The Scientific Association Dedicated to Analytical Excellence®

AOAC INTERNATIONAL Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN)

REVIEW TEAM MEETING

August 2016

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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

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[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.

AOAC®
INTERNATIONAL

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

AOAC®
INTERNATIONAL

The Scientific Association Dedicated to Analytical Excellence®

Policy

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

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

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

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

Instructions

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

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

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

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

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

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

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

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

   Copies of <u>all</u> 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

The Scientific Association Dedicated to Analytical Excellence®

AOAC INTERNATIONAL

POLICY AND PROCEDURES ON

VOLUNTEER CONFLICT OF INTEREST

Statement of Policy

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

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

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

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

Illustrations of Conflicts of Interest

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

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

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

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

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

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

Do's and Don'ts

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

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

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

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

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

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

Procedures

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

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

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

* * * * * *

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

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

AOAC SPIFAN REVIEW MEETING DRAFT AGENDA

(Method Submissions: Deadline August 15, 2016)

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1) **WELCOME & INTRODUCTIONS** *(Sullivan)*

2) **DISCUSS METHOD(S) SUBMITTED FOR FIRST ACTION *OFFICIAL METHODS*^{SM}**
    a) **Carotenoids**
    b) **Fructans (FOS)**
    c) **GOS**
    d) **Vitamin B_3**

3) **NEXT STEPS**

Conference numbers/code:

1-877-647-3411 US/Canada

Pass code: **774 157 5179#**

To view a list of toll-free international dial-in numbers **Click Here**.

AOAC SMPR 2015.004

Standard Method Performance Requirements^{SM} (SMPRs) for Total Vitamin B_3 (Niacin) in Infant and Adult/ Pediatric Nutritional Formula

Intended Use: Reference Method for Dispute Resolution

1 Applicability

Determinations of total vitamin B_3 (niacin) in all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates). Total B_3 defined as the sum of nicotinic acid (CAS No. 59-67-6) and nicotinamide (CAS No. 99586-65-9), and reported as niacin.

2 Analytical Technique

Any analytical technique that meets the following method performance requirements is acceptable.

3 Definitions

Accuracy (corresponds to the VIM definition for "trueness").—The closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value.

Adult/pediatric formula.—Nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment, made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.

Infant formula.—Breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding (Codex Standard 72-1981), made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.

Limit of detection (LOD).—The minimum concentration or mass of analyte that can be detected in a given matrix with no greater than 5% false-positive risk and 5% false-negative risk.

Limit of quantitation (LOQ).—The minimum concentration or mass of analyte in a given matrix that can be reported as a quantitative result.

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

Reproducibility.—The standard deviation or relative standard deviation calculated from among-laboratory data. Expressed as the reproducibility relative standard deviation (SD_R); or % reproducibility relative standard deviation ($\%RSD_R$).

Vitamin B_3 (niacin).—Refers to two compounds: nicotinic acid and nicotinamide. Both forms of the vitamin are equally biological active. Nicotinic acid is chemically pyridine 3-carboxylic acid, CAS No. 59-67-6. Nicotinamide is pyridine 3-carboxcylic acid amide, CAS No. 99586-65-9.

Table 1. Method performance requirements[a]

Analytical range	200–10000[b]
Limit of quantitation (LOQ)	≤200[b]
Recovery	90–110%
Repeatability (RSD_r)	≤5%
Reproducibility (RSD_R)	≤10%

[a] Concentrations apply to: (a) "ready-to-feed" liquids "as is"; (b) reconstituted powders (25 g into 200 g water); and (c) liquid concentrates diluted 1:1 by weight.

[b] µg/100 g reconstituted final product.

4 Method Performance Requirements

See Table 1.

5 System Suitability Tests and/or Analytical Quality Control

Suitable methods will include blank check samples, and check standards at the lowest point and midrange point of the analytical range.

6 Reference Material(s)

NIST Standard Reference Material® (SRM) 1849a Infant/Adult Nutritional Formula or equivalent. The SRM is a milk-based, hybrid infant/adult nutritional powder prepared by a manufacturer of infant formula and adult nutritional products. A unit of SRM 1849 consists of 10 packets, each containing approximately 10 g of material. Certified value of NIST 1849a is 109 ± 10 mg/kg niacin.

7 Validation Guidance

Recommended level of validation: *Official Methods of Analysis*^{SM}.

8 Maximum Time-to-Result

No maximum time.

Approved by AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). Final Version Date: April 17, 2015. Effective Date: April 17, 2015.

CHEMISTRY METHOD
DRAFT

A LC-MS/MS method for Analysis of Total Nicotinic acid and Nicotinamide in infant formula and adult nutritional samples

QA-

Effective: 2, March 2016

1. OVERVIEW

Purpose and background

Niacin (vitamin B3) is essential for the normal functioning of the human and animal body. Niacin is a water soluble vitamin. Niacin (vitamin B3) is a water-soluble vitamin that is essential for converting food to energy. It supports the function of the digestive system, skin and nerves, and improves circulation. Niacin deficiency causes pellagra which is characterized by a group of symptoms known as “the four Ds,” referring to dermatitis, dementia, diarrhea, and death. Niacin plays a critical role in the metabolism of fat, carbohydrates and amino acids. It functions in the body primarily in the form of two coenzymes: NAD and NADPH. NAD is used in the breakdown of sugar and the oxidation of fatty acids for energy production while NADPH is used in folate metabolism and the synthesis of fatty acids, cholesterol, steroid hormones and important precursors of DNA. Outside of its functions as a coenzyme, niacin is also involved in DNA repair and gene stability.

Principle

Free as well as bound nicotinic acid and nicotinamide are extracted using an ammonium formate buffers employing protease and taka-diastase treatments. The sample extracts are analyzed using liquid chromatography (LC) with tandem mass spectrometric detection.

Analyte Scope of the Method:

The method is applicable for the analysis of total Nicotinic acid, Nicotinamide, Total vitamin B3 in the sample can be calculated based on the results of the analysis.

Matrix Scope:

Infant formula and adult nutrition formula and pet foods.

Analytical Range:

2 – 4375 mcg/100g (with no dilution of the extract).

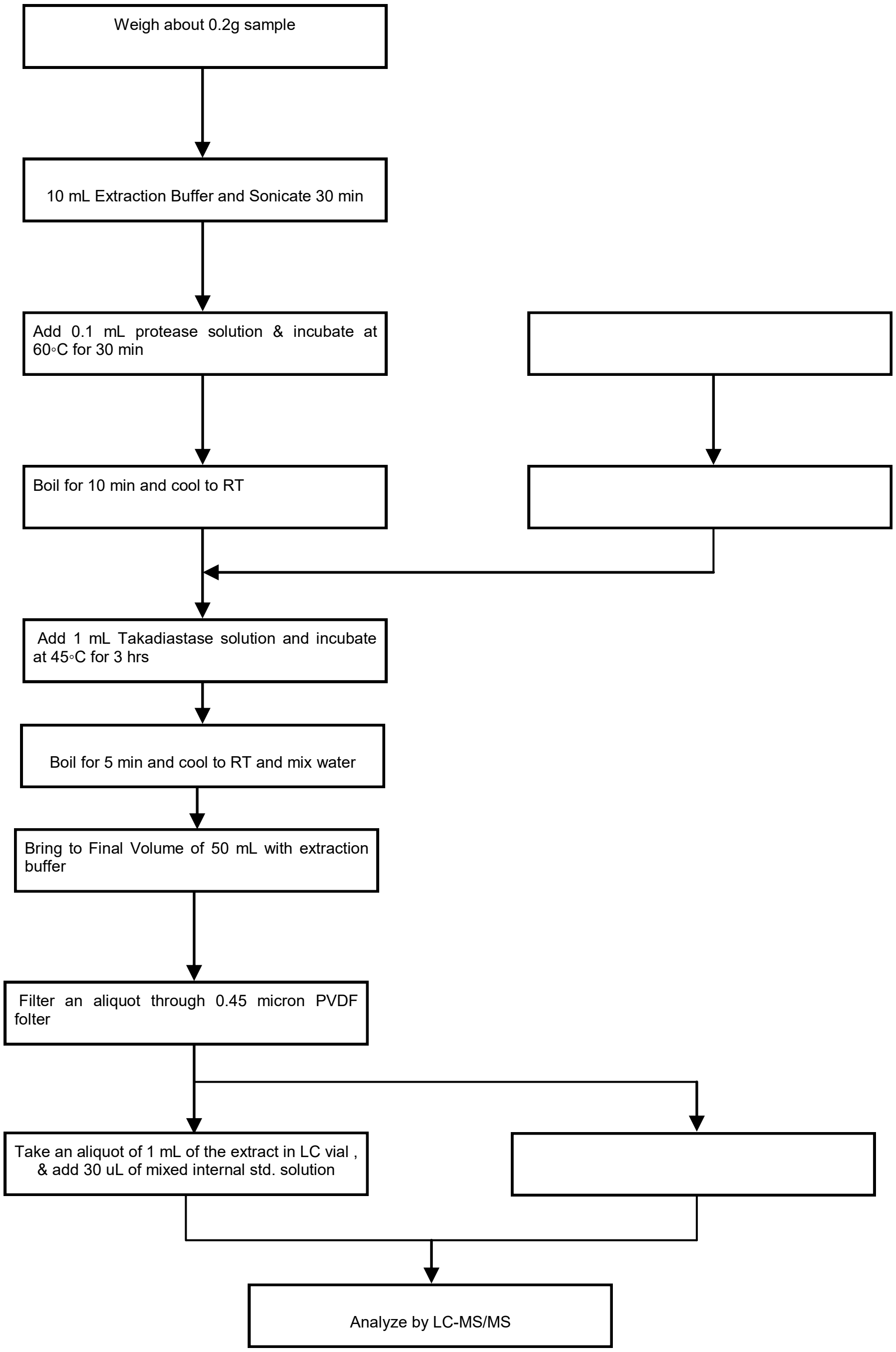
2-10,000 mcg/100g (with 2.3 x dilution of extracts of samples containing niacin levels higher than 4000 mcg/100g).

LOD and LOQ of the method:

LOD and LOQ of the Method of total Vitamin B3 (niacin)analysis:

Vitamin		***Total NIacin***
LOD	mcg/100 g	60
LOQ	mcg/100 g	195

2. Flow diagram



3. QUICK REFERENCE

3.1 Weigh about 0.2g -2.3 g of sample in a 50 mL centrifuge tube.
3.2 Add 10 mL Extraction Buffer & sonicate for 30 min.
3.4 Add 0.1 mL protease and incubate at 60C for 30 min.
3.5 Boil for 10 min, cool to room temperature (RT)
3.6 Add 1 mL takadiastase solution and incubate at 45 C for 3 hours.
3.7 Boil for 5min and cool to RT and make volume to 40 mL with extraction buffer.
3.8 Filter a portion of extract through 0.45 micron PVDF syringe filtrer.
3.9 Pipet 1 mL of sample extract in a HPLC vial and add 30uL of mixed internal std. solution and mix.internal standard.
4.0 Analyze by LC-MS/MS

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4. FULL METHOD

4.1 Apparatus

(Apparatus listed was used in method validation. Any necessary substitutions should be made based on technical equivalence, and verified with CRM's or sample spikes.)

1. Chromatographic system
2. Dionex Ultimate 3000 RS Pump, Autosampler, Column Compartment &Degasser Thermo Fisher / Agilent 1290 Infinity Pump, Autosampler, column compartment & Degasser
3. Agilent 1290 Infinity Pump, Autosample, column compartment & Degasser
4. The Detection (triple quard mass Spec) System: .— ABSciex 5500 QTrap / ABSciex 6500 QTrap
5. Software.—AbSciex Analyst and MultiQuant chromatography data system
6. Analytical column.— Thermo Acclaim C30 3um 2.1 x 150mm; (P/N 075725)

4.2 Labwares:

1. Autosampler vials.—Agilent 2.0ml amber vials, Fisher P/N 50-929-364)
2. Storage bottles.—Thermo Scientific Nalgene™ narrow-mouth packaging bottles; natural HPDE with PP closures, 250 mL, 24-415 closure, bulk pack (P/N 312089-0008, or equivalent).
3. Centrifuge tubes. —BD Falcon™ 50 mL conical, polypropylene; 17 (od) x 115 (length) mm; 25/bag (Fisher Scientific; P/N 14-432-22).
4. Filters. —Fisher PVDF auto vials (P/N 09-916A, or equivalent).
5. Analytical balance. —Mettler Toledo
6. 1000mL volumetric flasks
7. 10, and 100 mL volumetric flasks (Class A)

4.3 Reagents and Standards

4.3.1 Reagents

a) Deionized (DI) water.—Type I reagent grade, 18 MΩ-cm resistance or better.
b) Ammonium Hydroxide.— Fisher Scientific; P/N A669-500, or equivalent
c) Extraction Buffer - 12 mM Ammonium Formate in water; pH = 5.0
d) Ammonium Formate – Optima LC/MS Fisher Scientific A115-50g
e) Formic Acid – Optima LC/MS Fisher Scientific A117-50mL
f) Acetonitrile – Optima LCMS grade A955-4
g) Protease – Sigma P3910
h) Takadiastase – Pfaltz & Bauer P/N:T00040

4.3. 2 Calibration Standard Chemicals

Standards:

a) B3: Nicotinic acid -- 99% reagent grade or better (USP P/N #1461003)
b) B3: Nicotinamide -- 99% reagent grade or better (USP P/N #1462006)

4.3. 3 Calibration Internal Standard Chemicals

a) Internal Standard (IS) Nicotinic Acid-[13C3, 15N] – IsoSciences P/N 13257; 1mg
b) Internal Standard (IS) Nicotinamide-[13C3, 15N] – IsoSciences P/N 14327; 1mg

4.4 Internal standard Solutions and mixtures

a) IS Stock Solution – Add 1mL water to each individual vials of niacin and niacinamide IS Chemicals. Stock solution Concentrations are as follows:

Name	B Vitamin	mass (mg) of IS	volume H_2O (mL)	ppm (µg/mL)	B Vitamin
Nicotinic Acid $^{13}C_3$	B3-acid	1	1.00	1000.0	B3-acid
Nicotinamide $^{13}C_3$	B3-NH	1	1.00	1000.0	B3-NH

b) Internal Standard Mix(IS Mix) – Take various volumes of each stock as shown in table below and make up to 10mL with Extraction buffer.

Name	volume Stock (uL)	Final Vol (mL)	ppb (ng/mL)
Nicotinic Acid $^{13}C_3$	10	10	1000.0
Nicotinamide $^{13}C_3$	40		4000.0

4.5 Standard Solutions and mixtures

c) *100 mcg/mL B-Vitamin Stock Solution.* — Weigh 10mg of nicotinic acid as well as nicotinamide in individual 100mL volumetric flask. Dilute to volume with Milli-Q water to make a 100 mcg/mL stock solution. Sonicate to ensure completely dissolved. Transfer to amber storage bottle. The standard is stable for 1 month when stored at -20 °C. (Adjust for purity)

d) *500 ng/mL nicotinic acid and nicotinamide intermediate Solution.* — Take 0.05mL (50uL) of nicotinic acid as well as nicotinamide Stock solutions in a 10mL volumetric flask and dilute to volume with Extraction Buffer pH 5.0. Make Fresh each batch.

e) *Nicotinic acid and nicotinamide Working Standard Solutions.* — See table below for dilutions of the working calibration curve. The 12mM ammonium formate Extraction buffer pH 5.0 is used as the diluent. Place 1mL of each standard in an HPLC vial and add 30uL of IS Mix Std.

Std Level	Std Used	Vol. of Interm Mix Std (mL)	Final Vol (mL)	Final Concen. (ng/mL, ppb)
6	Interm	3.50	10.00	175
5	Interm	2.00	10.00	100
4	Interm	0.90	10.00	45
3	Std Level 6	1.00	10.00	17.5
2	Std Level 5	0.50	10.00	5.0
1	Std Level 4	0.20	10.00	0.9

4.4.3 Mixture of nicotinic acid and nicotinamide standard Solutions with Internal Standard:

Take accurately 1 mL of all the calibration standards (levels 1 through 6) in separate HPLC vials. To 1mL of each calibration standard solution add 0.03 mL of the internal standard mix solution (IS-Mix). The details of the preparations of different calibration standard mixture with mixed-internal standard solution is provided in the following Table.

Preparation of mixture of all calibration standards with the mixed internal standard (IS-Mix).

Std Level	Final Concen. (ng/mL, ppb)	Vol. of Std (mL)	Internal Std Mix Solution (IS-Mix) (mL)	Final Concen. (ng/mL, ppb)
6	175	1.00	0.030	175
5	100	1.00	0.030	100
4	45	1.00	0.030	45
3	17.5	1.00	0.030	17.5
2	5.0	1.00	0.030	5.0
1	0.9	1.00	0.030	0.9

The net concentration of different internal standards in the calibration standards is provided in the following Table.

The net concentration of different nicotinic acid and nicotinamide internal standards in the calibration standards are as the following.

Name	ppb (ng/mL)	Added to each Calibration std. (mL)	Internal std. final conc. in each calibration std. (ng/mL)
Nicotinic Acid $^{13}C_3$	1000.0	0.03	30.0
Nicotinamide $^{13}C_3$	4000.0		120.0

4.6 Reagent Solutions and mixtures

a) *Extraction Buffer 12.0 mM Ammonium Formate; pH 5.0.* — In a 1L Solvent reservoir bottle add 0.771g Ammonium formate and 1L Milli-Q water. Stir until dissolved completely. Adjust pH with formic acid to pH 5.0. (***1-2 drops***)

b) *Pro* Protease Solution – 50mg/mL. Weigh 100 mg of protease in a test tube and add 2 mL of water. Gently mix to dissolve/suspend and make sure no foaming. 0.1mL/sample needed. Make fresh for each batch.

c) *T*akadiastase – weigh 3g of taka-diastase and add 30ml 10mM ammonium formate pH 5.0 extraction buffer (1mL/sample needed) Make fresh each batch.

d) *Mobile Phase A 12.0 mM Ammonium Formate; pH 3.0.* — *I*n a 1L Solvent reservoir bottle add 0.771g Ammonium formate and 1L Milli-Q water. Stir until dissolved completely. Adjust pH with formic acid to pH 3.0

e) *Mobile Phase B* — 100% Acetonitrile

4.7 Procedure:

4.7.1 Sample Preparation.

Samples should be completely homogenous and well blended . NIST 1849a can be prepared as a slurry before the analysis 25 g and 200 mL of water and mixed to uniform mixture.

