stock Standard Solution into a 100 ml volumetric flask. Make to volume with ethanol. Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Record absorbance and calculate concentration. Make fresh daily.

(g) Vitamin D_3 Non-Labelled Purity Standard Solution (~10 µg/mL)—Pipette 1.0 mL of Vitamin D_3 Non-Labelled Stock Standard Solution into a 100 mL volumetric flask. Make to volume with ethanol. Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Record absorbance and calculate concentration. Make fresh daily.

(h) Non-labelled Working Standard Solution (~1 µg/ml)—Pipette 1.0 mL of Vitamin D_2 non-labelled purity standard solution and/or 1.0 mL of Vitamin D_3 Non-Labelled Purity Standard Solution into a 10 mL volumetric flask. Make to volume with acetonitrile and mix thoroughly. Make fresh daily.

(i) Calibration Standards— See Table 2016.05A for nominal vitamin D concentrations of the Calibration Standard Solutions. Make fresh daily.

(1) Calibration Standard 1—Pipette 10 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

(2) Calibration Standard 2—Pipette 50 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

(3) Calibration Standard 3—Pipette 250 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

(4) Calibration Standard 4—Pipette 500 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

(5) Calibration Standard 5—Pipette 1250 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

Table 2016.05A. Nominal concentration of Calibration Standards

Calibration Standard	Vitamin D Concentration (ng/mL)	d6-Vitamin D Concentration (µg/mL)
1	0.4	10
2	2.0	10
3	10	10
4	20	10
5	50	10

F. Sample Preparation

(a) Powder sample preparation

(1) Accurately weigh 1.8–2.2 g of powder sample into a boiling tube. Record weight.

(b) Powder sample preparation

(1) Accurately weigh 19.0–21.0 g of powder to a disposable slurry container. Record weight.

(2) Accurately weigh ~80 mL water to container. Record weight.

(3) Shake thoroughly until mixed. Place in dark at room temperature for 15 min and shake to mix every 5 min.

(4) Accurately weigh 9.5–10.5 g of slurry or reconstituted powder sample into a boiling tube. Record weight.

(c) Liquid sample preparation

(1) Accurately weigh 10.0 mL of liquid milk into a boiling tube. Record weight.

G. Extraction and Derivatization

(a) To powder, slurry, or liquid sample in a boiling tube, add 10 mL Ethanolic Pyrogallol Solution, add 0.50 mL of Stable Isotope Labelled Internal Standard Solution, cap and vortex mix.

(b) Add 2 mL of Potassium Hydroxide Solution to boiling tube; cap and vortex mix.

(c) Place boiling tube in water bath at 70°C for 1 h, vortex mix every 15 min.

(d) Place boiling tube in water bath at 70°C until cool.

(e) Add 10 mL isooctane to boiling tube; cap boiling tube tightly and place on horizontal shaker for 10 min.

(f) Add 20 mL of water to boiling tube and invert tube 10 times; place in centrifuge at ≥ 250 × *g* for 15 min.

(g) Transfer a 5 mL aliquot of the upper isooctane layer into a 15 mL centrifuge tube using a Pasteur pipette, taking care NOT to transfer any of the lower layer (discard boiling tube with lower layer).

(h) Add 5 mL of water to centrifuge tube; cap and vortex mix; place in centrifuge at 2000 × *g* for 5 min.

(i) Transfer 4–5 ml of upper isooctane layer to a new 15 mL disposable centrifuge tube using a disposable pipette, taking care NOT to transfer any of the lower layer (discard centrifuge tube with lower layer).

(j) Add 75 µL of PTAD solution to centrifuge tube; cap and immediately vortex mix.

(k) Allow to stand in dark for 5 min to allow for derivatization reaction to complete.

(l) Add 1 mL acetonitrile to centrifuge tube, cap and vortex mix; place in centrifuge at 2000 × *g* for 5 min.

(m) Using a variable volume pipette, transfer 500 µL of the lower layer into an Eppendorf vial taking care not to transfer any of the upper layer.

(n) Add 167 µL of water to the Eppendorf vial; cap and vortex mix.

(o) Using a syringe filter, transfer an aliquot from Eppendorf vial to an amber HPLC vial; cap ready for analysis.

H. Chromatography

(a) Set-up the UHPLC system with the following configuration shown in Table 2016.05B.

Table 2016.05B. Chromatographic instrument settings

Instrument Parameter	Value
mobile phase	A = formic acid, 0.1% B = methanol, 100%
column	Kinetex C_{18} core-shell, 2.6 μm, 2.1 × 50 mm
oven temperature	40 °C
chiller temperature	15 °C
injection volume:	3 μL
initial flow rate:	0.6 mL/min (see gradient details below)

(b) Form high pressure gradients by mixing of the two mobile phases, A and B, using the procedure given in Table 2016.05C.

Table 2016.05C. Gradient procedure for chromatographic separation

Time (min)	Flow rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)
0	0.6	25	75
3.3	0.6	0	100
3.7	1.0	0	100
4.8	1.0	0	100
4.9	0.6	25	75
5.5	0.6	25	75

I. Mass Spectrometry

(a) Set-up the mass spectrometer with the instrument setting shown in Table 2016.05D.

Table 2016.05D. Mass spectrometer settings

Instrument Parameter	Value
ionization mode	ESI+
curtain gas	30
nebulizer gas GS1	40
heater gas GS2	40
collision gas	N_2
source temperature	300 °C
ion spray voltage	5500 V

(b) Compound specific parameters to be used are shown in Table 2016.05E and Table 2016.05F.

Table 2016.05E. Compound parameters (vitamin D_2 instrument method only)

Vitamin D_2 ion[a]	Precursor	Product Ion	DP[b] (V)	EP[c] (V)	CE[d] (V)	CXP[e] (V)	Dwell Time
analyte quantifier	572.2	298.0	81	10	23	22	120
analyte qualifier	572.2	280.0			39	16	80
internal standard quantifier	578.2	298.0			23	22	120
internal standard qualifier	578.2	280.0			39	16	80

[a] Analyte = vitamin D_2-PTAD adduct, Internal standard ion = *d6*-vitamin D2-PTAD adduct
[b] DP = declustering potential
[c] EP = entrance potential
[d] CE = collision energy
[e] CXP = collision cell exit potential

Table 2016.05F. Compound parameters (vitamin D_3 instrument method only)

Vitamin D_3 ion[a]	Precursor	Product Ion	DP[b] (V)	EP[c] (V)	CE[d] (V)	CXP[e] (V)	Dwell Time
analyte quantifier	560.2	298.0	151	10	21	18	120
analyte qualifier	560.2	280.0			37	18	80

internal standard quantifier	566.2	298.0	21	18	120
internal standard qualifier	566.2	280.0	37	18	80

[a] Analyte = vitamin D_3-PTAD adduct, Internal standard ion = *d6*-vitamin D3-PTAD adduct
[b] DP = declustering potential
[c] EP = entrance potential
[d] CE = collision energy
[e] CXP = collision cell exit potential