4.7.2 Sample Extraction

1. Weigh approximately 0.2g – 2.3 g in 50mL Centrifuge tube
2. Add 10mL Extraction Buffer (12mM Ammonium Formate pH 5.0)
3. Sonicate 30min (quick vortex every 10 min)
4. Add 0.1mL Protease Solution (50mg/mL)
5. Incubate in 60^{o}C shaking water bath 30min
6. Boil 10min ; cool to R.T
7. Add 1mL Takadiastase Solution (1% in Extraction Buffer)
8. Incubate 3Hrs @ 45^{o}C shaking water
9. Boil 5min; Cool to R.T
10. Bring to Final Volume 40mL with Extraction Buffer
11. Centrifuge 5min 8500rpm
12. PVDF filter into 16x25 disposable glass culture tube
13. Pipette 1mL into HPLC vial and add 30u: of IS Mix
14. Inject 2uL on LCMS/MS

4.7.3 Method Blank: Process 0.5 mL of water just like sample as described in the section 4.7.2.

4.7.4 Standard as a Sample: Process 0.8 mL of mixed intermediate standard just like a sample as described in the section 4.7.2.

4.7.5 Analysis of the Calibration, Blank, standard as a sample and sample solutions:

1. Analyze by HPLC MS/MS.

4.8 LC-MS/MS Analysis

4.8.1. LC Analysis:

1. Use HPLC as described in the Apparatus section.
2. Detetor Mass spectrometer, API5500 Qtrap or 6500 Qtrap
3. Column: Thermo Acclaim C30 3um 2.1 x 150mm
4. HPLC Column Temperature: 30°C
5. Autosampler Temperature: 4-5°C
6. Injection Volume: 2 µL
7. Mobile Phase: A:
8. M.P.A: 12 mM ammonium formate (Optima) in water pH = 3.0
9. M.P.B: 100% ACN
10. ***Instrument HPLC Conditions***

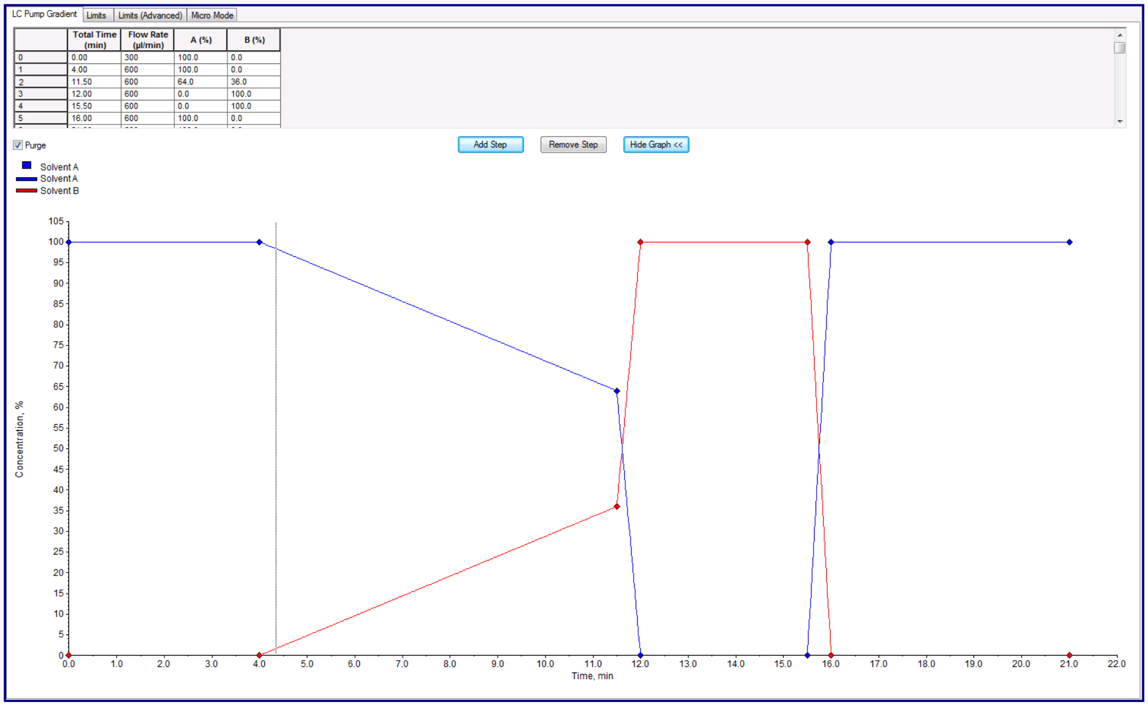
11. ***Dionex Ultimate 3000 / Agilent 1290 System***

12. (**a**) *Columns.*— Thermo Acclaim C30 3um 2.1 x 150mm
13. (**b**) *Mobile Phase.* —
14. M.P.A: 12 mM ammonium formate (Optima) in water pH = 3.0
15. M.P.B: 100% ACN

16. (**c**) *Gradient.* —

#	Time (min)	Flow Rate (uL/min)	A%	B%
0	0.0	300	100	0
1	4.0	600	100	0
2	11.5	600	64	36
3	12.0	600	0	100
4	15.5	600	0	100
5	16.0	600	100	0
6	21.0	600	100	0

17.



	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	300	100.0	0.0
1	4.00	600	100.0	0.0
2	11.50	600	64.0	36.0
3	12.00	600	0.0	100.0
4	15.50	600	0.0	100.0
5	16.00	600	100.0	0.0

18. (**d**) *Flow rate.* — 0.30 - 0.60 mL/min.
19. (**e**) *Injection volume.* — 2.0µL.
20. (**f**) *Temperature.* — 20 °C.
21. (**g**) *Autosampler temperature.* — 5.0 °C.
22. (**h**) *Run time.* — 21 min.

23. ***ABSciex 5500 QTrap / ABSciex 6500 QTrap***
24.
25. CUR – 30
26. CAD – medium
27. IonSpray Voltage IS – 4250
28. Temperature – 550
29. GS1 – 70
30. GS2 – 50

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
B3-acid_Quant	124.3	78.1	20	50	10	28	9
B3-acid_Qual1	124.3	80	20	50	10	28	10
B3-acid_Qual2	124.3	53	20	50	10	29	10
B3-amide_Quant	123.3	80.1	20	45	10	27	10

B3-amide_Qual1	123.3	78.1	20	45	10	34	10
B3-amide_Qual2	123.3	53	20	45	10	35	10
B3-acid IS1	128.1	83	20	50	10	28	10
B3-acid IS2	128.1	54	20	50	10	29	10
B3-acid IS3	128.1	81	20	50	10	28	10
B3-amide IS1	127	80	20	45	10	27	10
B3-amide IS 2	127	82	20	45	10	34	10
B3-amide IS 3	127	55	20	45	10	35	10

31. MS_Start.On Duration=0.5
32. MS_Start.Off Duration=12.00

4.9 Calculations and reporting:

4.9.1 Preparation of Calibration Curve:

1. Draw calibration curve of each analyte using the following relation.

X axis = ratio of ng/mL of the analyte vs concentration (ng/mL) of the internal std.

Y axis = ratio of peak area of the analyte vs the peak area of the respective internal std.
. Linear regression is

performed. By the MS software.

4.9.2. Calculation of each of the analytes in the samples, Blank and Std. as a sample:

1. Determine of peak area of the analyte vs the peak area of the respective internal std. for each sample extract and std. as a sample solution.
2. Determine ng/mL of the analyte in each sample extract std. as a sample solution.
3. Calculate ng/mL of each analyte in sample extracts as well as blank and std. as a sample solutions. based on the respective calibration curve.
4. Adjust analyte concentration in sample extract and std. as a sample solution by deducting respective analyte concentration estimated in the blank.

Adjusted analyte conc. (ng/mL) in sample or std. as asample = analyte in conc. sample or std. as a sample (ng/mL) – Respective analyte level in the blank (ng/mL).

4.9.3. Calculation of each of the analyte in the samples:

1. Concentration of each of the analyte is calculated in each sample is calculated by the following relationship:

   Conc. of the analyte in the sample (mg/Kg) = Adjusted analyte concentration (ng/mL) in the sample extract x Dilution of the extract (fold) x Final Volume (mL) of the extract/ (Sample Aliquot (g) x 1000).

   B3 (niacin and niacinamide) calculated in the sample are for the free base form.

4.9.6 Calculation of total vitamin B3 (Niacin) in samples:

The niacinamide estimated in the sample is calculated as niacin by multiplying niacinamide (mg/kg) estimated value by the factor provided in the Table Niacinamide in the sample is calculated as niacin by the following relationship.
Niacinamde as Niacin (mg/kg) = Niacinamide mg/kg x 1.008

Vitamin B3 Conversion Factors

Vitamin	Abbreviation	Salt to make standard	Mol. Wt of used std.	to report Nicotinamide as Nicotinic acid - Multiply by	to report total niacin as nicotinic acid
Nicotinic Acid	Vitamin B3-Acid	Free Base	123.11	1.0	sum Niacin + Niacinamide*1.008
Nicotinamide	Vitamin B3-NH	Free Base	122.13	1.0080	

Total Niacin (mg/kg) in the sample is calculated by sum of of niacin mg/kg and niacinamide calculate as niacin (mg/kg. This is also expressed ion the following relationship.

Total Noacin in the sample (mg/kg) = Niacin (mg/kg) + Niacinamide calculated as niacin (mg/kg).

6. SAFETY

- Safety glasses and gloves must be worn at all times as harmful reagents are used throughout the procedure.
- Acetonitrile is flammable and toxic use with the required precautions.
- Acids must not contact the skin or eyes. If contact does occur, flush the contacted area with large amount of water.
-

7. REFERENCE

AOAC 960.46 and AOAC 944.13

Silliker, Inc. Chemistry Research Center Report

August 8, 2016

Validation of A LC-MS/MS method of Analysis of Total Nicotinic acid and Nicotinamide in infant formula and adult nutritional samples

Prepared for:

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Principle

Free as well as bound nicotinic acid and nicotinamide are extracted using a slightly acidic buffer employing protease and takadiastase treatments. The sample extracts are analyzed using liquid chromatography (LC) with tandem mass spectrometric detection.

Reagents

a) Deionized (DI) water.—Type I reagent grade, 18 MΩ-cm resistance or better.
b) Ammonium Hydroxide.— Fisher Scientific; P/N A669-500, or equivalent
c) Extraction Buffer - 12 mM Ammonium formate in water; pH = 5.0
d) Ammonium Formate – Optima LC/MS Fisher Scientific A115-50g
e) Formic Acid – Optima LC/MS Fisher Scientific A117-50mL
f) Acetonitrile – Optima LCMS grade A955-4
g) Protease – Sigma P3910
h) Takadiastase – Pfaltz & Bauer P/N:T00040

Standards

a) Nicotinic acid (B3-acid) -- 99% reagent grade or better (USP P/N #1461003)
b) Nicotinamide (B3-amide) -- 99% reagent grade or better (USP P/N #1462006)

Internal Standards

a) Internal Standard (IS) Nicotinic Acid-[13C3, 15N] – IsoSciences P/N 13257; 1mg
b) Internal Standard (IS) Nicotinamide-[13C3, 15N] – IsoSciences P/N 14327; 1mg

Instrumentation

a) Chromatographic system.— ABSciex 5500 QTrap / ABSciex 6500 QTrap
b) Dionex Ultimate 3000 RS Pump, Autosampler, Column Compartment &Degasser Thermo Fisher / Agilent 1290 Infinity Pump, Autosampler, column compartment & Degasser
c) Agilent 1290 Infinity Pump, Autosample, column compartment & Degasser
d) Software.—AbSciex Analyst and MultiQuant chromatography data system
e) Analytical column.— Thermo Acclaim C30 3um 2.1 x 150mm; (P/N 075725)

Labwares

a) Autosampler vials.—Agilent 2.0ml amber vials, Fisher P/N 50-929-364)

b) Storage bottles.—Thermo Scientific Nalgene™ narrow-mouth packaging bottles; natural HPDE with PP closures, 250 mL, 24-415 closure, bulk pack (P/N 312089-0008, or equivalent).
c) Centrifuge tubes. — BD Falcon™ 50 mL conical, polypropylene; 17 (od) x 115 (length) mm; 25/bag (Fisher Scientific; P/N 14-432-22).
d) Disposable glass culture tubes 15x125
e) Filters. —Fisher PVDF auto vials (P/N 09-916A, or equivalent).
f) Analytical balance. —Mettler Toledo
g) 10, 100 and 1000mL volumetric flasks

Solutions and Reagents Preparation

a) Internal standard (IS) Stock Solution – Add 1mL water to each individual vials of nicotinic acid and nicotinamide IS Chemicals. Stock solution Concentrations are as follows:

Name	**B Vitamin**	**mass (mg) of IS**	**volume H_2O (mL)**	**ppm (µg/mL)**	**B Vitamin**
Nicotinic Acid $^{13}C_3$	B3-acid	1	1.00	1000.0	B3-acid
Nicotinamide $^{13}C_3$	B3-NH	1	1.00	1000.0	B3-NH

b) Internal Standard Mix(IS Mix) – Take various volumes of each stock as shown in table below and make up to 10mL with Extraction buffer.

Name	**volume Stock (uL)**	**Final Vol (mL)**	**ppb (ng/mL)**
Nicotinic Acid $^{13}C_3$	10	10	1000.0
Nicotinamide $^{13}C_3$	40		4000.0

c) *100 mcg/mL Stock Solutions.* — Weigh 10mg of nicotinic acid as well as nicotinamide in individual 100mL volumetric flask. Dilute to volume with Milli-Q water to make a 100 mcg/mL stock solution. Sonicate to ensure completely dissolved. Transfer to amber storage bottle. The standard is stable for 3 month when stored at -20 ^{o}C. (Adjust for purity)

d) *500 ng/mL nicotinic acid and nicotinamide intermediate Solution.* — Take 0.05mL (50uL) of nicotinic acid as well as nicotinamide Stock solutions in a 10mL volumetric flask and dilute to volume with Extraction Buffer pH 5.0. Make fresh each batch.

e) *Nicotinic acid and nicotinamide Working Standard Solutions.* — See table below for dilutions of the working calibration curve. The 12mM ammonium formate Extraction buffer pH 5.0 is used as the diluent. Place 1mL of each standard in an HPLC vial and add 30uL of IS Mix Std.

Std Level	Std Used	Vol. of Interm Mix Std (mL)	Final Vol (mL)	Final Concen. (ng/mL, ppb)
6	Interm	3.50	10.00	175
5	Interm	2.00	10.00	100
4	Interm	0.90	10.00	45
3	Std Level 6	1.00	10.00	17.5
2	Std Level 5	0.50	10.00	5.0
1	Std Level 4	0.20	10.00	0.9

f) *Extraction Buffer 12.0 mM Ammonium Formate; pH 5.0.* — In a 1L Solvent reservoir bottle add 0.771g Ammonium formate and 1L Milli-Q water. Stir until dissolved completely. Adjust pH with formic acid to pH 5.0. (***1-2 drops***)

g) Protease Solution (50mg/mL) – Weigh 100 mg and add 2 mL of water. Gently mix to dissolve/suspend and make sure no foaming. 0.1mL/sample needed. Make fresh for each batch.

h) Takadiaistase – weigh 3gram of taka-diastase and add 30ml 12mM ammonium formate pH 5.0 extraction buffer (1mL/sample needed) Make fresh each batch.

i) *Mobile Phase A 12.0 mM Ammonium Formate; pH 3.0.* — In a 1L Solvent reservoir bottle add 0.771g Ammonium formate and 1L Milli-Q water. Stir until dissolved completely. Adjust pH with formic acid to pH 3.0.

j) *Mobile Phase B* — 100% Acetonitrile

Sample Preparation

Samples should be completely homogenous and well blended . NIST 1849a can be prepared as a slurry before the analysis 25 g and 200 mL of water and mixed to uniform mixture.