J. Calculations

(a) Concentration of d6-vitamin D_2 in Vitamin D_2 Stable Isotope Labelled Stock Standard Solution:

$$SILD_2SS_{D2conc} = \frac{SILD_2SS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where: $SILD_2SS_{D2conc}$ = concentration of SIL *d6*-vitamin D_2 in stock standard (units: µg/mL)
$SILD_2SS_{abs(\lambda max)}$ = UV absorbance of stock standard at 265 nm (units: none)
$E^{1\%}_{1cm}$= extinction coefficient for vitamin D_2 in ethanol [461] (units: dL/g.cm)
10000 = concentration conversion factor (units: g/dL to µg/mL

(b) Concentration of *d6*-vitamin D_3 in Vitamin D_3 Stable Isotope Labelled Stock Standard Solution:

$$SILD_3SS_{D2conc} = \frac{SILD_3SS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where: $SILD_3SS_{D3conc}$ = concentration of SIL *d6*-vitamin D_3 in stock standard (units: µg/mL)
$SILD_3SS_{abs(\lambda max)}$ = UV absorbance of stock standard at 265 nm (units: none)
$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_3 in ethanol [485] (units: dL/g.cm)
10000 = concentration conversion factor (units: g/dL to µg/mL

(c) Concentration of *d6*-vitamin D_2 in Internal Standard:

$$SILIS_{D2conc} = SILD_2SS_{D2conc} \times \frac{1.0}{10} \times 1000$$

where: $SILIS_{D2conc}$ = concentration of SIL *d6*-vitamin D_2 in internal standard (units: ng/mL)
$SILD_2SS_{D3conc}$ = concentration of SIL *d6*-vitamin D_2 in stock standard (units: µg/mL)
1000 = concentration conversion factor (units: µg/mL to ng/mL)

(d) Concentration of *d6*-vitamin D_3 in Internal Standard:

$$SILIS_{D3conc} = SILD_3SS_{D3conc} \times \frac{1.0}{10} \times 1000$$

where: $SILIS_{D3conc}$ = concentration of SIL *d6*-vitamin D_3 in internal standard (units: ng/mL)
$SILD_3SS_{D3conc}$ = concentration of SIL *d6*-vitamin D_3 in stock standard (units: µg/mL)
1000 = concentration conversion factor (units: µg/mL to ng/mL)

(e) Concentration of non-labelled vitamin D_2 in Vitamin D_2 Non-Labelled Purity Standard Solution:

$$NLD_2PS_{D2conc} = \frac{NLD_2PS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where: NLD_2PS_{D2conc} = concentration of vitamin D_2 in purity standard (units: µg/mL)
$NLD_2PS_{abs(\lambda max)}$ = UV absorbance of purity standard at 265 nm (units: none)
$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_2 in ethanol [461] (units: dL/g.cm)
10000 = concentration conversion factor (units: g/dL to µg/mL)

(f) Concentration of non-labelled vitamin D_3 in Vitamin D_3 Non-Labelled Purity Standard Solution:

$$NLD_3PS_{D3conc} = \frac{NLD_3PS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where: NLD_3PS_{D3conc} = concentration of vitamin D_3 in purity standard (units: µg/mL)
$NLD_3PS_{abs(\lambda max)}$ = UV absorbance of purity standard at 265 nm (units: none)
$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_3 in ethanol [485] (units: dL/g.cm)
10000 = concentration conversion factor (units: g/dL to µg/mL)

(g) Concentration of non-labelled vitamin D_2 in Non-Labelled Working Standard Solution:

$$NLWS_{D2conc} = NLD_2PS_{D2conc} \times \frac{1.0}{10} \times 1000$$

where: $NLWS_{D2conc}$ = concentration of vitamin D_2 in working standard (units: ng/mL)
NLD_2PS_{D2conc} = concentration of vitamin D_2 in purity standard (units: µg/mL)
1000 = concentration conversion factor (units: µg/mL to ng/mL)

(h) Concentration of non-labelled vitamin D_3 in Non-Labelled Working Standard Solution:

$$NLWS_{D3conc} = NLD_3PS_{D3conc} \times \frac{1.0}{10} \times 1000$$

where: $NLWS_{D3conc}$ = concentration of vitamin D_3 in working standard (units: ng/mL)
NLD_3PS_{D3conc} = concentration of vitamin D_3 in purity standard (units: µg/mL)
1000 = concentration conversion factor (units: µg/mL to ng/mL)

(i) Concentration of vitamin D_2 and vitamin D_3 in Calibration Standards:

$$CS1_{D2conc} = NLWS_{D2conc} \times \frac{0.01}{25} \qquad CS1_{D3conc} = NLWS_{D3conc} \times \frac{0.01}{25}$$

$$CS2_{D2conc} = NLWS_{D2conc} \times \frac{0.05}{25} \qquad CS2_{D3conc} = NLWS_{D3conc} \times \frac{0.05}{25}$$

$$CS3_{D2conc} = NLWS_{D2conc} \times \frac{0.25}{25} \qquad CS3_{D3conc} = NLWS_{D3conc} \times \frac{0.25}{25}$$

$$CS4_{D2conc} = NLWS_{D2conc} \times \frac{0.5}{25} \qquad CS4_{D3conc} = NLWS_{D3conc} \times \frac{0.5}{25}$$

$$CS5_{D2conc} = NLWS_{D2conc} \times \frac{1.25}{25} \qquad CS5_{D3conc} = NLWS_{D3conc} \times \frac{1.25}{25}$$

where: $CS1\text{-}5_{D2conc}$ = concentration of vitamin D_2 in Calibration Standards (units: ng/mL)
$CS1\text{-}5_{D3conc}$ = concentration of vitamin D_3 in Calibration Standards (units: ng/mL)
$NLWS_{D2conc}$ = concentration of vitamin D_2 in Working Standard (units: ng/mL)
$NLWS_{D3conc}$ = concentration of vitamin D_3 in Working Standard (units: ng/mL)

(j) Concentration of stable isotope labelled vitamin D3 in Calibration Standards:

$$CS1\text{-}5_{D2conc} = SILIS_{D2conc} \times \frac{0.25}{25} \qquad CS1\text{-}5_{D3conc} = SILIS_{D3conc} \times \frac{0.25}{25}$$

where: $CS1\text{-}5_{D2conc}$ = concentration of SIL *d6*-vitamin D_2 in calibration standards (units: ng/mL)
$CS1\text{-}5_{D3conc}$ = concentration of SIL *d6*-vitamin D_3 in calibration standards (units: ng/mL)
$SILIS_{D2conc}$ = concentration of SIL *d6*-vitamin D_2 in internal standard (units: ng/mL)
$SILIS_{D3conc}$ = concentration of SIL *d6*-vitamin D*3* in internal standard (units: ng/mL)

(k) Determine the linear regression curve y = mx + c (using the "least squares" method) for the ratio of peaks areas (non-labelled vitamin D/stable isotope labelled vitamin D) vs. the ratio of concentrations (non-labelled vitamin D/stable isotope labelled vitamin D) for five calibration standards with the y-intercept forced through zero.