Extraction of Niacin (Vitamin B3) in samples:

- Weigh approximately 0.2g -2.3 g in 50mL Centrifuge tube

- Add 10mL Extraction Buffer (12mM Ammonium Formate pH 5.0)
- Sonicate 30min (quick vortex every 10 min)
- Add 0.1mL Protease Solution (50mg/mL)
- Incubate in 60°C shaking water bath 30min
- Boil 10min ; cool to R.T
- Add 1mL Taka Diastase Solution (1% in Extraction Buffer)
- Incubate 3Hrs @ 45°C shaking water
- Boil 5min; Cool to R.T
- Bring to Final Volume 50mL with Extraction Buffer
- Centrifuge 5min 8500rpm
- PVDF filter into 16x25 disposable glass culture tube
- Pipette 1mL into HPLC vial and add 30u: of IS Mix
- Inject 2uL on LCMS/MS

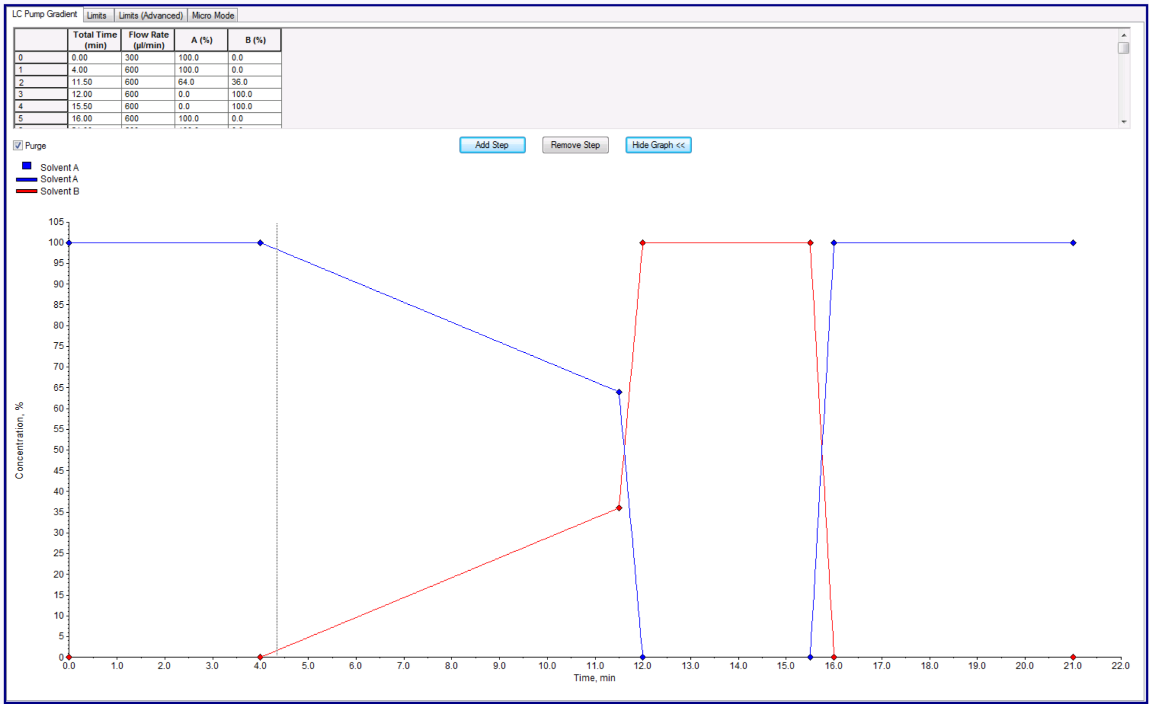
Instrument HPLC Conditions

Dionex Ultimate 3000 / Agilent 1290 System

(**a**) *Columns.*— Thermo Acclaim C30 3um 2.1 x 150mm
(**b**) *Mobile Phase.* —
M.P.A: 12 mM ammonium formate (Optima) in water pH = 3.0
M.P.B: 100% ACN

(**c**) *Gradient.* —

#	Time (min)	Flow Rate (uL/min)	A%	B%
0	0.0	300	100	0
1	4.0	600	100	0
2	11.5	600	64	36
3	12.0	600	0	100
4	15.5	600	0	100
5	16.0	600	100	0
6	21.0	600	100	0



	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	300	100.0	0.0
1	4.00	600	100.0	0.0
2	11.50	600	64.0	36.0
3	12.00	600	0.0	100.0
4	15.50	600	0.0	100.0
5	16.00	600	100.0	0.0

☑ Purge

(d) *Flow rate.* — 0.30 - 0.60 mL/min.
(e) *Injection volume.* — 2.0µL.
(f) *Temperature.* — 20 ^{o}C.
(g) *Autosampler temperature.* — 5.0 ^{o}C.
(h) *Run time.* — 21 min.

ABSciex 5500 QTrap / ABSciex 6500 QTrap

CUR – 30
CAD – medium
IonSpray Voltage IS – 4250
Temperature – 550
GS1 – 70
GS2 – 50

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
B3-acid_Quant	124.3	78.1	20	50	10	28	9
B3-acid_Qual1	124.3	80	20	50	10	28	10
B3-acid_Qual2	124.3	53	20	50	10	29	10
B3-amide_Quant	123.3	80.1	20	45	10	27	10
B3-amide_Qual1	123.3	78.1	20	45	10	34	10
B3-amide_Qual2	123.3	53	20	45	10	35	10
B3-acid IS1	128.1	83	20	50	10	28	10

B3-acid IS2	128.1	54	20	50	10	29	10
B3-acid IS3	128.1	81	20	50	10	28	10
B3-amide IS1	127	80	20	45	10	27	10
B3-amide IS 2	127	82	20	45	10	34	10
B3-amide IS 3	127	55	20	45	10	35	10

MS_Start.On Duration=0.5

MS_Start.Off Duration=12.00

Results

A LC-MS/MS method for analysis of nicotinic acid and nicotinamide and calculation of total niacin (vitamin B3) in infant and adult nutrition formula and pet food matrices was developed and validated. Results are presented in the following sections.

(a) Results of Chromatogrphy by the method: The chromatographic part of the method could separate nicotinic acid and nicotinamide in the samples from other water soluble B vitamins may present and also from any matrix interferences. Peak shapes of the nicotinic acid as well as nicotinamide were good (fig 1).

1.) Chromatogram of a Mixture vitamin B Standards – approx 20ppb

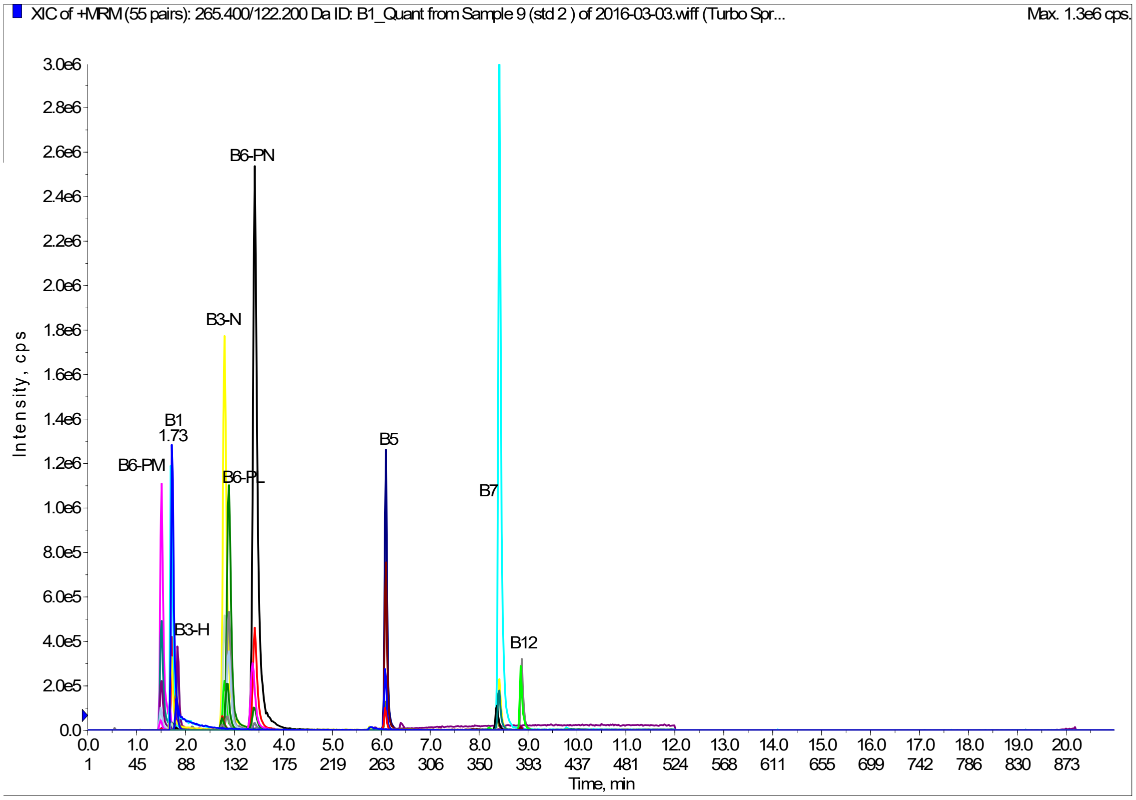


Fig 1: Chromatogram of a mixture vitamin B Standards – approx 20 ppb. B6-PM (pyridoxamine), B1 (thiamine), B3-H (nicotinic acid), B3-N (nicotinamide), B6-PL (pyridoxal), B6-PN (pyridoxine), B5 (pantothenic acid), B7 (biotin) and B12 (cyanocobalamin).

A blank solution containing only internal standards extracted like a sample through the process and analyzed by LC-MS/MS did not provide any interference in analysis of nicotinic acid and nicotinamide by the method. Peak shape of the internal standards was good.

2.) Chromatogram of a Sample – Blank (contains IS)

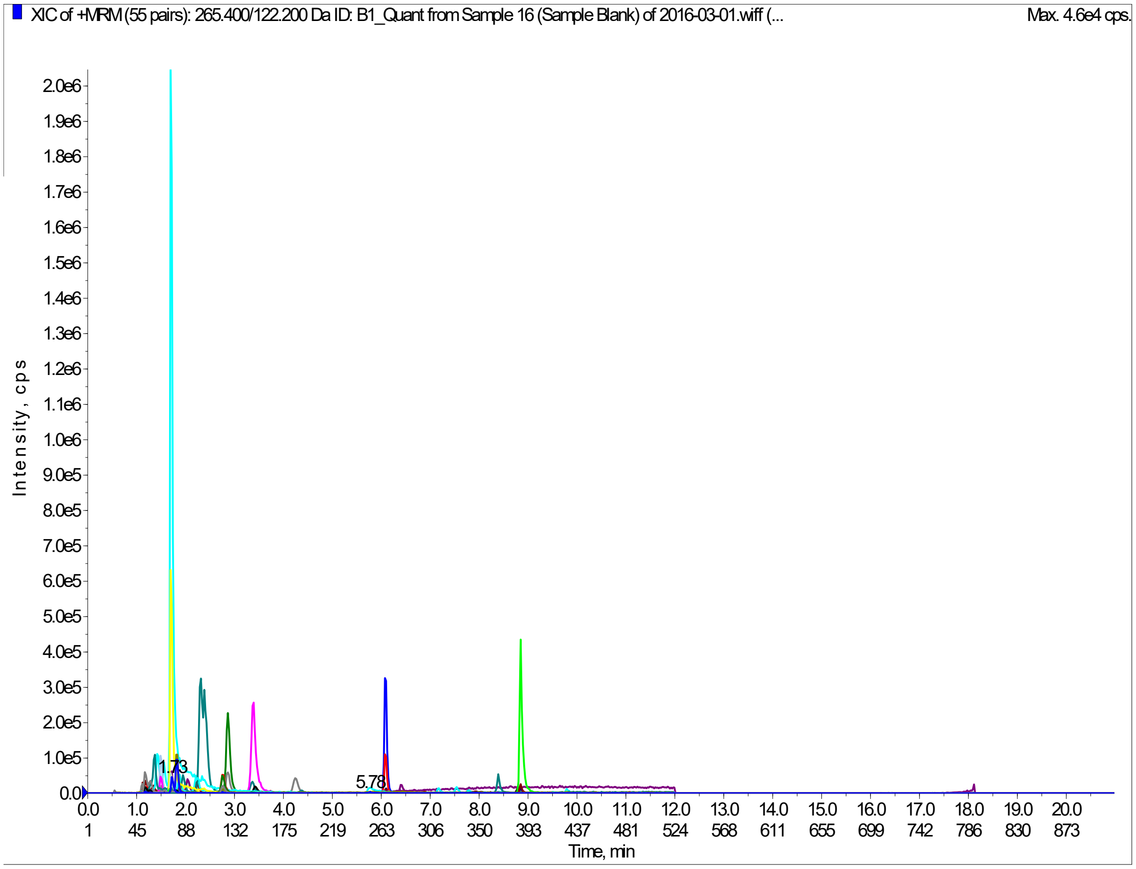


Fig 2: Chromatogram of a blank (contains IS) extracted like a sample through the process and analyzed by LC-MS/MS.

An aliquot of a standard extracted and analyzed like a sample provided no interference in the estmation of nicotinic acid and nicotinamide by the method (Fig 3).

3.) Chromatogram of an aliquot of a standard extracted and analyzed like a sample.

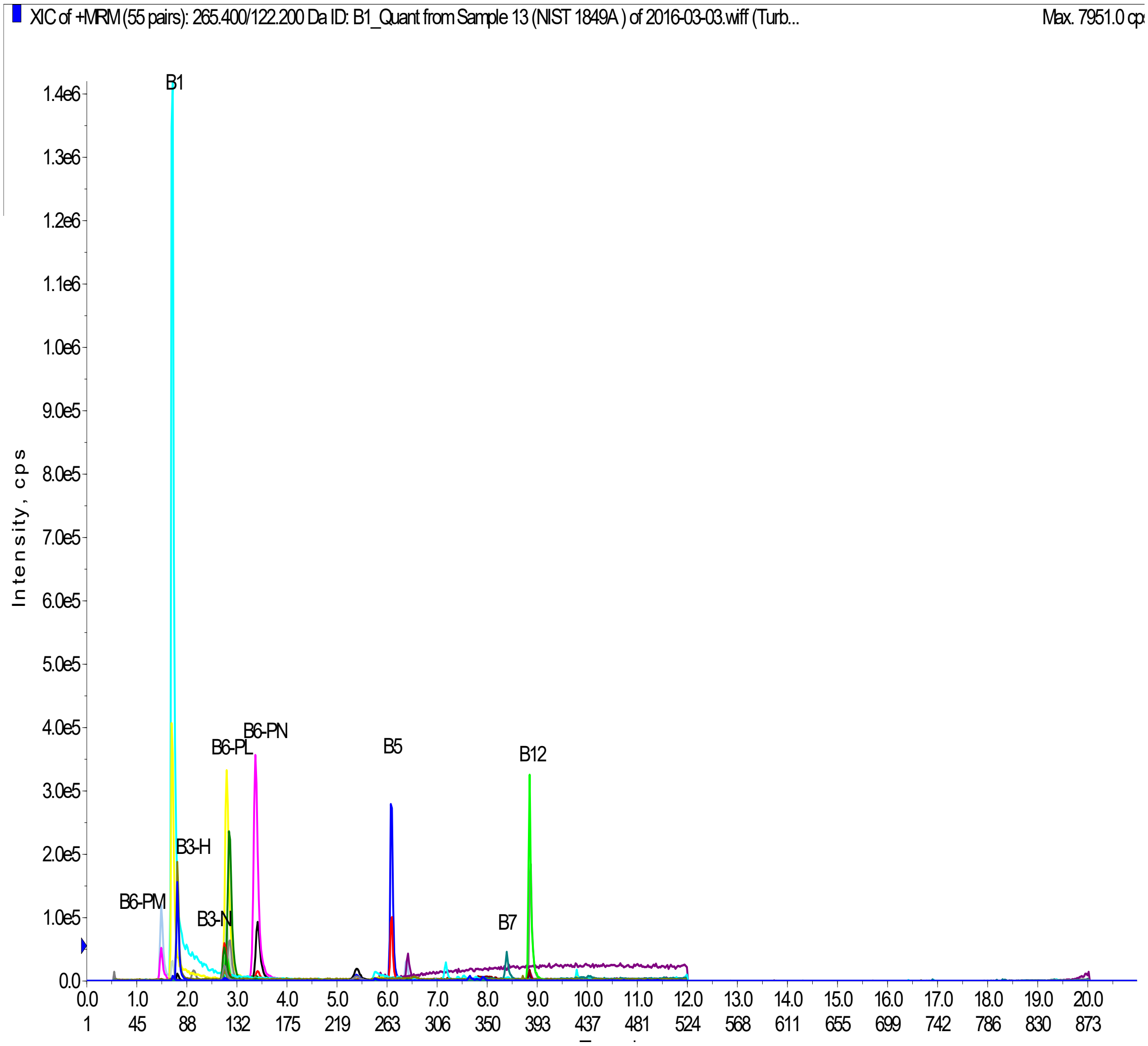


Fig 3: Chromatogram of a mixture vitamin B Standards processed through the method like a sample. B6-PM (pyridoxamine), B1 (thiamine), B3-H (nicotinic acid), B3-N (nicotinamide), B6-PL (pyridoxal), B6-PN (pyridoxine), B5 (pantothenic acid), B7 (biotin) and B12 (cyanocobalamin).

Chromatogram of the NIST 1849 Infant/adult nutrition formula provide no matrix interference in analysis of nicotinic acid and nicotinamide in the sample (fig 4).

Chromatogram of the NIST 1849 Infant/adult nutrition formula

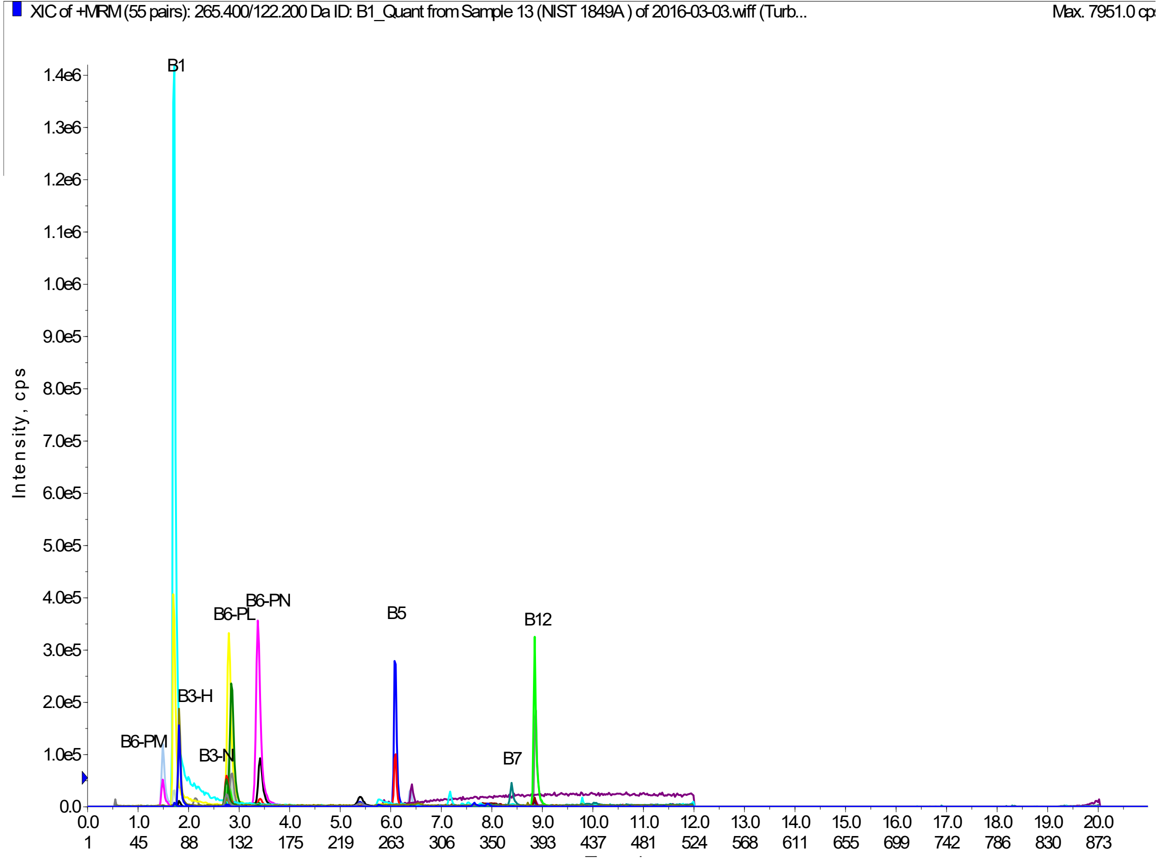


Fig 4: Chromatogram of NIST SRM 1849a (infant and adult nutrition formula) processed through by the method for analysis of nicotinic acid and nicotinamide analysis and estimation of total niacin. B6-PM (pyridoxamine), B1 (thiamine), B3-H (nicotinic acid), B3-N (nicotinamide), B6-PL (pyridoxal), B6-PN (pyridoxine), B5 (pantothenic acid), B7 (biotin) and B12 (cyanocobalamin).

(b) *Calibration Curves obtained by the method* –

The calibration standards provided a good linear relationship between concentration of the standards and their detector response for nicotinic acid as well as nicotinamide with R^2 values > 0.999.

Fig 5: Nicotinic acid Calibration Curve

Fig

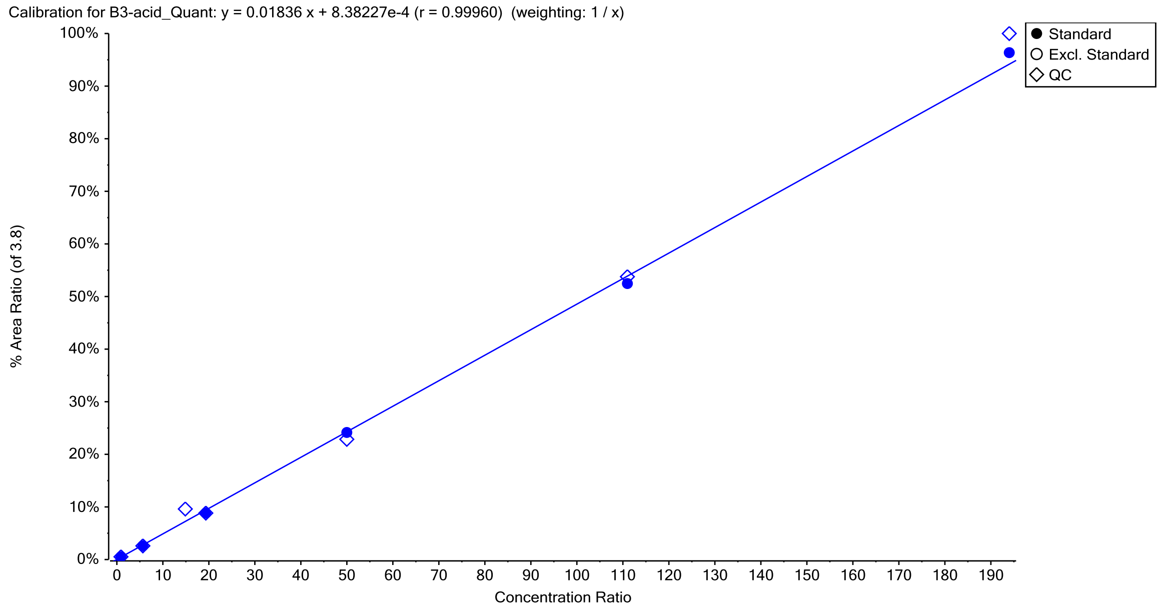
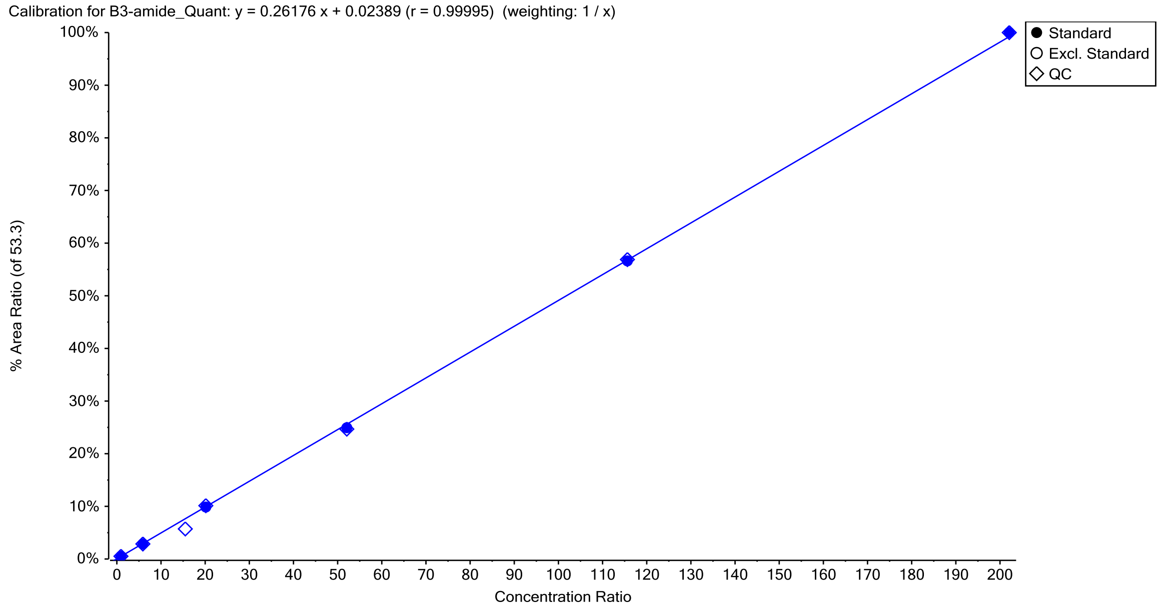


Fig 6. Nicotinamide Calibration Curve



Analytical Range of the method:

2 – 4375 mcg/100g (with no dilution of the extract).

2-10,000 mcg/100g (with 2.3 x dilution of extracts of samples containing niacin levels higher than 4000 mcg/100g).

Accuracy and Precision Evaluation of the method

Accuracy of the method in estimation of total niacin (vitamin B3) was evaluated by analysis of NIST SRM 1849a a milk based infant/adult nutrition formula and NIST SRM 1546A a meat sample. The results are presented in the following Table 1. The method provided results which were within the NIST specifications for vitamin B3 for both of analyzed NIST samples. The precision of the analysis of the replicates of the samples analyzed on multiple days (3 days) in terms of % RSD was found to be 4.8% and 4.4% for infant/adult nutrition formula sample and the meat sample respectively.

Table 1: Results of the analysis of the reference materials NIST 1849A Infant/Adult Nutritional Formula & NIST 1546A Meat in replicates on multiple days (3 days) for vitamin B3 contents

	Total Vitamin B3 **mcg/g**
	NIST 1849A Adult/Infant Nutritional Formula
NIST Value	**108.13 +/- 9.92**
Average:	**105.4**
% of NIST Value	**97.4**
%RSD:	4.8
n:	7
	NIST 1546A Meat Homogenate
NIST Value	**41 +/- 4.8**
Average:	**43.336**
% of NIST Value	**105.7**
%RSD:	4.4
n:	7

Analysis of SPIFAN Samples

Samples Details:

Samples analyzed in this study are listed in the following Table 2.

Table 2: Details of the SPIFAN Samples analyzed in the study

#	**Samples for precision and accuracy evaluation**	**Silliker Lab ID**
6	SRM 1849a	357621588
7	Adult Nutritional Powder Milk Protein based	357621615

8	Infant Formula Powder Partially Hydrolyzed Milk based	357621671
9	Infant Formula Powder Partially Hydrolyzed Soy based	357621721
10	Adult Nutritional Powder Low Fat	357621752
11	Child Formula Powder	357621769
12	Infant Elemental Powder	357621776
13	Infant Formula Powder Milk based	357621792
14	Infant Formula Powder Soy based	357621812
15	Infant Formula RTF Milk based (SPIFAN Control Milk)	357621827
16	Adult Nutritional RTF High Protein	357621840
17	Adult Nutritional RTF High Fat	357621876
#	**Samples for spike recovery evaluation**	
1	Child Formula Powder	357621487

Reconstitution of powder samples for the analysis:

25 g of the powdered samples were taken into 200g water and mixed. The reconstituted beverage was mixed thoroughly just before the analysis.

Analysis of the RTF sample:

The RTF samples were mixed and analyzed as is.

Results of the Niacin analysis in the SPIFAN Samples.

Total Nicotinic acid and Nicotinamide were analyzed in all the listed SPIFAN samples. The powder samples were tested after their reconstitution as beverage as detailed earlier. The RTF samples were tested as is. The total niacin in the samples was calculated based on total nicotinic acid and nicotinamide in the samples. The samples were analyzed in triplicates on two days (total n=6) in all the SPIFAN samples. NIST SRM was analyzed in duplicated on two days (total n=40. It may be mentioned that the additional results for the NIST SRM 1849a are provided in additional report provided along with this report.

The results are expressed as mcg of total niacin as nicotinic acid per 100 g of the reconstituted or as is RTF samples. The results in case of the NIST SRM 1849a (one of the SPIFAN samples) were calculated as mcg of total niacin as nicotinic acid per g of the powdered sample so that results can be compared with the NIST specs for the sample.

The results of the SPIFAN samples for total niacin (as nicotinic acid) are presented in the following Tables 3 and 4.

Table 3: Results of total Niacin as nicotinic acid estimated in SPIFAN samples based on the analysis of total nicotinic acid and nicotinamide, part I,

	Total Niacin as Nicotinic acid
	mcg/g of the powder
#6 - NIST SRM 1849A Adult/Infant Nutritional Formula	
	108.13 +/- 9.92
Average:	**107.2**
% Recov:	**99.1 (Results % of NIST Expected value)**
%RSD:	5.0
n:	4
	Total Niacin as Nicotinic acid
	mcg/100g of the reconstituted beverage or RTF
#7 - Adult Nutritional Powder Milk Protein based	
Average:	**710.2**
%RSD:	3.5
n:	6
# 8 - Infant Formula Powder Partially Hydrolyzed Milk based	
Average:	**754.4**
%RSD:	5.4
n:	6
# 9 - Infant Formula Powder Partially Hydrolyzed Soy based	
Average:	**913.8**
%RSD:	5.4
n:	6
# 10 - Adult Nutritional Powder Low Fat	
Average:	**1577.7**
%RSD:	6.0
n:	6
# 11 - Child Formula Powder	
Average:	**581.5**
%RSD:	6.0
n:	6

The Total niacin values calculated based on the results of total nicotinic acid and nictoinamide in the samples. The samples were analyzed in triplicates (NIST in duplicates0 independently on two days.

Table 4: Results of total Niacin as nicotinic acid estimated in SPIFAN samples based on the analysis of total nicotinic acid and nicotinamide, part II

	Total Niacin as Nicotinic acid
	mcg/100g of the reconstituted beverage or RTF
# 12 - Infant Elemental Powder	
Average:	**1489.6**
%RSD:	5.8
n:	6
# 13 - Infant Formula Powder Milk based	
Average:	**968.8**
%RSD:	5.0
n:	6
# 14 - Infant Formula Powder Soy based	
Average:	**1023.8**
%RSD:	5.0
n:	6
# 15 -Infant Formula RTF Milk based	
Average:	**871.8**
%RSD:	5.6
n:	6
# 16 - Adult Nutritional RTF High Protein	
Average:	**3065.1**
%RSD:	5.5
n:	6
# 17 - Adult Nutritional RTF High Fat	
Average:	**4019.7**
%RSD:	5.1
n:	6

The Total niacin values calculated based on the results of total nicotinic acid and nicotinamide in the samples. The samples were analyzed in triplicates independently on two days.

Accuracy of the method in estimation of total niacin (vitamin B3) assessed by analysis of NIST SRM 1849a a milk based infant/adult nutrition formula sample. The results are presented in the above Table 3. The method provided results which are within the NIST specifications for total niacin for the NIST SRM sample. The precision of the analysis of the replicates of the sample analyzed on multiple days (2 days) in terms of % RSD was found to be 5.0%.

The inter-day precision of the total niacin estimation in replicates (n=6 except NIST SRM with n=4) on two days by the method in the SPIFAN samples in terms of % RSD varied in the range of 3.5% to 6.0%. The inter-day precision was slightly higher than 5% specified in the SMPR in some of the samples though the value presented in the study are for the inter-day precision (Tables 3 and 4).

Spike Recovery Evaluation in SPIFAN Sample:

Spike Recovery of nicotinic acid as well as nicotinamide was evaluated in a SPIFAN sample (Child Formula powder) at three different concentrations. . The summary of the spike recovery results is presented in the following Table.

Table 5: Spike recovery evaluation of nicotinic acid and nicotinamide in a SPIFAN Sample at different concentrations.

	Average % Spike Recovery (n = 3)		
	Spike amount mcg/100g of the reconstituted sample		
Spike Level -->	**250 (n=3)**	**500 (n=3)**	**1000 (n=3)**
Nicotinic acid	99.5	99.5	99.5
Nicotinamide	99.4	99.4	99.4
Total Niacin	99.5	99.5	99.5

Child Formula powder sample was analyzed in replicates wth and without spikes of different concentrations of nicotinic acid as well as nicotinamide and their spike recovery was estimated.

The % spike recovery of the nicotic acid and nicotinamide at all the concentrations studied was found to be within 99.4 - 99.5%.

LOD / LOQ Determination of the Method –

The LOD and LOQ of the method were determined by replicate analysis of one of the SPIFAN sample (*Adult Nutritional Powder Milk Protein based).* The sample was analyzed in replicates (n=3) on multiple days (2 days) (total n=6). LOD & LOQ are calc by 3*Stdev and 10*Stdev for total vitamin B3 (niacin) and the result are summarized in the following Table 6.

Table 6: LOD and LOQ of the Method of total Vitamin B3 (niacin)analysis based on total nicotinic acid nicotinamide in a SPIFAN sample

Vitamin		***Total Niacin***
LOD	mcg/100g	60
LOQ	mcg/100g	195

Conclusions –

A LC-MS/MS method for analysis of nicotinic acid and nicotinamide and calculation of total niacin (vitamin B3) in SPIFAN samples was validated. The chromatographic part of the method could separate nicotinic acid and nicotinamide in the samples from other water soluble B vitamins and also from any matrix interferences. The calibration standards provided a good linear relationship between concentration of the standards and their detector response for nicotinic acid as well as nicotinamide with R^2 values > 0.999.

Accuracy of the method in estimation of total niacin (vitamin B3) was evaluated by analysis of NIST SRM 1849, a milk based infant/adult nutrition formula, and NIST SRM 1546A a meat sample. The method provided results which were within the NIST specifications for vitamin B3 for both of analyzed NIST samples. The precision of the analysis of the replicates of the samples analyzed on multiple days (3 days) in terms of % RSD was found to be 4.8% and 4.4% for infant/adult nutrition formula sample and the meat sample respectively.

The inter-day precision of the analysis of the replicates of the samples analyzed on multiple days (2 days) in terms of % RSD was found to be in the range of 3.5-6.0%. In some of the samples % RSD obtained was slightly higher than 5% specified in the SMPR though the value presented in the study are for the inter-day precision.

Spike recovery of nicotinic acid and nicotinamide was evaluated to further measure the method accuracy. Nicotinic acid and Nicotinamide spike recoveries in a SPIFAN sample averaged around 99.4 – 99.5%.

LOD and LOQ of the Method for analysis for total Vitamin B3 (niacin) analysis was estimated in a reconstituted SPIFAN sample and was found to be 60 and 195 mcg/100 *g*. respectively.

Evaluation of Method Performance vs. SMPR requirements.