(l) The concentration of vitamin D2 in the sample is calculated as:

$$\text{Result } D_2 = \frac{PA_{NLD2}}{PA_{SILD2}} \times \frac{SILIS_{D2conc}}{L} \times \frac{SILIS_{vol}}{S_{mass}} \times \frac{100}{1000}$$

where: Result D_2 = vitamin D_2 concentration in sample (units: µg/hg)
PA_{NLD2} = peak area of vitamin D_2 in sample (units: none)
PA_{SILD2} = peak area of SIL *d6*-vitamin D_2 in sample (units: none)
$SILIS_{D2conc}$ = concentration of *d6*-vitamin D_2 in Internal Standard (units: ng/mL)
L = slope of calibration curve (units: none)
$SILIS_{vol}$ = volume of Internal Standard spiked to sample [0.5] (units: mL)
1000 = mass conversion factor (units: µg/g to mg/g)
100 = mass conversion factor (units: mg/g to mg/hg)
S_{mass} = mass of sample (units: g)

(m) The concentration of vitamin D3 in the sample is calculated as:

$$\text{Result } D_3 = \frac{PA_{NLD3}}{PA_{SILD2}} \times \frac{SILIS_{D3conc}}{L} \times \frac{SILIS_{vol}}{S_{mass}} \times \frac{100}{1000}$$

where: Result D_3 = vitamin D_3 concentration in sample (units: µg/hg)

PA_{NLD3} = peak area of vitamin D_3 in sample (units: none)

PA_{SILD3} = peak area of SIL *d6*-vitamin D_3 in sample (units: none)

$SILIS_{D3conc}$ = concentration of *d6*-vitamin D_3 in Internal Standard (units: ng/mL)

L = slope of calibration curve (units: none)

$SILIS_{vol}$ = volume of Internal Standard spiked to sample [0.5] (units: mL)

1000 = mass conversion factor (units: µg/g to mg/g)

100 = mass conversion factor (units: mg/g to mg/hg)

S_{mass} = mass of sample (units: g)

(n) The concentration of vitamin D_3 in the sample is calculated as:

$$\text{Result (IU/hg)} = \text{Result } (\mu\text{g/hg}) \times 40$$

where: 40 = dietary conversion factor (units: µg/hg to IU/hg)

K. Data Handling

Report result as µg/hg to 1 decimal place or IU/hg to 0 decimal places.

L. References

(1) Gill, B.D.; Zhu, X.; Indyk, H.E. (2015) A rapid method for the determination of vitamin D3 in milk and infant formula by liquid chromatography/tandem mass spectrometry. *Journal of AOAC International* 98, 431–435.

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Analysis of Vitamin D_2 and Vitamin D_3 in Infant and Adult Nutritional Formulas by Liquid Chromatography–Tandem Mass Spectrometry: A Multi-Laboratory Testing Study

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Abstract

A multi-laboratory testing study was conducted on AOAC First Action Method 2016.05: Analysis of Vitamin D_2 and Vitamin D_3 in Fortified Milk Powders, Infant Formulas, and Adult/Pediatric Nutritional Formulas by Liquid Chromatography–Tandem Mass Spectrometry. Nine laboratories participated in the analysis of duplicate samples of 20 nutritional products. The samples were saponified at high temperature with lipid-soluble components extracted into isooctane; an aliquot was washed, and vitamin D derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione to form a high molecular mass, easily ionizable adduct, extracted into acetonitrile and analyzed by reversed phase liquid chromatography-tandem mass spectrometry. Stable isotope-labeled internal standards were used for quantitation to correct for losses in extraction and variation in derivatization and ionization efficiencies. Acceptable precision as relative standard deviation (RSD) was demonstrated; repeatability ranged from 1.9 to 5.8% RSD_r and reproducibility values ranged from 6.4 to 12.7% RSD_R, with samples meeting the precision limits specified in the vitamin D Standard Method Performance Requirements and the guidelines recommended for the Horwitz ratio. Method accuracy was assessed using the NIST 1849a SRM, with the *p*-value of 0.32 indicating an absence of bias against the certified value. As expected, placebo samples not fortified with vitamin D returned negligible results.

Introduction

Vitamin D is not a true vitamin as individuals with adequate skin exposure to UV radiation produce vitamin D from a precursor, 7-dehydrocholesterol. However, dietary supplementation of vitamin D is necessary for many, with infant formulas typically fortified with vitamin D_3 and, less commonly, vitamin D_2. Both vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are metabolized in the liver to their respective 25-hydroxy vitamers, which are the dominant circulating forms in blood. The main biological function of vitamin D is calcium homeostasis, controlling the absorption, transport, and deposition of calcium and phosphorus as part of bone mineralization (1).

Rapid, high-throughput analytical methods for vitamin D are needed for routine testing to meet product specifications, and reference methods utilizing contemporary techniques are needed to demonstrate product compliance with strict global regulations. Given that the internationally accepted multi-dimensional LC–UV method for vitamin D, AOAC 2002.05 (2), has a long total analysis time, an updated reference method for vitamin D was identified by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) as a priority. We previously developed a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method that incorporates saponification prior to solvent extraction, with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) derivatization prior to instrumental analysis (3). The method subsequently underwent a comprehensive single laboratory validation (SLV) study using the SPIFAN kit (4), a set of infant formula and adult nutritional products that were selected as a representative sub-sample of the wide range of commercially available products, and the results were compared with the standard method performance requirements (SMPR) (5, 6). In March 2016, this LC–MS method was reviewed by the SPIFAN expert review panel (ERP), was approved for Official First Action status as AOAC Method 2016.05 (7), and was recommended to advance to a multi-laboratory testing (MLT) study for evaluation of reproducibility.

Multi-Laboratory Testing Study

The participating laboratories represented a wide range of food-testing laboratories including governmental agencies, infant formula manufacturers, and contract analytical services. Prior to commencement of the MLT study, each collaborator received a detailed study protocol to allow familiarization with the technique and an opportunity to communicate any difficulties.

The SPIFAN kit and a candidate Standard Reference Material (SRM) NIST 1869 (National Institute of Standards and Technology, Gaithersburg, MD), a soy-based infant/adult nutritional formula fortified with both vitamin D_2 and vitamin D_3, were used in this study. A practice sample, NIST 1849a, was run by participants and, when acceptable results had been obtained, approval to proceed to the analysis of the SPIFAN kit samples was given. The SPIFAN kit was tested over 2 separate days as blind-coded duplicate pairs.

All data were statistically analyzed using the harmonized guidelines for collaborative studies to establish overall mean, intra-laboratory repeatability (S_r), repeatability relative standard deviation (RSD_r), inter-laboratory reproducibility (S_R), reproducibility relative standard deviation (RSD_R), and Horwitz ratio (HorRat) (8). Cochran (p = 0.025, one-tail) and Grubbs (single and double, p = 0.025, two-tail) tests were utilized to determine outliers.

Method

The method protocol sent to the collaborating laboratories was as described in AOAC First Action Method 2016.05 with the minor modification of removal of the drying step for acetone.

AOAC Official Method 2016.05

Analysis of Vitamin D_2 and Vitamin D_3 in Fortified Milk Powders, Infant Formulas, and Adult/Pediatric Nutritional Formulas by Liquid Chromatography–Tandem Mass Spectrometry

Final Action 2017

[Applicable to the determination of vitamin D_2 and vitamin D_3 in fortified milk powders, infant formulas, and adult/pediatric nutritional formulas.]

Caution: Refer to the Material Safety Data Sheets for all chemicals prior to use. Use all appropriate personal protective equipment and follow good laboratory practices.