AOAC SMPR: …………. 2015.004

Method Reference #	
Method title:	**A LC-MS/MS Method for Analysis of Total Niacin (vitamin B3) based on Nicotinic Acid and**
Principle of the method:	Free as well as bound nicotinic acid and nicotinamide are extracted employing protease and taka-diastase

Parameter	**SMPR Requirement**		**Method Performance** **Please report in units as stated in SMPR!**	**Weighting factor for parameter**	**Suitability Ranking (1-3-5)** (select from drop-down list, 5 = best)
SPIFAN matrices		Applicable to all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates). Any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.	**Applicable to all forms of infant, adult and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates. Any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.**	1	
All analytes defined in the applicability statement are measured.		Total Niacin based on analysis of nicotinic acid and nicotinamide	**Total Niacin based on analysis of nicotinic acid and nicotinamide**	1	
Analytical Range.		200-10000	195-10000 (2.3 x dilution of the extract >4000 mcg/100g)	1	
Limit of detection (LOD)	Analyte 1	Total Niacin	60 mcg/100g (RTF or Reconstituted)	1	
	Analyte 2				
	Analyte 3				
	Analyte 4				
	Analyte 5				
Limit of quantification (LOQ)	Analyte 1	Total Niacin (< 200 mcg/100g)	195 mcg/100g (RTF or Reconstituted)	2	
	Analyte 2				
	Analyte 3				
	Analyte 4				
	Analyte 5				
Accuracy/Recovery	Spike recovery (%)	90-110%	99.50%	2	
	Bias vs SRM	NIST SRM 1849 a Expected = 108.13 ± 9.92 mg/kg as niacinamide	104.5 ± 5.0 mg/kg, (% of NIST SRM Value = 96.7), n=7 , as niacinamide	3	
Repeatability (RSDr)				3	
		<5%	0.6-4.9% (21 values)		
			(3 exceptions = 6.4%, 7.2% and 7.6%)		
Intermediate Reproducibility (RSDiR)				1	
		<5%	3.5-6.0%		
			(all samples analyzed in triplicates on two days, total n=6)		
Reproducibility (RSDR)				1	

ADDITIONAL EVALUATION PARAMETERS

Adequate proof of performance via system suitability			
Feedback from Users of the Method since being awarded First Action *Official Methods* Status			
Did Method Author Consider ERP's Method Specific Recommendations (See web link to specific method comments):			
Bias against established method	**Is there a bias Yes/No ?**		
Analytical equipment	**Analytical equipment is commonly available in most labs.**		
Proprietary equipment	**No unique proprietary equipment/accessories are required.**		
Laboratory safety	**Method does not require any special safety precautions e.g. personal protection from highly toxic solvents.**		
Other Considerations			
Overall Score		0	
Decision by ERP			
Recommendation of ERP 2 years after First Action Status	move to Final Action/repeal/remove/expand 2 year term		

Notes:

[a] *Concentrations apply to (1) "ready to feed liquids" "as-is"; (2) reconstituted powders (25 g into 200 g water); and (3) liquid concentrates diluted 1:1 by weight.*

[b] *Units*

Table 1 Accuracy - Spike Recovery

Product	SPIFAN Code	Analyte	Nr. replicates (n)	Spike Recovery				
				Native level	+ Spike Level 1		+ Spike Level 2	
					Avg (%)	RSD %	Avg (%)	RSD (%)
Child Formula Powder	Lot # 00411RF00	Total NICOTINIC Acid	3		99.5	0.03	99.5	0.02
		Total Nicotinamide	3		99.4	0.01	99.4	0.03
		Total Niacin (calculated)			99.5		99.5	
				Global Mean %	99.5	0.0	99.5	0.0

Table 2 Accuracy - Analytical bias compared to reference material

Certified Reference Material					Measured Results					
CRM product	Analyte	Certified value	Uncertainty	Certified range	Concentration	Difference from certified value	Bias			Confidence interval
							Nominal	T_{stat}	*p* -value	
NIST SRM 1849a	Niacin	108.13	9.92	98.21 - 118.05	104.5	3.6	-3.6	#DIV/0!	#DIV/0!	#NUM! - #NUM!
				Mean	104.5	3.6				
				SD	#DIV/0!	#DIV/0!				

$$T_{stat} = \frac{\bar{d}}{sd \,/\, \sqrt{n}}$$

where: $\bar{d}$ = mean of the differences between measured results and reference value
sd = standard deviation of the differences between results and reference value
n = number of replicate analyses of SRM

H_0: $\bar{d}$ = 0, The null hypothesis is that there is no difference between the measured results and the certified value:

p -value: is the probability (at the 95% levels of confidence) of obtaining the test statistic assuming that the null hypothesis is true
p -value < 0.05 indicate a difference of statistical signifance between measured results and SRM

95% confidence interval: The true population of the measured value expected to fall within the confidence interval estimate 95% of the time

lower limit = mean $- t_{crit} \frac{sd}{\sqrt{n}}$ upper limit = mean + $t_{crit} \frac{sd}{\sqrt{n}}$

where: t_{crit} = critical values of the t-distribution
sd = standard deviation of the differences between results an reference value
n = number of replicate analyses of SRM

Table 3

Precision - repeatability.

Product	SPIFAN Code	Analyte	Nr. replicates (n)	Conc. Level	RSDr	RSD iR
NIST SRM 1849a	CLC10-b	Niacin	6	107.2 mcg/g pwd	1.5; 4.9	5.0
Adlt. Nutr. Pwd. Mlk Prtn based	11750017V3	Niacin	6	710.2 mcg/100g	4.4; 0.9	3.5
Inf. Form. Pwd Part Hydlzd Mlk based	1172572116	Niacin	6	754.4 mcg/100g	1.4; 7.6	5.4
Inf. Form. Pwd Part Hydlzd Soy based	117257651Z	Niacin	6	913.8 mcg/100g	4.2; 7.2	5.4
Adlt Nutr Pwd Low Fat	00394RF00	Niacin	6	1577.7 mcg/100g	4.2; 1.5	6.0
Child Form. Powder	00412RF00	Niacin	6	581.5 mcg/100g	6.4; 2.7	6.0
Inf. Form. Elemental Powder	00403RF00	Niacin	6	1489.6 mcg/100g	4.1; 0.7	5.8
Inf. Form. Powder Milk based	D04HTCVV	Niacin	6	968.8 mcg/100g	4.5; 1.0	5.0
Inf. Form. Powder Soy based	E29JVLV	Niacin	6	1023.8 mcg/100g	0.6; 0.8	5.0
Inf. Form. RTF Milk based	EV4H2R	Niacin	6	871.8 mcg/100g	2.5; 2.6	5.6
Adult Nutr RTF High Protein	00730RF00	Niacin	6	3065.1 mcg/100g	2.3; 3.0	5.5
Adult Nutr RTF High Fat	00729RFOO	Niacin	6	4019.7 mcg/100g	1.7; 0.8	5.1
				Global Mean %	#DIV/0!	5.3

REQUIREMENTS SMPR	Conc. Level	RSDr	RSD iR
	X	≤ Z %	≤ W %
Analyte 1		≤5%	
Analyte 2			
Analyte 3			
Analyte 4			
Analyte 5			
Analyte 1			
Analyte 2			
Analyte 3			
Analyte 4			
Analyte 5			
Analyte 1			
Analyte 2			
Analyte 3			
Analyte 4			
Analyte 5			
Analyte 1			
Analyte 2			
Analyte 3			
Analyte 4			
Analyte 5			

Table 4

Precision - reproducibility

Product	SPIFAN Code	Analyte	Nr. replicates (n)	Conc. Level	RSD_R
				Global Mean %	#DIV/0!

REQUIREMENTS SMPR	Conc. Level	RSD_R
	X	≤ Z %
Analyte 1		
Analyte 2		
Analyte 3		
Analyte 4		
Analyte 5		
Analyte 1		
Analyte 2		
Analyte 3		
Analyte 4		
Analyte 5		
Analyte 1		
Analyte 2		
Analyte 3		
Analyte 4		
Analyte 5		
Analyte 1		
Analyte 2		
Analyte 3		
Analyte 4		
Analyte 5		

AOAC SMPR 2014.014

Standard Method Performance Requirements for Determination of Selected Carotenoids in Infant and Adult/Pediatric Nutritional Formula

Intended Use: Reference Method for Dispute Resolution

1 Applicability

Determinations of all-*trans* α-carotene (CAS 7488-99-5), *cis* isomers of α-carotene, all-*trans* β-carotene (CAS 7235-40-7), *cis* isomers of β-carotene, all-*trans* lutein (CAS 127-40-2), *cis* isomers of lutein, and lycopene in all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates).

2 Analytical Technique

Any analytical technique that meets the following method performance requirements is acceptable.

3 Definitions

Accuracy (corresponds to the VIM definition for "trueness").—The closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value.

Adult/pediatric formula.—Nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment [AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN); 2010], made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.

α-Carotene.—All-*trans* α-carotene (IUPAC name: 1,3,3-trimethyl-2-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)-3,7,12,16-tetramethyl-18-(2,6,6-trimethylcyclohex-2-en-1-yl) octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]cyclohexene, CAS No.: 7488-99-5) and its *cis* isomers (Figure 1).

β-Carotene.—All-*trans* ß-carotene (IUPAC name: 1,3,3-trimethyl-2-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)-3,7,12,16-tetramethyl-18-(2,6,6-trimethylcyclohexen-1-yl) octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]cyclohexene, CAS No.: 7235-40-7) and its *cis* isomers (Figure 2).

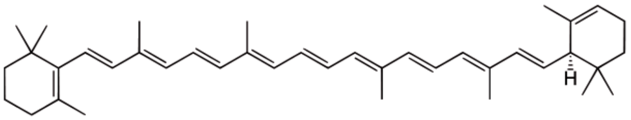


Figure 1. Molecular structure of all-*trans* α-carotene.

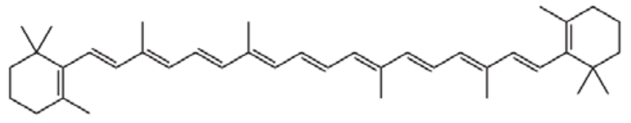


Figure 2. Molecular structure of all-*trans* β-carotene.

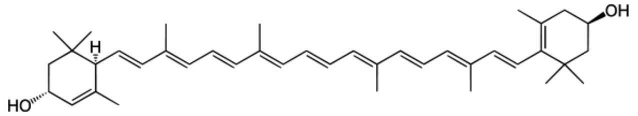


Figure 3. Molecular structure of all-*trans* lutein.

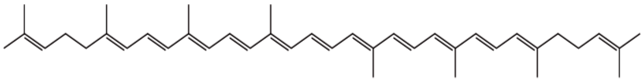


Figure 4. Molecular structure of lycopene.

Carotenoids.—A class of organic pigments consisting of four 10-carbon terpene units, which in turn are formed from eight 5-carbon isoprene units. For the purposes of this standard, only the four carotenoids listed in the Applicability section are considered biologically important enough for consideration in this standard for in infant formula/adult nutritionals.

Infant formula.—Breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding (Codex Standard 72–1981), made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.

Limit of detection (LOD).—The minimum concentration or mass of analyte that can be detected in a given matrix with no greater than 5% false-positive risk and 5% false-negative risk.

Limit of quantitation (LOQ).—The minimum concentration or mass of analyte in a given matrix that can be reported as a quantitative result.

Lutein.—*All*-trans lutein (IUPAC name: (1R)-4-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)-18-[(1R,4R)-4-hydroxy-2,6,6-trimethylcyclohex-2-en-1-yl]-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-3,5,5-trimethylcyclohex-3-en-1-ol, CAS No.: 127-40-2) and its *cis* isomers (Figure 3).

Lycopene.—IUPAC name: (6E,8E,10E,12E,14E,16E,18E,20E,22E,24E,26E)-2,6,10,14,19,23,27,31-octamethyldotriaconta-2,6,8,10,12,14,16,18,20,22,24,26,30-tridecaene, CAS No.: 502-65-8 (Figure 4).

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

Reproducibility.—The standard deviation or relative standard deviation calculated from among-laboratory data. Expressed as the reproducibility standard deviation (SD_R); or % reproducibility relative standard deviation ($\%RSD_R$).

4 Method Performance Requirements

See Table 1.

Table 1. Method performance requirements[a]

Parameter	Minimum acceptable criteria	
Analytical range	1–1300[b]	
Limit of quantitation (LOQ)	≤1[b]	
Recovery	90–110%	
Repeatability (RSD_r)	1–100	8%
	>100–1300	5%
Reproducibility (RSD_R)	1–100	15%
	>100–1300	10%

[a] Concentrations apply to: (*a*) ‘ready-to-feed” liquids “as is”; (*b*) reconstituted powders (25 g into 200 g water); and (*c*) liquid concentrates diluted 1:1 by weight.

[b] µg/100 g reconstituted final product; range and LOQ are based on total of *cis*+*trans* isomers.

5 System Suitability Tests and/or Analytical Quality Control

Suitable methods will include blank check samples, and check standards at the lowest point and midrange point of the analytical range. Methods must be capable of resolving lutein from zeaxanthin.

6 Reference Material(s)

Neither NIST nor IRMM produce a certified reference material for carotenoids in infant formula. The carotenoid content of SRM 1849a has not been determined (as of November 2014).

7 Validation Guidance

Recommended level of validation: *Official Methods of Analysis*^{SM}.

8 Maximum Time-to-Result

No maximum time.

Approved by Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). Final Version Date: September 6, 2014. Effective Date: November 13, 2014.

Determination of Lutein and β-Carotene in Infant Formula and Adult Nutritionals

Reversed-Phase Ultra-High Performance Liquid Chromatography

(Applicable to the determination of all-*trans*-lutein, *cis* isomers of lutein, all-*trans*-β-carotene, and *cis* isomers of β-carotene in infant formula and adult nutritionals.)

A. Principle

Powder samples are reconstituted in water, and liquid sample is first spiked with an internal standard and saponified with potassium hydroxide. Samples are then extracted with MTBE and THF, followed by hexane. The supernatants from the liquid-liquid extraction are dried under nitrogen and reconstituted in IPA. Separation is done by reversed-phase chromatography on a C30 column. All-*trans* lutein and β-carotene are separated from their major *cis* isomers, as well as zeaxanthin, α-carotene, and lycopene.

B. Apparatus

(a) UHPLC System. – Consisting of pump, autosampler, thermostatted column compartment, UV-Vis detector with 60 mm flow cell, and data acquisition software
(b) Analytical column. – C30 Carotenoid column, 3 µm, 2.0 x 250 mm (YMC p/n CT99S03-2502WT)
(c) Guard column. – C30 Guard column, 3 µm, 2.1 x 10 mm (YMC p/n CT99S03-01Q1GC)
(d) Guard cartridge holder. – YMC p/n XPGCH-Q1
(e) Spectrophotometer. – Wavelength range of 200-700 nm, with 1 cm quartz cells
(f) Top loading balance. – Capable of weighing to 0.1 g
(g) Analytical balance. – Capable of weighing to 0.1 mg
(h) Ultrasonic water bath. – 40 kHz
(i) Reciprocating shaker. – Capable of 200 rpm
(j) Evaporator. – With pure nitrogen supply
(k) Laboratory centrifuge. – With adapters for 50 mL centrifuge tubes
(l) Centrifuge tubes. – 50 mL, polypropylene
(m) Syringes. – 1 mL, disposable
(n) Syringe filters. – 0.2 µm, PTFE
(o) Class A volumetric flasks. – Various sizes, clear and amber
(p) Scintillation vials. – 12 mL, amber
(q) HPLC vials – amber, with 300 µL inserts
(r) Class A volumetric pipettes. – Various sizes

C. Reagents

Note: Reagent volumes may be scaled up or down provided good laboratory practices are followed.

(a) Deionized water. – >18 MΩ·cm
(b) Methanol (MeOH). – HPLC grade
(c) Methyl tert butyl ether (MTBE). – HPLC grade
(d) n-Hexane. – HPLC grade

(e) Potassium hydroxide (KOH). – pellets, ACS grade

(f) Reagent alcohol (ROH). – denatured ethanol, HPLC grade

(g) α-Tocopherol (Vitamin E). – 95%

(h) Pyrogallic acid (Pyrogallol). – ACS grade

(i) 2-Propanol (IPA). – HPLC grade

(j) Tetrahydrofuran (THF). – 99.9%, stabilized with BHT

(k) Ammonium acetate. – HPLC grade, 98%

(l) Potassium hydroxide solution, 50% (w/w)

Add 50 mL water to a 250 mL beaker. Weigh 50 g KOH and slowly transfer to the beaker under constant stirring. When dissolved and cooled, transfer to a media bottle.

(m) MTBE/Vitamin E

Dissolve 2.2 g α-tocopherol in 500 mL MTBE. Store in refrigerator.

(n) Pyrogallol solution (0.2 M Pyrogallic Acid in Ethanol)

Dissolve 6.3 g pyrogallic acid in 250 mL ROH. Store in refrigerator. Solution should be clear; discard if colored.

(o) Extraction solution (10 mM Vitamin E in 1:1 MTBE/THF)

Dissolve 2.2 g α-tocopherol in 250 mL MTBE and 250 mL THF. Store in refrigerator.

(p) Sample Solvent (10 mM Vitamin E in IPA)

Dissolve 4.4 g α-tocopherol in 1000 mL IPA. Store in refrigerator.

(q) Mobile Phase for LC System

(1) Phase A. – 20 mM Ammonium Acetate in 98:2 Methanol/Water

Combine 980 mL MeOH, 20 mL water, and 1.54 g ammonium acetate and mix.

(2) Phase B. - MTBE

D. Standards

(a) Lutein. – ChromaDex p/n ASB-00012453 or equivalent

(b) β-Carotene. – USP p/n 1065480 or equivalent

(c) Apocarotenal (β-8′-Apocarotenal). – USP p/n 1040854 or equivalent

(d) Lutein containing ca. 10% zeaxanthin. – USP p/n 1370804

(e) β-Carotene System Suitability Reference Standard. – USP p/n 1065491

E. Standards Preparation

(a) Carotenoid Stock Solutions

Weigh (to 0.1 mg) approximately 10 mg each of lutein, β-carotene and apocarotenal reference standard into separate 50 mL volumetric flasks. Add ca. 40 mL MTBE/Vitamin E to each, sonicate 2-3 minutes, and dilute to volume.

(b) Standard Measuring Solutions for UV-Visible Spectroscopy

Transfer 1.0 mL of each standard stock solution to separate 100 mL volumetric flasks and dilute each to volume with MTBE. Measure against an MTBE blank.

(c) Individual Carotenoid Working Solutions

For lutein and β-carotene: Transfer 100 µL of each standard stock solution to separate 10 mL volumetric flasks and dilute each to volume with sample solvent.