A. Principle

Samples are saponified at high temperature; then lipid-soluble components are extracted into isooctane. A portion of the isooctane layer is transferred and washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is added to derivatize vitamin D to form a high-molecular mass, easily ionizable adduct. The vitamin D adduct is then extracted into a small volume of acetonitrile and analyzed by reverse-phase liquid chromatography (LC). Detection is by tandem mass spectrometry (MS/MS) using multiple reaction monitoring. Stable isotope-labeled (SIL) d6-vitamin D_2 and d6-vitamin D_3 internal standards are used for quantitation to correct for losses in extraction and any variation in derivatization and ionization efficiencies.

B. Apparatus

(a) *Ultra-high performance LC (UHPLC) system.*—Nexera (Shimadzu, Kyoto, Japan) or equivalent LC system consisting of a dual pump system, a sample injector unit, a degasser unit, and a column oven.

(b) *Triple-quadrupole mass spectrometer.*—Triple Quad 6500 (Sciex, Framingham, MA) or equivalent MS/MS instrument.

(c) *Column.*—Kinetex C_{18} core-shell, 2.6 µm, 2.1 mm × 50 mm (Phenomenex, Torrance, CA) or equivalent.

(d) *UV spectrophotometer.*—Capable of digital readout to 3 decimal places.

(e) *Centrifuge tubes.*—Polypropylene, 15 mL.

(f) *Boiling tubes.*—Glass, 60 mL.

(g) *Water baths.*—Cold 20°C, hot 70°C.

(h) *Disposable syringes.*—1 mL.

(i) *Syringe filters.*—PTFE, 0.2 µm, 13 mm.

(j) *Centrifuge.*—Suitable for 60 mL boiling tubes and 15 mL centrifuge tubes.

(k) *Pasteur pipette.*—Glass, ~140 mm.

(l) *Horizontal shaker.*

(m) *Microcentrifuge vials.*—2 mL.

(n) *Filter membranes.*—0.45 µm nylon.

(o) *Cryogenic vials.*—2 mL.

(p) *Schott bottles.*—1 L.

(q) *HPLC vials, septa, and caps.*

C. Reagents

(a) *Vitamin D_2 (ergocalciferol).*—CAS No. 50-14-6, purity: ≥99%.

(b) *Vitamin D_3 (cholecalciferol).*—CAS No. 67-97-0, purity: ≥99%.

(c) *d6-Vitamin D_2.*— (26,26,26,27,27,27-d6 ergocalciferol), CAS No. 1311259-89-8, enrichment: ≥99%, purity: ≥99%.

(d) *d6-Vitamin D_3.*— (26,26,26,27,27,27-d6 cholecalciferol), CAS No. 118584-54-6, enrichment: ≥99%, purity: ≥99%.

(e) *PTAD (4-phenyl-1,2,4-triazoline-3,5-dione).*—Reagent grade (store in desiccator at 2–8°C).

(f) *Formic acid.*—LC–MS grade.

(g) *Potassium hydroxide.*—Reagent grade.

(h) *Pyrogallol.*—Reagent grade.

(i) *Ethanol.*—LC grade.

(j) *Methanol.*—LC–MS grade.

(k) *Isooctane (2,2,4-trimethylpentane).*—LC grade.

(l) *Acetone.*—LC grade.

(m) *Acetonitrile.*—LC–MS grade.

(n) *Water.*—Purified with resistivity ≥18 MΩ.

D. Reagent Preparation

(a) *PTAD solution (10 mg mL^{-1}).*—To a 5 mL volumetric flask, add 50 mg PTAD, then add 4 mL acetone, and dissolve; dilute to volume with acetone. Expiry: 1 day.

(b) *Potassium hydroxide solution (50%, w/v).*—Dissolve 100 g potassium hydroxide in 200 mL water. Expiry: 1 month.

(c) *Ethanolic pyrogallol solution (1%, w/v).*— Dissolve 5 g pyrogallol in 500 mL ethanol. Expiry: 1 day.

(d) *Mobile phase A (formic acid; 0.1%, v/v).*—To 500 mL water, add 0.5 mL formic acid. Expiry: 1 week.

(e) *Mobile phase B (methanol; 100%, v/v).*—500 mL methanol. Expiry: 1 month.

E. Standard Preparation

Vitamin D is sensitive to light; perform all steps under UV-shielded lighting. If vitamin D_3 is exclusively required for analysis, then standards pertaining to vitamin D_2 need not be used and vice versa.

(a) *Stable isotope-labeled vitamin D_2 or vitamin D_3 stock standard ($SILD_2SS$ or $SILD_3SS$; ~10 $\mu g\ mL^{-1}$).*—

(1) Dispense the contents of a 1 mg vial of d6-vitamin D_2 or a 1 mg vial of d6-vitamin D_3 into separate 100 mL volumetric flasks.

(2) Dissolve in ~90 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol.

(3) Measure the absorbance of an aliquot of $SILD_2SS$ or $SILD_3SS$ at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record the concentration.

(4) Immediately dispense aliquots of $SILD_2SS$ or $SILD_3SS$ (~1.3 mL) into cryogenic vials and freeze at ≤15°C.

(b) *Stable isotope-labeled internal standard (SILIS; ~1 $\mu g\ mL^{-1}$).*—

(1) Prepare an adequate volume of SILIS for the daily sample numbers. For every 15 samples (or part thereof) in an analytical run, remove one vial of $SILD_2SS$ and one vial of $SILD_3SS$ from the freezer and allow to warm to room temperature.

(2) Pipette 1.0 mL each of $SILD_2SS$ and $SILD_3SS$ into the same 10 mL volumetric flask (use a separate 10 mL volumetric flask for each set of 15 samples). Dilute to volume with acetonitrile and mix thoroughly.

(3) Pool all 10 mL volumetric flasks together and mix thoroughly.

(4) Make fresh daily.

(c) *Non-labeled vitamin D_2 or vitamin D_3 stock standard (NLD_2SS or NLD_3SS; ~1 $mg\ mL^{-1}$).*—

(1) Accurately weigh approximately 50 mg vitamin D_2 or vitamin D_3 into separate 50 mL volumetric flasks.

(2) Dissolve in ~40 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol. Store in a freezer at ≤15°C for a maximum of 3 months.

(d) *Non-labeled vitamin D_2 or vitamin D_3 purity standard (NLD_2PS or NLD_3PS; ~10 $\mu g\ mL^{-1}$).*—

(1) Pipette 1.0 mL NLD_2SS or NLD_3SS into separate 100 mL volumetric flasks. Dilute to volume with ethanol.

(2) Measure the absorbance of an aliquot of each solution at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Record the absorbance and calculate the concentration.

(3) Make fresh daily.

(e) *Non-labeled working standard (NLWS; ~1 μg mL^{-1}).*—

(1) Pipette 1.0 mL NLD_2PS and 1.0 mL NLD_3PS into a single 10 mL volumetric flask. Dilute to volume with acetonitrile.

(2) Make fresh daily.

(f) *Calibration standards (CS).*—Make fresh daily. See **Table 2016.05A** for concentrations of the calibration standard solutions.—

(1) *Calibration standard 1 (CS1).*—Pipette 10 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.

(2) *Calibration standard 2 (CS2).*—Pipette 50 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.

(3) *Calibration standard 3 (CS3).*—Pipette 250 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.

(4) *Calibration standard 4 (CS4).*—Pipette 500 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.

(5) *Calibration standard 5 (CS5).*—Pipette 1250 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.

(6) To each calibration standard, add 5 mL acetonitrile and 75 μL PTAD solution; shake to mix.

(7) Leave the calibration standards in the dark for 5 min.

(8) Add 6.25 mL water to each calibration standard and then dilute to volume with acetonitrile; shake to mix.

(9) Transfer ~1 mL of each calibration standard to an HPLC vial ready for analysis.

Table 2016.05A Nominal concentrations of calibration standards

Calibration standard	Concentration, ng mL^{-1}	
	Vitamin D	SIL d6-vitamin D
CS1	0.4	10
CS2	2.0	10

CS3	10	10
CS4	20	10
CS5	50	10

F. Sample Preparation

Vitamin D is sensitive to light; perform all steps under UV-shielded lighting. Sample preparation step (b) is optional for powder samples for which homogeneity may be an issue.

(a) *Powder sample preparation.*—Accurately weigh 1.8–2.2 g powder sample into a boiling tube. Record the weight.

(b) *Slurry sample preparation.*—

(1) Accurately weigh 19.0–21.0 g powder into a disposable slurry container. Record the weight.

(2) Accurately weigh ~80 mL water into the container. Record the weight.

(3) Shake thoroughly until mixed. Place in the dark at room temperature for 15 min and shake to mix every 5 min.

(4) Accurately weigh 9.5–10.5 g slurry or reconstituted powder sample into a boiling tube. Record the weight.

(c) *Liquid sample preparation.*—Accurately weigh 10.0 mL liquid milk into a boiling tube. Record the weight.

G. Extraction and Derivatization

(a) To a powder, slurry, or liquid sample in a boiling tube, add 10 mL ethanolic pyrogallol solution, then add 0.5 mL SILIS, and then cap and vortex mix.

(b) Add 2 mL potassium hydroxide solution to the boiling tube; cap and vortex mix.

(c) Place the boiling tube in a water bath at 70°C for 1 h; vortex mix every 15 min.

(d) Place the boiling tube in a water bath at room temperature until cool.

(e) Add 10 mL isooctane to the boiling tube; cap the boiling tube tightly and place on a horizontal shaker for 10 min.

(f) Add 20 mL water to the boiling tube and invert the tube 10 times; place in a centrifuge at ≥250 × *g* for 15 min.

(g) Transfer a 5 mL aliquot of the upper isooctane layer into a 15 mL centrifuge tube using a Pasteur pipette, taking care not to transfer any of the lower layer.

(h) Add 5 mL water to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 2000 × *g* for 5 min.

(i) Transfer 4–5 mL upper isooctane layer to a new 15 mL disposable centrifuge tube using a disposable pipette, taking care not to transfer any of the lower layer.

(j) Add 75 µL PTAD solution to the centrifuge tube; cap and immediately vortex mix.

(k) Allow to stand in the dark for 5 min to allow the derivatization reaction to complete.

(l) Add 1 mL acetonitrile to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 2000 × *g* for 5 min.

(m) Using a variable volume pipette, transfer 500 µL lower layer into a microcentrifuge vial, taking care not to transfer any of the upper layer.

(n) Add 167 µL water to the microcentrifuge vial; cap and vortex mix.

(o) Using a syringe filter, transfer an aliquot from the microcentrifuge vial to an amber HPLC vial; then cap.

H. Chromatography

(a) Set up the UHPLC system with the configuration shown in **Table 2016.05B.**

Table 2016.05B Chromatographic instrument settings

Instrument parameter	Value
Mobile phase A	Formic acid, 0.1%
Mobile phase B	Methanol, 100%
Column	Kinetex C_{18}
Oven temperature	40°C
Chiller temperature	15°C
Injection volume	3 µL
Initial flow rate	0.6 mL min^{-1}

(b) Form gradients by high-pressure mixing of the two mobile phases, A and B, using the procedure shown in **Table 2016.05C**.

Table 2016.05C Gradient procedure for chromatographic separation

Time, min	Flow rate, mL min^{-1}	Mobile phase composition	
		% A	% B
0	0.6	25	75
3.3	0.6	0	100
3.7	1.0	0	100

4.8	1.0	0	100
4.9	0.6	25	75
5.5	0.6	25	75

I. Mass Spectrometry

(a) Set up the mass spectrometer with the instrument settings shown in **Table 2016.05D**.

Table 2016.05D Mass spectrometer instrument settings[a]

Instrument parameter	Value
Ionization mode	ESI^+
Curtain gas	30 psi
Nebulizer gas GS1	40 psi
Heater gas GS2	40 psi
Collision gas	N_2
Source temperature	300°C
Ion spray voltage	5500 V

[a] These settings are suitable for the 6500 triple-quadrupole mass spectrometer (Sciex). Optimal settings on alternative instruments may differ.

(b) The specific compound parameters to be used are shown in **Tables 2016.05E** and **2016.05F**.

Table 2016.05E Compound parameters (vitamin D_2 instrument method only)

Vitamin D_2 ion[a]	Precursor ion, m/z	Product ion, m/z	DP[b], V	EP[c], V	CE[d], V	CXP[e], V	Dwell time, ms
Analyte quantifier	572.2	298.0	81	10	23	22	120
Analyte qualifier	572.2	280.0			39	16	80
Internal standard quantifier	578.2	298.0			23	22	120
Internal standard qualifier	578.2	280.0			39	16	80

[a] Analyte = vitamin D_2–PTAD adduct, Internal standard ion = d6-vitamin D_2–PTAD adduct.
[b] DP = declustering potential, [c] EP = entrance potential, [d] CE = collision energy, [e] CXP = collision cell exit potential.

Table 2016.05F Compound parameters (vitamin D_3 instrument method only)

Vitamin D_3 ion[a]	Precursor ion, m/z	Product ion, m/z	DP[b], V	EP[c], V	CE[d], V	CXP[e], V	Dwell time, ms
Analyte quantifier	560.2	298.0	151	10	21	18	120
Analyte qualifier	560.2	280.0			37	18	80
Internal standard quantifier	566.2	298.0			21	18	120
Internal standard qualifier	566.2	280.0			37	18	80

[a] Analyte = vitamin D_3–PTAD adduct, Internal standard ion = d6-vitamin D_3–PTAD adduct.
[b] DP = declustering potential, [c] EP = entrance potential, [d] CE = collision energy, [e] CXP = collision cell exit potential.

J. Calculations

(a) *Concentration of stable isotope-labeled vitamin D_2 in stock standard SILD$_2$SS.*—

$$SILD_2SS_{D2conc} = \frac{SILD_2SS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where: $SILD_2SS_{D2conc}$ = concentration of d6-vitamin D_2 in stock standard (µg mL^{-1}); $SILD_2SS_{abs(\lambda max)}$ = UV absorbance of stock standard at 265 nm (cm^{-1}); $E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_2 in ethanol (461 dL g.cm^{-1}); 10000 = concentration conversion factor (g dL^{-1} to µg mL^{-1}).