For apocarotenal: Transfer 1.0 mL standard stock solution to a 100 mL volumetric flask and dilute to volume with sample solvent.

(d) Apocarotenal Intermediate Solution

Transfer 3.0 mL apocarotenal stock solution to a 50 mL volumetric flask and dilute to volume with sample solvent.

(e) Mixed Carotenoid Intermediate Solution

Combine 2.0 mL each of lutein and β-carotene standard stock solutions in a 100 mL volumetric flask and dilute to volume with sample solvent.

(f) Calibration Solutions

Transfer apocarotenal intermediate solution and mixed carotenoid intermediate solution to volumetric flasks according to the table below and dilute to volume with sample solvent.

Calibration Solution	Apocarotenal Intermediate (mL)	Mixed Carotenoid Intermediate (mL)	Total volume (mL)	Lutein conc (µg/100 mL)	Apocarotenal conc (µg/100 mL)	β-Carotene conc (µg/100 mL)
C1	2.0	15.0	25	240	96	240
C2	2.0	8.0	25	128	96	128
C3	2.0	5.0	25	80	96	80
C4	2.0	2.0	25	32	96	32
C5	8.0	1.0	100	4	96	4

(g) β-Carotene System Suitability Solution

Transfer 20 mg β-carotene system suitability reference standard to a 50 mL volumetric flask. Add 1 mL water and 4 mL THF and sonicate 5 minutes. Dilute to volume with IPA and sonicate 5 minutes. Cool to room temperature, filter the cloudy suspension through 0.2 µm PTFE, and dilute the clear filtrate 1:4 with IPA. Store in refrigerator.

(h) Lutein System Suitability Solution

Transfer 10 mg USP lutein to a 100 mL volumetric flask, dilute to volume with ROH, and mix. Transfer 1 mL to a 50 mL volumetric flask. Add ca. 35 mL IPA, stopper loosely, and heat in a water bath at 80 °C for two hours. Cool to room temperature, add 250 µL apocarotenal stock solution, and dilute to volume with IPA. Store in refrigerator.

(i) Internal Standard Solution (ISTD)

Prepare immediately before use.

(1) For infant formula and samples with low carotenoid concentrations (up to 100 µg of an individual carotenoid per 100 g as fed):

Transfer 4.0 mL apocarotenal working solution to a 50 mL volumetric flask and dilute to volume with pyrogallol solution. This is enough solution for 9 samples.

(2) For samples with individual carotenoid concentrations > 100 µg/100 g:

Transfer 4.0 mL apocarotenal intermediate solution to a 50 mL volumetric flask and dilute to volume with pyrogallol solution.

F. Sample Preparation

Note on range: While this method can quantify carotenoids in the range of 1-1300 µg/100 g, it is recommended to only quantify a 100-fold difference with a single preparation. For example, the range of 1-100 µg/100 g works well for infant formula, but the range of 15-1500 µg/100 g would work best for samples with the highest carotenoid concentrations.

(a) Prepare up to 9 samples at a time.
(b) Weights – Weigh all samples (powders and liquids) to 0.1 mg.
    (1) Powders – Weigh approximately 625 mg powder sample into a 50 mL centrifuge tube. Add 5 mL water, cap, and vortex until dissolved. Let sit up to 15 min at room temperature.
    (2) Liquid ready-to-feed (RTF) with individual carotenoid concentrations ≤200 µg/100 g – Shake bottle or can on a reciprocating shaker 10 min before opening. Pipette 5 mL sample into a 50 mL centrifuge tube.
    (3) Infant formula concentrate – Shake bottle or can on a reciprocating shaker 10 min before opening. Pipette 2.5 mL sample into a 50 mL centrifuge tube. Add 2.5 mL water, cap, and vortex 10 sec. Let sit up to 15 min at room temperature.
    (4) RTF sample with individual carotenoid concentrations >200 µg/100 g – Shake bottle or can on a reciprocating shaker 10 min before opening. Pipette 2 mL sample into a 50 mL centrifuge tube. Add 3 mL water, cap, and vortex 10 sec. Let sit up to 15 min at room temperature.
(c) Pipette 5.0 mL ISTD solution to each tube.
(d) Add 1.5 mL KOH solution to each tube with a repeater pipette.
(e) Shake on reciprocating shaker 5 min.
(f) Add 8 mL extraction solution to each tube with a repeater pipette.
(g) Shake 10 min.
(h) With a repeater pipette or dispenser, add 10 mL water and 10 mL hexane to each tube.
(i) Shake 1 min.
(j) Centrifuge at 1000 rpm for 5 min.
(k) Transfer a portion of the supernatant to a 12 mL scintillation vial.
    (1) Samples with individual carotenoid concentrations ≤ 50 µg/100 g – use 10 mL supernatant
    (2) Samples with individual carotenoid concentrations > 50 µg/100 g – use 3 mL supernatant
(l) Dry under nitrogen at ≤40 °C.
(m) Reconstitute dried extract in sample solvent and vortex to mix.
    (1) Samples with individual carotenoid concentrations ≤ 100 µg/100 g – add 0.5 mL
    (2) Samples with individual carotenoid concentrations 100-500 µg/100 g – add 1 mL
    (3) Samples with individual carotenoid concentrations 500-1000 µg/100 g – add 2 mL
    (4) Samples with individual carotenoid concentrations 1000-1500 µg/100 g – add 3 mL
(n) Filter through 0.2 µm PTFE syringe filter prior to injection.

G. Chromatography

(a) Conditions

<table>
<tr><td>Analytical column</td><td colspan="2">YMC C30 3 µm, 250 x 2.0 mm</td></tr>
<tr><td>Guard column</td><td colspan="2">YMC C30 3 µm, 10 x 2.0 mm</td></tr>
<tr><td>Column temperature</td><td colspan="2">30 °C</td></tr>
<tr><td>Mobile phases</td><td colspan="2">A: 20 mM ammonium acetate in 98:2 MeOH/Water
B: MTBE</td></tr>
<tr><td rowspan="7">Gradient</td><td>Time (min)</td><td>%B</td></tr>
<tr><td>0</td><td>5</td></tr>
<tr><td>1</td><td>8</td></tr>
<tr><td>8</td><td>15</td></tr>
<tr><td>25</td><td>100</td></tr>
<tr><td>25.5</td><td>5</td></tr>
<tr><td>32</td><td>5</td></tr>
<tr><td>Flow rate</td><td colspan="2">0.25 mL/min</td></tr>
<tr><td>Backpressure</td><td colspan="2">ca. 185 bar</td></tr>
<tr><td>Injection volume</td><td colspan="2">5 µL</td></tr>
<tr><td>UV/Visible detection</td><td colspan="2">450 nm, ref = off</td></tr>
</table>

(b) Resolution between isomers

(1) Resolution between lutein *cis* and *trans* isomers. – Inject the lutein system suitability solution and determine the resolution between the two major *cis* isomers and all-*trans*-lutein. Typical resolution between 13-*cis* and 13′-*cis* lutein is ≥1.5, between 13′-*cis* and all-*trans*-lutein is ≥2.6.

(2) Resolution between all-*trans*-lutein, zeaxanthin, and apocarotenal. – From the chromatogram of the lutein system suitability solution, determine the resolution between all-*trans*-lutein, zeaxanthin, and apocarotenal. Typical resolution between all-*trans*-lutein and zeaxanthin is ≥3.7, between zeaxanthin and apocarotenal is ≥3.0.

(3) Resolution between β-carotene *cis* and *trans* isomers and α-carotene. – Inject the β-carotene system suitability solution and determine the resolution between the two major *cis* isomers, all-*trans*-β-carotene, and α-carotene. Typical resolution between 13-*cis*-β-carotene and *cis/trans* α-carotene is ≥1.7, between all-*trans*- β-carotene and 9-*cis*- β-carotene is ≥2.6.

(c) Calibration. – Inject the calibration solutions before and after each set of sample injections (up to 12 samples in each set). Average the peak areas from the two standard curves and calculate the slope relative to the internal standard, using zero as the y-intercept. See H.(d) for calculation. The slopes from the two curves should not differ by more than 2.0%.

H. Calculations

(a) Determine the purity of lutein and β-carotene standards by first determining the spectrophotometric purity and then the chromatographic purity of each. The overall purity is calculated as the product of the two measured purities

(1) Spectrophotometric Purity (SP)

Measure each standard measuring solution from E.(b) against an MTBE blank at its absorbance maximum (444 nm for lutein and 450 nm for β-carotene). Calculate the spectrophotometric purity of each reference standard as the observed absorbance over the expected absorbance:

$SP = (Abs_{MS} \times 100 \times 1000)/(E_{1\%,1cm} \times W)$

Where Abs_{MS} = absorbance of the standard measuring solution
100 = dilution factor for stock solution to standard measuring solution
1000 = factor for g to mg
$E_{1\%,1cm}$ = extinction coefficient (from Craft and Soares, 1992)
Lutein in MTBE: 2589 at 444 nm
β-Carotene in MTBE: 2588 at 450 nm
W = weight of reference standard, in mg

(2) Chromatographic Purity (CP)

Inject standard working solutions E.(c) at least three times. The CP is calculated as:

CP = (area of the all-trans-carotenoid peak)/(sum of areas of all relevant peaks)

Relevant peaks include all peaks in the HPLC chromatogram with the exception of solvent peaks.

(3) Reference Standard Purity (P)

Calculate the purity of each reference standard:

$P(\%) = SP \times CP \times 100$

Where 100 is the conversion of decimal to percent

(b) Calculate the concentration of each carotenoid analyte (e.g. lutein, C_L) in the all-*trans* form, in µg/100 mL, in the working solution:

$C_L = W_{Lut} \times P_{Lut} \times (1000/10{,}000)$

Where W_{Lut} = the weight of lutein used to make the stock solution, in mg
P_{Lut} = the reference standard purity of all-*trans*-lutein calculated in section H.(a) above
1000 = the conversion of mg to µg
10,000 = the factor for the dilution of 1 mL stock in 100 mL working solution and for the conversion of percent to decimal

(c) Calculate the concentration of the apocarotenal internal standard (C_A), in µg/100 mL, in the working solution:

$C_A = W_A \times CP_A \times (1000/100)$

Where W_A = the weight of apocarotenal used to make the stock solution, in mg
CP_A = the chromatographic purity of apocarotenal
1000 = the conversion of mg to µg
100 = the factor for the dilution of 1 mL stock in 100 mL working solution

(d) Plot the relative responses for each analyte (e.g. RR_{Lut}) vs. relative concentration (e.g. RC_{Lut}) in the five calibration solutions

$RR_{Lut} = (A_{Lut}/A_A)$

Where A_{Lut} = the peak area of all-*trans* lutein in the calibration solution (AU)
A_A = the peak area of apocarotenal in the calibration solution (AU)

$RC_{Lut} = (C_{Lut}/C_A)$

Where C_{Lut} = the concentration of all-trans-lutein in the calibration solution (µg/100 mL)
C_A = the concentration of apocarotenal in the calibration solution (µg/100 mL)

Determine the slope of the curve (response factor [RF]) for each carotenoid based on linear regression, forcing the intercept through zero.

(e) Calculate the mass of apocarotenal (M_A), in µg, added to the test samples:

$M_A = (C_A \times V_A) \times (4/50)$

Where C_A = the concentration of apocarotenal in the intermediate or WS (µg/100 mL)
V_A = the volume of ISTD solution added to each sample, in mL
4 = the volume of apocarotenal intermediate or WS used in the ISTD solution, in mL
50 = the total volume of ISTD solution made, in mL

(f) Calculate the contents of all-*trans* lutein, *cis* isomers of lutein, and total lutein in the test samples:

$Lut_{trans} = (M_A/M_S) \times (A_{Lut}/A_A) \times (100/RF_{Lut})$

Where Lut_{trans} = the all-*trans* lutein in the sample (µg/100 g)
M_A = the mass of apocarotenal added to the test sample, in µg

M_S = the sample weight, in g
A_{Lut} = the peak area of all-*trans*-lutein in the sample chromatogram (AU)
A_A = the peak area of apocarotenal in the sample chromatogram (AU)
RF_{Lut} = the response factor for all-*trans*-lutein

$$Lut_{cis} = (M_A/M_S) \text{ x } ([A_{13cisLut} + A_{13'cisLut} + A_{9cisLut} + A_{9'cisLut}]/A_A) \text{ x } (100/RF_{Lut})$$

Where Lut_{cis} = the *cis* isomers of lutein in the sample (µg/100 g)
M_A = the mass of apocarotenal added to the test sample, in µg
M_S = the sample weight, in g
$A_{13cisLut}$ = the peak area of 13-*cis* lutein in the sample chromatogram (AU)
$A_{13'cisLut}$ = the peak area of 13′-*cis* lutein in the sample chromatogram (AU)
$A_{9cisLut}$ = the peak area of 9-*cis* lutein in the sample chromatogram (AU)
$A_{9'cisLut}$ = the peak area of 9′-*cis* lutein in the sample chromatogram (AU)
A_A = the peak area of apocarotenal in the sample chromatogram (AU)
RF_{Lut} = the response factor for all-*trans*-lutein

$$Lut_{Total} = Lut_{trans} + Lut_{cis}$$

(g) Calculate the contents of all-*trans*-β-carotene, *cis* isomers of β-carotene, and total β-carotene in the test samples:

$$BC_{trans} = (M_A/M_S) \text{ x } (A_{BC}/A_A) \text{ x } (100/ RF_{BC})$$

Where BC_{trans} = the all-*trans*-β-carotene in the sample (µg/100 g)
M_A = the mass of apocarotenal added to the test sample, in µg
M_S = the sample weight, in g
A_{BC} = the peak area of all-*trans*-β-carotene in the sample chromatogram (AU)
A_A = the peak area of apocarotenal in the sample chromatogram (AU)
RF_{BC} = the response factor for all-*trans*-β-carotene

$$BC_{cis} = RF_{BC} \text{ x } (M_A/M_S) \text{ x } ([(A_{15cisBC} \text{ x } 1.4) + (A_{13cisBC} \text{ x } 1.2) + A_{9cisBC} + A_{XcisBC}]/A_A) \text{ x } (100/ RF_{BC})$$

Where BC_{cis} = the *cis* isomers of β-carotene in the sample (µg/100 g)
RF_{BC} = the response factor for all-*trans*-β-carotene
M_A = the mass of apocarotenal added to the test sample, in µg
M_S = the sample weight, in g
A_{BC} = the peak area of all-*trans*-β-carotene in the sample chromatogram (AU)
$A_{15cisBC}$ = the peak area of 15-*cis*-β-carotene in the sample chromatogram (AU)
$A_{13cisBC}$ = the peak area of 13-*cis*-β-carotene in the sample chromatogram (AU)
A_{9cisBC} = the peak area of 9-*cis*-β-carotene in the sample chromatogram (AU)

A_{XcisBC} = the peak area of unidentified *cis* isomers of β-carotene in the sample chromatogram (AU)
A_A = the peak area of apocarotenal in the sample chromatogram (AU)
RF_{BC} = the response factor for all-*trans*-β-carotene

$$BC_{Total} = BC_{trans} + BC_{cis}$$

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(i) Szpylka and DeVries (2005). *J. AOAC Int*. 88(5): 1279-1291.
(j) The United States Pharmacopeial Convention (2014). Revision Bulletin - Beta Carotene.
(k) Waysek et al. (2010). *J. AOAC Int*. 93(2): 499-509.

Method Validation for Determination of Lutein and β-Carotene in Infant Formula and Adult Nutritionals

For Review by the SPIFAN ERP

12 August 2016
Greg Hostetler, Senior Research Scientist
Perrigo Nutritionals

Summary

A UHPLC method for determination of lutein and β-carotene in infant formula and adult nutritionals was validated using the SPIFAN sample kit, including both unfortified and fortified samples. The data provided show that the method meets the requirements of the SMPR.

- Linearity
  Linearity of the calibration solutions correlates to approximately 0.8-45 µg/100 g as fed for samples prepared for the lowest sample concentrations. With dilutions specified in the method, the range can be extended to approximately 2250 µg/100 as fed. This extends beyond the range of 1-1300 µg/100 g specified in the SMPR. Linearity of the internal standard was also demonstrated.
- LOD/LOQ
  The LOD for lutein and β-carotene is 0.08 µg/100 g. The LOQ for both lutein and β-carotene (0.27) meet that of ≤1 µg/100 g specified in the SMPR.
- Specificity
  All experiments showed separation of all-*trans*-lutein and β-carotene from their major *cis* isomers, apocarotenal, α-carotene, lycopene, and zeaxanthin. Samples spiked with all-*trans*-lutein and β-carotene showed no isomerization during sample preparation.
- Precision
  For all measurements in the range of 1-100 µg/100 g, repeatability was ≤5.8% RSD for lutein and ≤5.1% RSD for β-carotene. For measurements >100 µg/100 g, repeatability was ≤1.1% RSD for lutein and ≤1.7% RSD for β-carotene. The method meets the precision requirements of the SMPR.
- Accuracy
  The SMPR calls for 90-110% recovery from spiked samples, and all data meets this requirement.

Linearity

Linearity of the relative responses of analyte concentrations was measured using a 5-point standard curve on three different days. Correlation coefficients, visual inspection, residuals, and relative errors of back-calculated concentrations were used for evaluation. Linearity of the internal standard was also demonstrated.

The ranges for lutein and β-carotene (4-240 μg/100 mL) correlate to approximately 0.8-45 μg/100 g as fed for samples prepared for the lowest sample concentrations. With dilutions specified in the method, the range can be extended to approximately 2250 μg/100 as fed. This extends beyond the range of 1-1300 μg/100 g specified in the SMPR.