(b) *Concentration of stable isotope-labeled vitamin D_3 in stock standard SILD$_3$SS.*—

$$SILD_3SS_{D3conc} = \frac{SILD_3SS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where: $SILD_3SS_{D3conc}$ = concentration of d6-vitamin D_3 in stock standard (µg mL^{-1}); $SILD_3SS_{abs(\lambda max)}$ = UV absorbance of stock standard at 265 nm (cm^{-1}); $E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_3 in ethanol (485 dL g.cm^{-1}); 10000 = concentration conversion factor (g dL^{-1} to µg mL^{-1}).

(c) *Concentration of stable isotope-labeled vitamin D_2 in internal standard SILIS.*—

$$SILIS_{D2conc} = SILD_2SS_{D2conc} \times \frac{1.0}{10} \times 1000$$

where: $SILIS_{D2conc}$ = concentration of d6-vitamin D_2 in internal standard (ng mL^{-1}); $SILD_2SS_{D2conc}$ = concentration of d6-vitamin D_2 in stock standard (µg mL^{-1}); 1000 = concentration conversion factor (µg mL^{-1} to ng mL^{-1}).

(d) *Concentration of stable isotope-labeled vitamin D_3 in internal standard SILIS.*—

$$SILIS_{D3conc} = SILD_3SS_{D3conc} \times \frac{1.0}{10} \times 1000$$

where: $SILIS_{D3conc}$ = concentration of d6-vitamin D_3 in internal standard (ng mL^{-1}); $SILD_3SS_{D3conc}$ = concentration of d6-vitamin D_3 in stock standard (µg mL^{-1}); 1000 = concentration conversion factor (µg mL^{-1} to ng mL^{-1}).

(e) *Concentration of non-labeled vitamin D_2 in purity standard NLD_2PS.*—

$$NLD_2PS_{D2conc} = \frac{NLD_2PS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where: NLD_2PS_{D2conc} = concentration of vitamin D_2 in purity standard (µg mL^{-1}); $NLD_2PS_{abs(\lambda max)}$ = UV absorbance of purity standard at 265 nm (cm^{-1}); $E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_2 in ethanol (461 dL $g.cm^{-1}$); 10000 = concentration conversion factor (g dL^{-1} to µg mL^{-1}).

(f) *Concentration of non-labeled vitamin D_3 in purity standard NLD_3PS.*—

$$NLD_3PS_{D3conc} = \frac{NLD_3PS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where: NLD_3PS_{D3conc} = concentration of vitamin D_3 in purity standard (µg mL^{-1}); $NLD_3PS_{abs(\lambda max)}$ = UV absorbance of purity standard at 265 nm (cm^{-1}); $E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_3 in ethanol (485 dL $g.cm^{-1}$); 10000 = concentration conversion factor (g dL^{-1} to µg mL^{-1}).

(g) *Concentration of non-labeled vitamin D_2 in working standard NLWS.*—

$$NLWS_{D2conc} = NLD_2PS_{D2conc} \times \frac{1.0}{10} \times 1000$$

where: $NLWS_{D2conc}$ = concentration of vitamin D_2 in working standard (ng mL^{-1}); NLD_2PS_{D2conc} = concentration of vitamin D_2 in purity standard (µg mL^{-1}); 1000 = concentration conversion factor (µg mL^{-1} to ng mL^{-1}).

(h) *Concentration of non-labeled vitamin D_3 in working standard NLWS.*—

$$NLWS_{D3conc} = NLD_3PS_{D3conc} \times \frac{1.0}{10} \times 1000$$

where: $NLWS_{D3conc}$ = concentration of vitamin D_3 in working standard (ng mL^{-1}); NLD_3PS_{D3conc} = concentration of vitamin D_3 in purity standard (µg mL^{-1}); 1000 = concentration conversion factor (µg mL^{-1} to ng mL^{-1}).

(i) *Concentrations of vitamin D_2 and vitamin D_3 in calibration standards CS1–CS5.*—

$$CS1_{Dconc} = NLWS_{Dconc} \times \frac{0.01}{25}$$

$$CS2_{Dconc} = NLWS_{Dconc} \times \frac{0.05}{25}$$

$$CS3_{Dconc} = NLWS_{Dconc} \times \frac{0.25}{25}$$

$$CS4_{Dconc} = NLWS_{Dconc} \times \frac{0.5}{25}$$

$$CS5_{Dconc} = NLWS_{Dconc} \times \frac{1.25}{25}$$

where: $CS1–CS5_{Dconc}$ = concentration of vitamin D_2 or vitamin D_3 in calibration standards (ng mL^{-1}); $NLWS_{Dconc}$ = concentration of vitamin D_2 or vitamin D_3 in working standard (ng mL^{-1}).

(j) *Concentrations of stable isotope-labeled d6-vitamin D_2 and d6-vitamin D_3 in calibration standards CS1–CS5.*—

$$CS1\text{-}5_{Dconc} = SILIS_{Dconc} \times \frac{0.25}{25}$$

where: $CS1–CS5_{Dconc}$ = concentration of d6-vitamin D_2 or d6-vitamin D_3 in calibration standards (ng mL^{-1}); $SILIS_{Dconc}$ = concentration of d6-vitamin D_2 or d6-vitamin D_3 in internal standard (ng mL^{-1}).

(k) *Mass of powder in slurried sample.*—

$$S_{mass} = \frac{D_{mass}}{(D_{mass} + W_{mass})} \times A_{mass}$$

where: S_{mass} = the mass of powder in slurried sample (g); D_{mass} = the mass of dry powder used to make the slurry (g); W_{mass} = the mass of water used to make the slurry (g); A_{mass} = the mass of the aliquot of slurried sample used in the analysis (g).

(l) Determine the linear regression curve $y = mx + c$ (using the "least squares" method) for the ratio of peak areas (non-labeled vitamin D/stable isotope-labeled d6-vitamin D) vs. the ratio of concentrations (non-labeled vitamin D/stable isotope-labeled d6-vitamin D) for five calibration standards with the y-intercept forced through zero.

(m) The concentration (*w/w*) of vitamin D_2 or vitamin D_3 in dry powders is calculated as:

$$\text{Result D} = \frac{PA_{NLD}}{PA_{SILD}} \times \frac{SILIS_{Dconc}}{L} \times \frac{SILIS_{alqt}}{S_{mass}} \times \frac{100}{1000}$$

where: Result D = vitamin D_2 or vitamin D_3 concentration in sample (µg hg^{-1}); PA_{NLD} = peak area of vitamin D_2 or vitamin D_3 in sample; PA_{SILD} = peak area of d6-vitamin D_2 or d6-vitamin D_3 in sample; $SILIS_{Dconc}$ = concentration of d6-vitamin D_2 or

d6-vitamin D_3 in SILIS (ng mL^{-1}); L = slope of calibration curve; $SILIS_{alqt}$ = volume of SILIS aliquot spiked into sample (0.5 mL); S_{mass} = mass of sample (g); 1000 = concentration conversion factor (ng g^{-1} to µg g^{-1}); 100 = concentration conversion factor (µg g^{-1} to µg hg^{-1}).