Table 1. Residuals for the internal standard

Apocarotenal	
Conc (μg/100 mL)	Residual
203.5	4.119148067
101.8	-7.399430255
40.7	-3.177165248
20.4	1.435593088
4.07	2.222225757
2.04	2.79962859

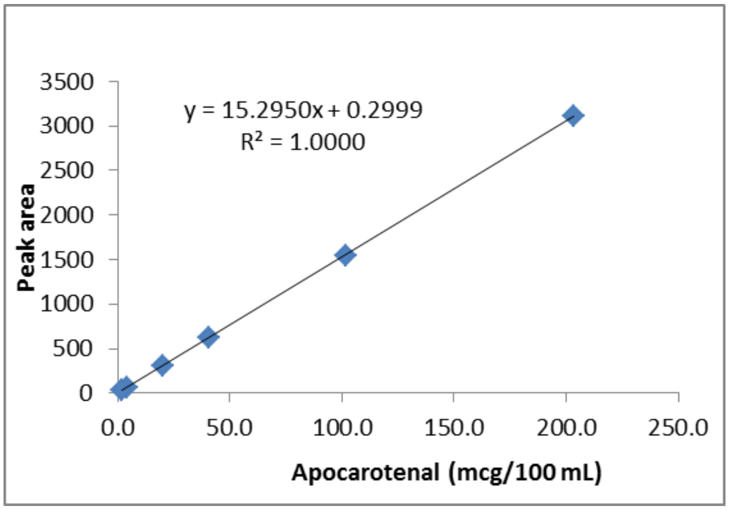

Figure 1. Linearity plot for apocarotenal.

Table 2. Residuals and error of back-calculated concentrations for all-*trans*-lutein standard curves.

	Experiment 1		Experiment 2		Experiment 3	
Conc (µg/100 mL)	Residual	Error	Residual	Error	Residual	Error
235.9	0.000368916	0.01%	-0.000497233	-0.02%	-6.16301E-05	0.00%
125.8	-0.003210486	0.24%	-0.00122698	0.09%	0.00221285	-0.16%
78.6	0.00379152	-0.44%	0.003308385	-0.38%	-0.004609309	0.54%
31.5	0.000817163	-0.24%	0.000644834	-0.18%	0.003230669	-0.93%
3.93	-0.001767113	3.78%	-0.002229005	4.65%	-0.00077258	1.65%

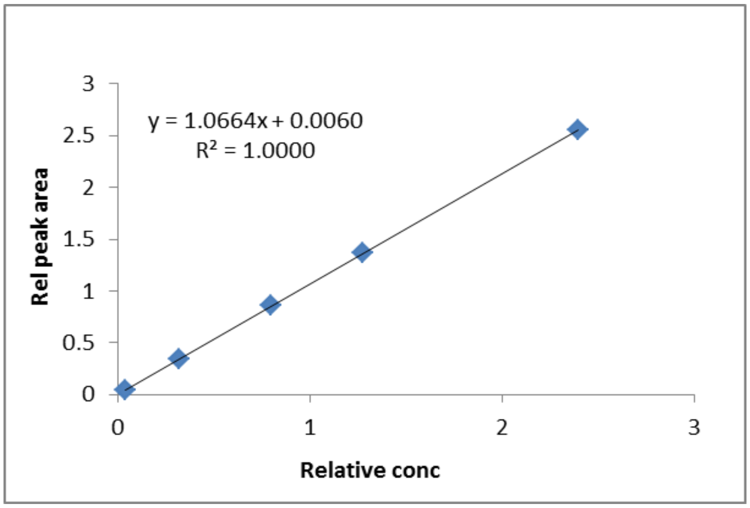

Figure 2. Linearity plot for lutein.

Table 3. Residuals and error of back-calculated concentrations for all-*trans*-β-carotene standard curves.

	Experiment 1		Experiment 2		Experiment 3	
Conc (µg/100 mL)	Residual	Error	Residual	Error	Residual	Error
227.4	0.00125077	0.05%	0.002290766	0.09%	-0.001591824	-0.06%
121.3	-0.002520458	-0.18%	-0.003680882	-0.27%	0.00318571	0.23%
75.8	-0.000366823	-0.04%	-0.00193471	-0.23%	-0.000338007	-0.04%
30.3	0.001615483	0.47%	0.002244522	0.65%	0.000226108	0.06%
3.79	2.10278E-05	0.04%	0.001080305	2.33%	-0.001481986	-2.97%

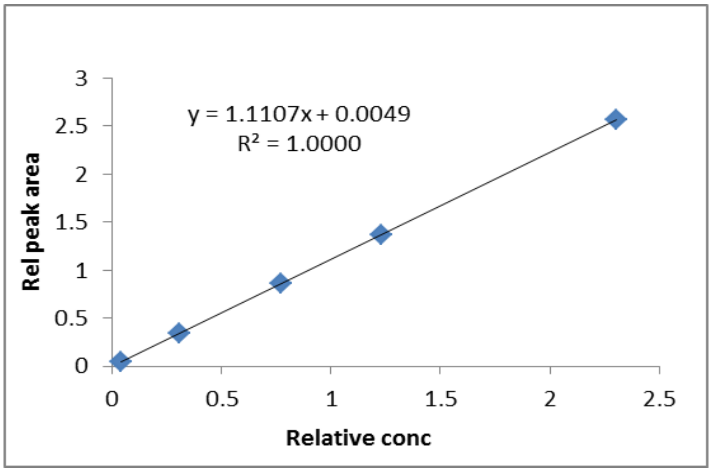

Figure 3. Linearity plot for β-carotene.

LOD/LOQ

The limit of detection (LOD) and limit of quantitation (LOQ) were extrapolated from the signal-noise ratio calculated in ChemStation software when measuring analyte concentrations of 1.4-1.7 mcg/100 g. LOD was calculated as (3 * measured conc)/signal-noise ratio. LOQ was calculated as (10 * measured conc)/signal-noise ratio. Results from three different spiked samples were averaged. The LOQ for both lutein and β-carotene meet that of ≤1 µg/100 g specified in the SMPR.

Table 4. LOD and LOQ for lutein in spiked SRM 1849a.

Sample	Measured conc (mcg/100 g as fed)	S/N	Extrapolated LOD (mcg/100 g)	Extrapolated LOQ (mcg/100 g)
1	1.40	68.6	0.06	0.20
2	1.46	70.4	0.06	0.21
3	1.43	37.0	0.12	0.39
		Avg	0.08	0.27

Table 5. LOD and LOQ for β-carotene in spiked SRM 1849a.

Sample	Measured conc (mcg/100 g as fed)	S/N	Extrapolated LOD (mcg/100 g)	Extrapolated LOQ (mcg/100 g)
1	1.62	61.6	0.08	0.26
2	1.70	61.2	0.08	0.28
3	1.66	61.0	0.08	0.27
		Avg	0.08	0.27

Specificity

The SMPR calls for the determination of all-*trans* and *cis* isomers of lutein and β-carotene, as well as separation of lutein from zeaxanthin. Specificity was evaluated with visual inspection of chromatograms and by measuring resolution of system suitability standard mixtures. Because apocarotenal is used as an internal standard, samples were prepared without internal standard to ensure there were no interfering peaks. Standard mixtures were isomerized with heat to identify major cis isomers.

Peak assignments were based on relative retention times from previous studies using C30 columns and methanol/MTBE as the mobile phase (Aman et al., 2005; Marx et al, 2000; Ping and Lian, 2006; Puspitasari-Nienaber, 2002). Samples spiked with all-*trans*-lutein and β-carotene showed no isomerization during sample preparation. All experiments showed separation of all-*trans*-lutein and β-carotene from their major *cis* isomers, apocarotenal, α-carotene, lycopene, and zeaxanthin.

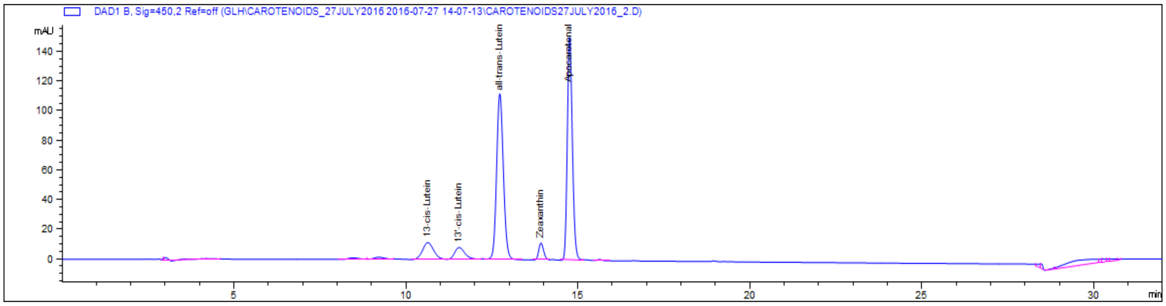

Figure 4. Lutein system suitability solution.

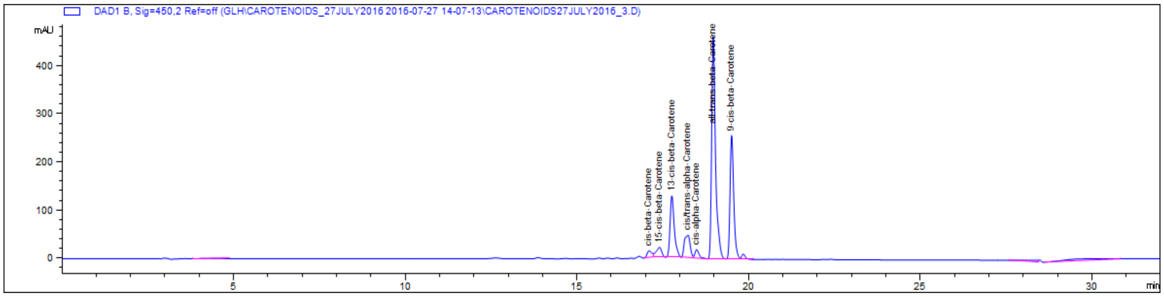

Figure 5. Beta-carotene system suitability solution.

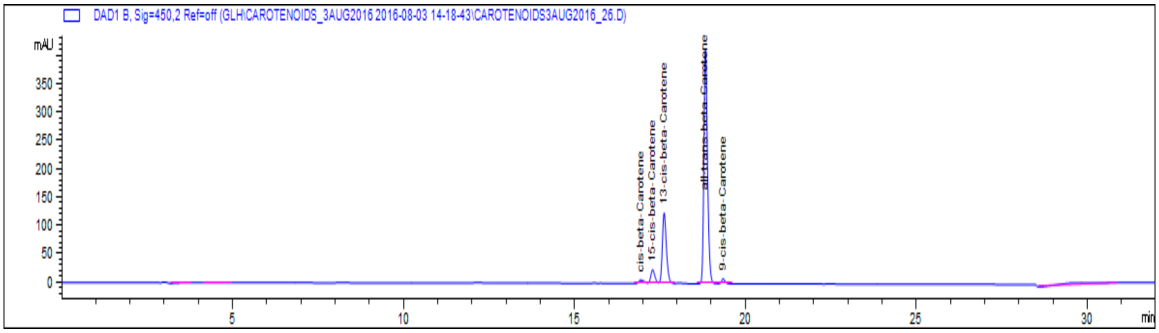

Figure 6. Heat-isomerized β-carotene solution.

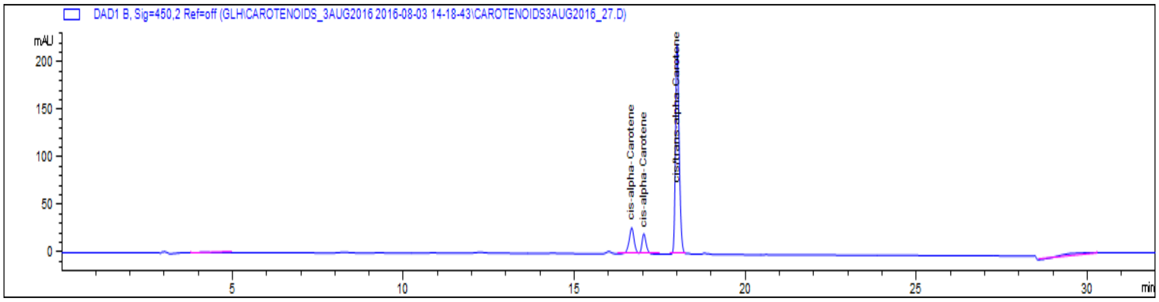

Figure 7. Heat-isomerized α-carotene solution.

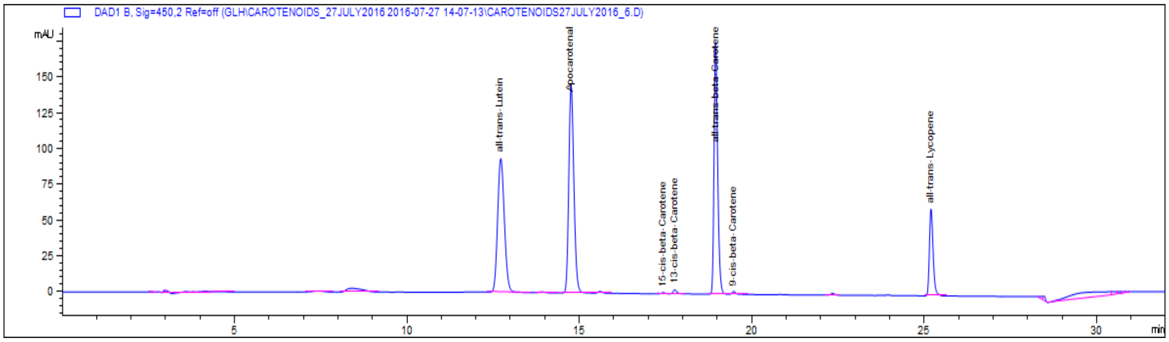

Figure 8. Calibration solution with addition of lycopene standard.

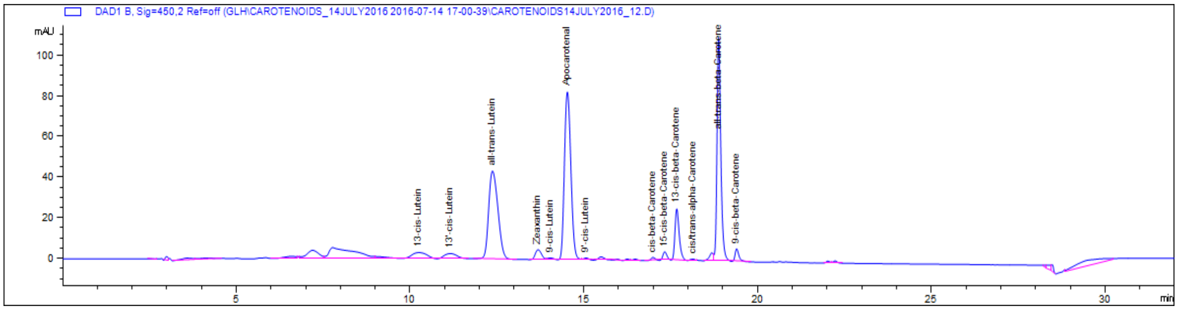

Figure 9. SPIFAN Milk-based infant formula.

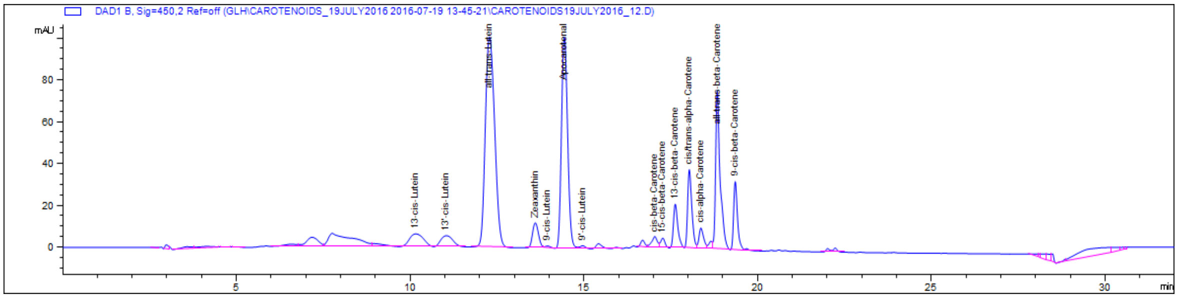

Figure 10. SPIFAN Toddler formula.

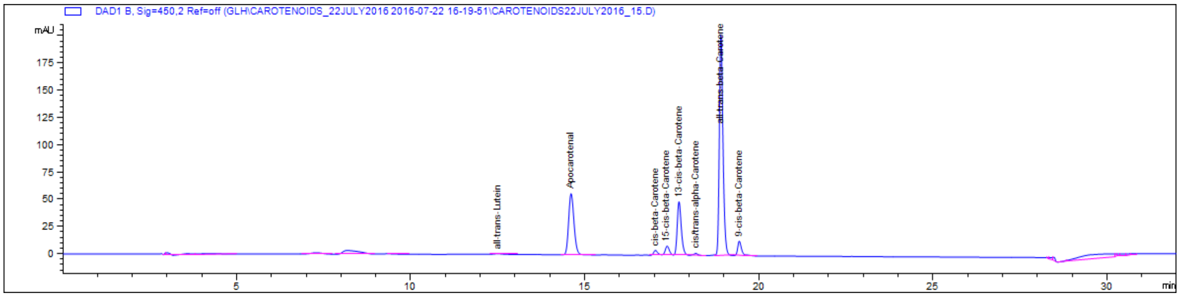

Figure 11. SPIFAN High protein adult nutritional RTF.

Precision

All samples were prepared in triplicate and the SPIFAN samples with substantial carotenoid concentrations were prepared a at least 12 times (3 replicates x 4 days). Intermediate precision (RSD_{iR}) was calculated as the relative standard deviation of all replicates. Repeatability (RSD_r) for triplicate samples was calculated as the relative standard deviation, and for larger samples sizes it was estimated by performing a one-way ANOVA on the intermediate precision data and calculating the standard deviation from the within-day variance. Although the SMPR does not specify criteria for intermediate precision, the requirements for repeatability and reproducibility are outlined in Table 6. For all measurements above 1 µg/100 g, the method meets the precision requirements of the SMPR.