(n) The concentration (*w/v*) of vitamin D_2 or vitamin D_3 in ready-to-feed (RTF) liquids is calculated as:

$$\text{Result D} = \frac{PA_{NLD}}{PA_{SILD}} \times \frac{SILIS_{Dconc}}{L} \times \frac{SILIS_{alqt}}{S_{vol}} \times \frac{100}{1000}$$

where: Result D = vitamin D_2 or vitamin D_3 concentration in sample (µg dL^{-1}); PA_{NLD} = peak area of vitamin D_2 or vitamin D_3 in sample; PA_{SILD} = peak area of d6-vitamin D_2 or d6-vitamin D_3 in sample; $SILIS_{Dconc}$ = concentration of d6-vitamin D_2 or d6-vitamin D_3 in SILIS (ng mL^{-1}); L = slope of calibration curve; $SILIS_{alqt}$ = volume of SILIS aliquot spiked into sample (0.5 mL); S_{vol} = volume of sample (mL); 1000 = concentration conversion factor (ng mL^{-1} to µg mL^{-1}); 100 = concentration conversion factor (µg mL^{-1} to µg dL^{-1}).

(o) The concentration of vitamin D_2 or vitamin D_3 as IU hg^{-1} in the sample is calculated as:

$$\text{Result(IU hg}^{-1}\text{)} = \text{Result(}\mu\text{g hg}^{-1}\text{)} \times 40$$

where: 40 = dietary conversion factor (µg hg^{-1} to IU hg^{-1}).

K. Data Handling

Report result as µg hg^{-1} to one decimal place or as IU hg^{-1} to zero decimal places.

Results and Discussion

The initial phase of method evaluation within the participating laboratories involved the analysis of a practice sample. The NIST 1849a SRM was selected as the practice sample as it provided confidence that the method was implemented appropriately within each laboratory and that accurate results could be obtained.

A total of 12 laboratories agreed to participate as part of this study; however, only 9 laboratories were able to submit data for evaluation prior to the submission deadline, with 2

laboratories not reporting any data and 1 laboratory unable to achieve acceptable results for the practice sample as defined by the SPIFAN ERP (4).

Upon completion of the analysis of all samples, each participating laboratory reported measured results as well as additional information such as sample identification, weights, volumes, UV absorbances, and peak areas. Participants were also asked to document any deviation from the method and any other pertinent comments based on their experiences in adapting the method into their laboratory. The results received from participants were tabulated and are summarized in Table 1. All 9 collaborating laboratories returned acceptable standard calibration parameters based on linear regression correlation coefficients (r^2: ≥ 0.998).

Only a single pair of results for vitamin D_2 from Lab 6 was excluded as Cochran outliers; no other outliers were identified and all other results were used in the generation of precision values.

Repeatability ranged from 1.9 to 5.8% RSD_r and reproducibility values ranged from 6.4 to 12.7% RSD_R (Table 2), with $HorRat_R$ values for the method ranging from 0.2 to 0.6 (expected range 0.5–2.0) (8).

Method accuracy was assessed in accordance with SPIFAN procedures (4), based on results from the NIST 1849a SRM (Table 3). The *p*-value of 0.32 indicates that no bias against the certified value was found. As expected, placebo samples not fortified with vitamin D returned negligible results.

The method has demonstrated its compliance with the applicability statement of vitamin D SMPR, 2011.004 (5), and has been demonstrated to be suitable for the analysis of vitamin D in a wide range of infant formulas and nutritional products, as illustrated with the range of different matrices used in the SLV study (6) and this MLT study.

A summary of each laboratory's performance was sent to participants, along with an invitation to make comments on the performance of the method in their laboratory. In general, comments were positive with respect to the ease of use of the method. Lab 3 and Lab 9 found that the centrifugation of the samples at 250 × *g* did not give a good separation between the two layers and recommended that a higher centrifuge speed be used. It was noted by Lab 4 that the drying step for acetone was not necessary and did not use it. This had also been noted by the authors and all participants were advised not to dry acetone during the MLT study. Lab 6 used vials in a heating block rather than boiling tubes in a water bath because of limitations of equipment and the results did not appear to have been compromised.

Safety concerns with this method were evaluated and there were no major hazards beyond those typically found in chemistry laboratories. Users of the method are directed to use appropriate safety equipment when handling acids, bases, and solvents, and to refer to Material Safety Data Sheets for detailed safety instructions for each chemical used.

The described protocol provides an accurate, precise, rapid, and robust method for the analysis of vitamin D that is suitable both for routine compliance testing and as a reference method to demonstrate product compliance against global regulations.

Conclusion

An MLT study of AOAC First Action 2016.05, an LC–MS/MS method for the analysis of vitamin D_2 and vitamin D_3 in infant formulas and nutritional products, was undertaken. The method was implemented in 9 laboratories and demonstrated acceptable precision and accuracy.

Recommendation

A study report summarizing the outcomes of this multi-laboratory collaborative study was submitted with the recommendation that AOAC First Action Method 2016.05 be accepted as

a SPIFAN endorsed AOAC Final Action method. The SPIFAN ERP evaluated the study data in March 2017 and endorsed the recommendation, which was subsequently approved by the AOAC Official Methods Board.

Acknowledgments

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Table 1. Tabulated raw data for replicate analyses of SPIFAN kit

Lab # [a]	Vitamin D_3, µg hg^{-1}											
	SRM NIST 1849a				Partially hydrolyzed soy-based infant formula powder		Infant elemental powder		High-protein adult nutritional ready-to-feed		Soy-based infant formula	
3	10.78	10.19	10.52	10.05	7.89	8.93	9.21	8.96	0.97	0.98	11.54	10.36
4	9.92	10.55	10.61	10.51	8.69	9.11	8.02	7.95	0.97	0.94	10.28	10.45
6	10.69	10.94	10.48	10.85	9.13	9.53	8.61	8.29	1.10	1.08	10.62	10.33
7	10.22	10.58	10.13	10.28	9.07	8.83	8.33	8.53	0.98	0.92	9.81	10.02
8	10.04	10.13	10.09	9.87	8.11	8.26	8.04	8.14	0.93	0.92	9.76	9.64
9	11.11	11.73	12.18	11.34	9.22	9.74	9.07	9.00	1.21	1.11	11.43	10.77
10	9.83	9.39	9.44	9.46	8.26	8.38	7.50	7.52	0.85	0.94	9.38	9.45
11	9.58	9.12	9.27	9.61	8.49	8.21	8.03	7.64	1.02	0.89	8.78	9.77
12	9.60	8.92	10.20	10.14	7.34	6.90	7.40	6.87	0.77	0.74	7.82	7.54

[a] Data for Labs 1, 2, and 5 not submitted prior to MLT submission deadline.

Table 1 (continued). Tabulated raw data for replicate analyses of SPIFAN kit

	Vitamin D_3, μg hg^{-1}											
Lab # [a]	High-protein adult nutritional ready-to-feed (unfortified)		Infant formula powder		High-fat adult nutritional ready-to-feed (unfortified)		Milk-based infant formula ready-to-feed		High-fat adult nutritional ready-to-feed		Milk-based infant formula ready-to-feed (unfortified)	
3	0.00	0.00	9.10	9.00	0.00	0.00	0.70	0.72	1.30	1.34	0.00	0.00
4	0.02	0.01	8.61	8.86	0.10	0.16	0.68	0.69	1.38	1.24	0.02	0.01
6	0.00	0.00	8.70	9.00	0.00	0.00	0.72	0.71	1.39	1.46	0.00	0.00
7	0.01	0.00	8.59	8.71	0.01	0.02	0.67	0.66	1.26	1.28	0.00	0.00
8	0.00	0.00	7.82	7.93	0.00	0.00	0.67	0.65	1.19	1.16	0.00	0.00
9	0.00	0.00	8.44	8.87	0.08	0.05	0.81	0.73	1.30	1.24	0.01	0.01
10	0.00	0.00	7.95	7.92	0.02	0.02	0.59	0.59	1.23	1.25	0.00	0.00
11	0.00	0.00	8.57	7.44	0.00	0.00	0.63	0.68	1.23	1.27	0.00	0.00
12	0.00	0.00	7.66	6.84	0.00	0.00	0.51	0.52	1.18	1.13	0.00	0.00

[a] Data for Labs 1, 2, and 5 not submitted prior to MLT submission deadline.

Table 1 (continued). Tabulated raw data for replicate analyses of SPIFAN kit

Lab # [a]	Vitamin D_3, µg hg^{-1}											
	Milk-based child formula powder		Partially hydrolyzed milk-based infant formula powder		Child elemental powder		Milk-based infant formula powder		FOS/GOS[b]-based infant formula powder		Infant elemental powder (unfortified)	
3	7.93	7.75	9.05	8.86	9.60	9.36	10.35	10.63	7.16	7.44	0.00	0.00
4	8.32	8.09	9.29	9.27	8.94	9.28	10.36	10.25	7.17	7.00	0.50	0.30
6	8.21	8.65	9.25	9.35	9.36	9.15	10.58	10.67	7.01	6.83	0.00	0.00
7	7.36	7.37	9.04	8.76	9.06	9.24	10.11	10.23	6.95	6.66	0.01	0.04
8	7.78	7.78	8.32	8.41	8.72	8.44	9.77	10.03	6.70	6.77	0.00	0.00
9	7.26	7.52	9.90	9.63	9.87	9.67	11.86	10.78	8.09	7.93	0.03	0.03
10	7.50	7.50	8.47	8.39	8.40	8.32	9.48	9.61	6.57	6.49	0.01	0.00
11	6.82	6.77	8.85	7.75	7.02	7.45	8.78	9.79	5.75	6.23	0.00	0.00
12	7.43	7.64	8.32	8.29	8.09	7.41	8.10	9.66	7.04	5.80	0.00	0.00

[a] Data for Labs 1, 2, and 5 not submitted prior to MLT submission deadline.

[b] FOS—Fructooligosaccharide, GOS—Galactooligosaccharide.

Table 1 (continued). Tabulated raw data for replicate analyses of SPIFAN kit

Lab # [a]	Vitamin D_3, µg hg^{-1}				Vitamin D_2, µg hg^{-1}			
	Milk-based child formula powder (unfortified)		Low-fat adult nutritional powder		Candidate SRM NIST 1869			
3	0.00	0.00	3.20	3.02	15.07	14.76	13.14	14.05
4	0.06	0.04	3.74	3.55	13.62	14.49	13.75	14.44
6	0.00	0.00	3.28	3.56	*21 09	*18.01	14.24	14.10
7	0.01	0.02	3.35	3.45	12.79	12.80	13.19	12.72
8	0.00	0.00	2.64	2.83	13 20	13.34	12.60	12.64
9	0.03	0.04	4.06	3.81	16.75	17.54	17.61	17.34
10	0.00	0.01	3.15	3.13	14.30	13.30	13.30	13.40
11	0.00	0.00	2.98	2.83	15.44	15.49	13.06	11.93
12	0.00	0.00	2.89	3.25	10.49	11.14	12.21	11.99

[a] Data for Labs 1, 2, and 5 not submitted prior to MLT submission deadline.

* Results removed as Cochran outlier prior to precision calculation.

AOAC INTERNATIONAL

Stakeholder Panel on
Infant Formula and Adult Nutritionals

(2016.05 – Vitamin D Comments)

Method	Method Title	Comments
OMA# 2016.05	*AOAC 2016.05: Analysis of Vitamin D2 and Vitamin D3 by LC MS/MS in Milk Powders, Infant Formulas, and Adult Nutritionals*	○ Comments received from ISO

AOAC INTERNATIONAL
SPIFAN Nutrients ERP
Final Action Review (June 19, 2017)

First Name	Last Name	E-mail Address	ERP Final Recommendation to the OMB	Comments:	ERP Final Recommendation to the OMB 2	Comments: 2
Martine	van Gool	martine.vangool@frieslandcampina.com	I recommend AOAC 2016.02 (final version)		I recommend AOAC 2016.05 (final version)	
Jinchuan	Yang	jinchuan_yang@waters.com	I recommend AOAC 2016.02 (final version)		I recommend AOAC 2016.05 (final version)	
Donald	Gilliland	don.gilliland@abbott.com	I recommend AOAC 2016.02 (final version)	No comments. The method document as written is acceptable as Final Action Method	I recommend AOAC 2016.05 (final version)	No comments. The method document as written is acceptable as Final Action Method
John	Austad	john.austad@covance.com	I recommend AOAC 2016.02 (final version)		I recommend AOAC 2016.05 (final version)	
Estela	Kneeteman	estelak@inti.gob.ar	I recommend AOAC 2016.02 (final version)		I recommend AOAC 2016.05 (final version)	
Esther	Campos Gimenez	esther.campos-gimenez@rdls.nestle.com	I recommend AOAC 2016.02 (final version)		I recommend AOAC 2016.05 (final version)	
Darryl	Suliivan	darryl.sullivan@covance.com	I recommend AOAC 2016.02 (final version)		I recommend AOAC 2016.05 (final version)	
Adrienne	Mc Mahon	adrienne.mcmahon@wyethnutrition.com	I recommend AOAC 2016.02 (final version)	Hi, I recommend this version following some minor editing for OMB review and approval. I have done some minor editing throughout the document but will send a copy to George and cc Delia Best Regards Adrienne	I recommend AOAC 2016.05 (final version)	Hi, I recommend this version for OMB review and approval. One comment - I would prefer if per 100g (powder or RTF) was used to report results rather than per hg-1 or dL-1. Best Regards Adrienne
Brendon	Gill	brendon.gill@fonterra.com	I recommend AOAC 2016.02 (final version)	Changes made to describe back-flushing in sample preparation appear in Final Action method in online web book (although not in email pdf attachment). This method completely MLT successfully and should be Final Action.	I recommend AOAC 2016.05 (final version)	There is no abstention button, so have clicked approve although it should be noted that this is our method.
Melissa	Phillips	melissa.phillips@nist.gov	I recommend AOAC 2016.02 (final version)		I recommend AOAC 2016.05 (final version)	
David	Woollard	DavidWoollard@eurofins.com	I recommend AOAC 2016.02 (final version)	2016.02 has proved itself through the MLT to be reliable and I must accept this. However, I have had problems running the test because of the detection issues of biotin at low wavelength. Equipment available to me was very old so this problem might not occur elsewhere. However, the ERP should be vigilant about the long-term robustness of the method. Options for the future are to add a derivatisation step with avidin to create a fluorescence or to use MS detection.	I recommend AOAC 2016.05 (final version)	This method is a major step forward in vitamin D analysis. Previous methodology using LC-UVD has been hampered by interferences, even when semi-prep clean-up is implemented.