Table 6. Precision requirements from SMPR 2014.014.

Sample conc (µg/100 g)	Repeatability	Reproducibility
1-100	8%	15%
>100-1300	5%	10%

Perrigo Nutritionals

Table 7. Precision data for lutein in SPIFAN matrices and select PN matrices (µg/100 g). For powder samples, results were calculated using a factor of 25 g powder/225 g reconstituted weight.

Formula	Code	n	cis-Lutein	RSDr	RSDiR	trans-Lutein	RSDr	RSDiR	Total Lutein	RSDr	RSDiR
Child Formula Placebo	00847RF00	3	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
Infant Elemental Placebo	00796RF	3	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
AND RTF High Protein Placebo	00821RF00	3	<	n/a	n/a	3.3	3.8%	n/a	3.3	3.8%	n/a
AND RTF High Fat Placebo	00820RF00	3	2.0	3.8	n/a	4.2	4.6%	n/a	6.2	4.1%	n/a
IF RTF Milk Based Placebo	EV4H2Q	3	<	n/a	n/a	0.5	5.7%	n/a	0.6	4.8%	n/a
SRM 1849a	CLC10-B	3	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
IF Partially Hydrolyzed Milk Based	410057652Z	3	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
IF Partially Hydrolyzed Soy Based	410057651Z	3	<	n/a	n/a	<	n/a	n/a	0.3	11.5%	n/a
Toddler Formula Milk Based	4052755861	12	3.7	1.2%	2.2%	21.9	3.6%	3.8%	25.5	3.1%	3.4%
IF Milk Based	4044755861	12	2.1	3.6%	3.7%	9.4	5.0%	5.9%	11.5	4.1%	5.1%
AND Powder	00859RF00	3	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
Child Formula Powder	00866RF00	3	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
IF Elemental	00795RF	12	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
IF w FOS/GOS	50350017W1	12	0.3	12.3%	14.3%	0.9	2.9%	5.2%	1.2	3.4%	4.2%
IF Milk Based (Control)	K16NTAV	39	1.6	3.3%	5.5%	4.5	1.2%	1.6%	6.1	1.6%	2.3%
IF Soy Based	E10NWZC	12	0.6	5.7%	8.0%	1.6	3.2%	3.4%	2.2	2.8%	3.1%
IF RTF Milk Based	EV4H2R	3	<	n/a	n/a	0.5	6.2%	n/a	0.7	16.9%	n/a
AND RTF High Protein	00730RF00	12	1.9	4.1%	4.4%	3.1	2.4%	3.0%	4.9	2.9%	3.4%
AND RTF High Fat	00729RF00	3	1.7	5.8%	n/a	3.5	3.3%	n/a	5.2	3.2%	n/a
PN AND High Lutein Powder		3	21.4	0.3%	n/a	82.1	0.1%	n/a	104	0.2%	n/a
PN Chocolate PND RTF		3	13.0	0.4%	n/a	127	1.1%	n/a	140	1.0%	n/a
PN Infant Formula RTF		3	1.5	3.8%	n/a	3.9	1.4%	n/a	5.5	2.0%	n/a

< Below LOQ

Table 8. Precision data for β-carotene in SPIFAN matrices and select PN matrices (μg/100 g). For powder samples, results were calculated using a factor of 25 g powder/225 g reconstituted weight.

Formula	Code		cis-BC	RSDr	RSDiR	trans-BC	RSDr	RSDiR	Total BC	RSDr	RSDiR
Child Formula Placebo	00847RF00	3	0.3	0.7%	n/a	0.6	2.6%	n/a	0.9	1.8%	n/a
Infant Elemental Placebo	00796RF	3	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
AND RTF High Protein Placebo	00821RF00	3	<	n/a	n/a	0.8	11.2%	n/a	0.8	11.2%	n/a
AND RTF High Fat Placebo	00820RF00	3	<	n/a	n/a	0.4	47.4%	n/a	0.6	48.5%	n/a
IF RTF Milk Based Placebo	EV4H2Q	3	0.5	3.3%	n/a	0.4	2.2%	n/a	0.9	2.6%	n/a
SRM 1849a	CLC10-B	3	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
IF Partially Hydrolyzed Milk Based	410057652Z	3	0.8	0.2%	n/a	1.0	2.0%	n/a	1.8	1.2%	n/a
IF Partially Hydrolyzed Soy Based	410057651Z	3	0.6	1.8%	n/a	0.6	6.7%	n/a	1.3	3.9%	n/a
Toddler Formula Milk Based	4052755861	12	6.5	1.0%	2.4%	7.4	0.7%	2.9%	13.9	0.8%	2.2%
IF Milk Based	4044755861	12	4.4	2.1%	2.7%	10.1	1.3%	2.7%	14.4	1.5%	2.4%
AND Powder	00859RF00	3	<	n/a	n/a	0.3	5.9%	n/a	0.3	5.9%	n/a
Child Formula Powder	00866RF00	3	0.3	15.3%	n/a	0.4	2.5%	n/a	0.7	5.4%	n/a
IF Elemental	00795RF	12	7.3	0.3%	0.7%	15.3	0.7%	0.7%	22.7	0.5%	0.6%
IF w FOS/GOS	50350017W1	12	1.3	5.1%	6.3%	3.4	3.0%	4.6%	4.8	2.3%	3.8%
IF Milk Based (Control)	K16NTAV	39	6.0	1.1%	2.7%	13.2	0.5%	2.2%	19.2	0.6%	1.7%
IF Soy Based	E10NWZC	12	6.5	2.6%	2.3%	11.0	2.0%	1.8%	17.5	2.2%	1.9%
IF RTF Milk Based	EV4H2R	3	0.6	6.9%	n/a	0.4	4.8%	n/a	1.0	4.6%	n/a
AND RTF High Protein	00730RF00	12	265	1.6%	2.7%	534	1.7%	2.3%	799	1.6%	2.4%
AND RTF High Fat	00729RF00	3	<	n/a	n/a	0.3	7.7%	n/a	0.4	5.9%	n/a
PN AND High Lutein		3	<	n/a	n/a	0.5	18.3%	n/a	0.5	18.3%	n/a
PN Chocolate PND RTF		3	<	n/a	n/a	<	n/a	n/a	<	n/a	n/a
PN Infant Formula RTF		3	9.1	0.3%	n/a	16.4	0.4%	n/a	25.5	0.4%	n/a

< Below LOQ

Accuracy

There is no certified reference material for carotenoids in infant formula and adult nutritionals. Accuracy was assessed by spike and recovery of all-*trans* lutein and β-carotene is various matrices. Spiked and unspiked samples were measured in triplicate. The SMPR calls for 90-110% recovery from spiked samples, and all data meets this requirement.

Table 9. Spike and recovery data for SRM 1849a. Samples were spiked with standards at the internal standard step of sample preparation.

	Native level (μg/100 g)	Spike (μg/100 g)	Recovery	RSD	Spike (μg/100 g)	Recovery	RSD
Lutein	ND	1.4	105.5%	0.8%	13.8	99.6%	0.2%
β-Carotene	0.2	1.3	107.5%	0.9%	13.6	103.2%	0.3%

ND Not detected

Table 10. Spike and recovery data for SRM 1849a. Samples were spiked with FloraGlo 5% lutein at the powder reconstitution step of sample preparation.

	Native level (μg/100 g)	Spike (μg/100 g)	Recovery	RSD	Spike (μg/100 g)	Recovery	RSD	Spike (μg/100 g)	Recovery	RSD
Lutein	ND	34.8	92.3%	0.8%	68.3	94.0%	1.9%	135.3	97.6%	1.6%

ND Not detected

Table 11. Spike and recovery data for AND High Protein Placebo. Samples were spiked with FloraGlo 5% lutein at the initial sample dilution step.

	Native level (μg/100 g)	Spike (μg/100 g)	Recovery	RSD	Spike (μg/100 g)	Recovery	RSD	Spike (μg/100 g)	Recovery	RSD
Lutein	3.3	62.7	96.7%	0.8%	148.8	100.1%	0.3%	297.6	101.0%	0.3%

Table 12. Spike and recovery data for AND High Protein Placebo. Samples were spiked with FloraGlo 5% lutein at the initial sample dilution step.

	Native level (µg/100 g)	Spike (µg/100 g)	Recovery	RSD	Spike (µg/100 g)	Recovery	RSD	Spike (µg/100 g)	Recovery	RSD
Lutein	3.3	486.4	101.1%	0.5%	708.8	101.2%	0.5%	1425.3	100.7%	0.2%

Table 13. Spike and recovery data for Child Formula Placebo. Samples were spiked with standards at the internal standard step of sample preparation.

	Native level (µg/100 g)	Spike (µg/100 g)	Recovery	RSD	Spike (µg/100 g)	Recovery	RSD
Lutein	0.1	5.5	101.8%	0.2%	16.8	100.8%	0.3%
β-Carotene	0.6	5.3	107.2%	0.2%	16.2	104.2%	0.3%

Table 14. Spike and recovery data for Elemental Formula Placebo. Samples were spiked with standards at the internal standard step of sample preparation.

	Native level (µg/100 g)	Spike (µg/100 g)	Recovery	RSD	Spike (µg/100 g)	Recovery	RSD
Lutein	ND	5.5	103.7%	0.2%	16.8	101.9%	0.6%
β-Carotene	0.1	5.3	104.1%	0.2%	16.2	103.5%	0.6%

ND Not detected

Table 15. Spike and recovery data for PN Infant Formula RTF. Samples were spiked with standards at the internal standard step of sample preparation.

	Native level (µg/100 g)	Spike (µg/100 g)	Recovery	RSD	Spike (µg/100 g)	Recovery	RSD
Lutein	3.9	6.2	98.7%	0.4%	18.4	98.6%	0.1%
β-Carotene	16.4	5.9	100.6%	0.9%	17.7	100.1%	0.6%

Acknowledgments

Shang-Jing Pan and Douglas Gordon of Abbott Nutrition for reviewing the method and providing feedback.
Steve Tennyson and Scott Christiansen of Perrigo Nutritionals for reviewing the method and validation data.

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Evaluation of Method Performance vs. SMPR requirements.

AOAC SMPR: 2014.014

Method Reference #	**Carot-02**
Method title:	**Determination of Lutein and β-Carotene in Infant Formula and Adult Nutritionals**
Principle of the method:	Powder samples are reconstituted in water, and liquid sample is first spiked with an internal standard and saponified with potassium hydroxide.

Parameter	**SMPR Requirement**		**Method Performance** **Please report in units as stated in SMPR!**	**Weighting factor for parameter**	**Suitability Ranking (1-3-5)** (select from drop-down list, 5 = best)
SPIFAN matrices		Applicable to all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates). Any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.	**All SPIFAN matrices used, as well as in-house infant formula and adult nutritional and pediatric formulas**	**1**	
All analytes defined in the applicability statement are measured.		all-trans-lutein, cis isomers of lutein, all-trans-beta-carotene, cis isomers of beta-carotene	**All forms of lutein and beta-carotene are measured.**	**1**	
Analytical Range.	Lutein β-carotene	1-1300 mcg/100 g as fed 1-1300 mcg/100 g as fed	0.8-2250 mcg/100 g as fed 0.8-2250 mcg/100 g as fed	**1**	
Limit of detection (LOD)	Lutein	n/a	0.08 mcg/100 g	**1**	
	β-carotene	n/a	0.08 mcg/100 g		
Limit of quantification (LOQ)	Lutein	1 mcg/100 g	0.27 mcg/100 g	**2**	
	β-carotene	1 mcg/100 g	0.27 mcg/100 g		
Accuracy/Recovery	Spike recovery (%)	90-110%	92-108%	**2**	
	Bias vs SRM	n/a	n/a	**3**	
Repeatability (RSDr)	1-100 mcg/100 g	8%	≤5.8%	**3**	
	100-1300 mcg/100 g	5%	≤1.7%		
Intermediate Reproducibility (RSDiR)	1-100 mcg/100 g	n/a	≤6.3%	**1**	
	100-1300 mcg/100 g	n/a	≤2.7%		
Reproducibility (RSDR)				**1**	

ADDITIONAL EVALUATION PARAMETERS

Adequate proof of performance via system suitability	**System suitability solutions are used in the method, and chromatograms show good separation of lutein and beta-carotene isomers as well as resolution from zeaxanthin, alpha-carotene, and lycopene.**		
Feedback from Users of the Method since being awarded First Action *Official Methods* Status			
Did Method Author Consider ERP's Method Specific Recommendations (See web link to specific method comments):	**carotene and lycopene be included in the method. While alpha-carotene is separated with this method, it was not included in the determination because only one SPIFAN matrix contained the analyte. Lycopene is also separated, but is excluded due to poor standard injection precision and poor standard stability.**		
Bias against established method	**No established method.**		
Analytical equipment	**Analytical equipment (UV/Vis spec, UHPLC, horizontal shaker) is commonly available in most labs.**		
Proprietary equipment	**No unique proprietary equipment/accessories are required.**		
Laboratory safety	**Method does not require any special safety precautions e.g. personal protection from highly toxic solvents. Halogenated solvents are not used.**		
Other Considerations			

Overall Score		0
Decision by ERP		
Recommendation of ERP 2 years after First Action Status	move to Final Action/repeal/remove/expand 2 year term	

Notes:

[a] *Concentrations apply to (1) "ready to feed liquids" "as-is"; (2) reconstituted powders (25 g into 200 g water); and (3) liquid concentrates diluted 1:1 by weight.*

[b] *Units*

Table 1 Accuracy - Spike Recovery

Product	SPIFAN Code	Analyte	Nr. replicates (n)	Spike Recovery				
				Native level	+ Spike Level 1		+ Spike Level 2	
					Avg (%)	RSD %	Avg (%)	RSD (%)
SRM 1849a	CLC10-B	all-trans-Lutein	3	0	105.5	0.8	99.6	0.2
SRM 1849a	CLC10-B	all-trans-beta-Carotene	3	0.2	107.5	0.9	103.2	0.3
SRM 1849a	CLC10-B	FloraGlo 5% Lutein	3	0	92.3	0.8	94.0	1.9
SRM 1849a	CLC10-B	FloraGlo 5% Lutein	3	0			97.6	1.6
AND RTF High Protein Placebo	00821RF00	FloraGlo 5% Lutein	3	3.3	96.7	0.8	100.1	0.3
AND RTF High Protein Placebo	00821RF00	FloraGlo 5% Lutein	3	3.3	101.0	0.3	101.1	0.5
AND RTF High Protein Placebo	00821RF00	FloraGlo 5% Lutein	3	3.3	101.2	0.5	100.7	0.2
Child Formula Placebo	00847RF00	all-trans-Lutein	3	0.1	101.8	0.2	100.8	0.3
Child Formula Placebo	00847RF00	all-trans-beta-Carotene	3	0.6	107.2	0.2	104.2	0.3
Infant Elemental Placebo	00796RF	all-trans-Lutein	3	0	103.7	0.2	101.9	0.6
Infant Elemental Placebo	00796RF	all-trans-beta-Carotene	3	0.1	104.1	0.2	103.5	0.6
PN Milk Based IF RTF		all-trans-Lutein	3	3.9	98.7	0.4	98.6	0.1
PN Milk Based IF RTF		all-trans-beta-Carotene	3	16.4	100.6	0.9	100.1	0.6
				Global Mean %	101.7	0.5	100.4	0.6

Table 2 Accuracy - Analytical bias compared to reference material

Certified Reference Material					**Measured Results**					
CRM product	Analyte	Certified value	Uncertainty	Certified range	Concentration	Difference from certified value	Bias			Confidence interval
							Nominal	T_{stat}	*p* -value	
				Mean						
				SD						

$$T_{stat} = \frac{\overline{d}}{sd \ / \sqrt{n}}$$

where: $\overline{d}$ = mean of the differences between measured results and reference valu
sd = standard deviation of the differences between results and reference valu
n = number of replicate analyses of SRM

H_0: $\overline{d} = 0$, The null hypothesis is that there is no difference between the measured results and the certified value:

p -value: is the probability (at the 95% levels of confidence) of obtaining the test statistic assuming that the null hypothesis is tru
p -value < 0.05 indicate a difference of statistical signifance between measured results and SRM

95% confidence interval: The true population of the measured value expected to fall within the confidence interval estimate 95% of the tim

lower limit = mean - $t_{crit} \frac{sd}{\sqrt{n}}$ upper limit = mean + $t_{crit} \frac{sd}{\sqrt{n}}$

where: t_{crit}= critical values of the t-distributio
sd = standard deviation of the differences between results an reference valu
n = number of replicate analyses of SRM

Table 3.1 Precision - repeatability.

Product	SPIFAN Code	Analyte	Nr. replicates (n)	Conc. (mcg/100 g)	RSDr	RSD iR
Child Formula Placebo	00847RF00	Total Lutein	3	<	n/a	n/a
Infant Elemental Placebo	00796RF	Total Lutein	3	<	n/a	n/a
AND RTF High Protein Placebo	00821RF00	Total Lutein	3	3.3	3.8%	n/a
AND RTF High Fat Placebo	00820RF00	Total Lutein	3	6.2	4.1%	n/a
IF RTF Milk Based Placebo	EV4H2Q	Total Lutein	3	0.6	4.8%	n/a
SRM 1849a	CLC10-B	Total Lutein	3	<	n/a	n/a
IF Partially Hydrolyzed Milk Based	410057652Z	Total Lutein	3	<	n/a	n/a
IF Partially Hydrolyzed Soy Based	410057651Z	Total Lutein	3	0.3	11.5%	n/a
Toddler Formula Milk Based	4052755861	Total Lutein	12	25.5	3.1%	3.4%
IF Milk Based	4044755861	Total Lutein	12	11.5	4.1%	5.1%
AND Powder	00859RF00	Total Lutein	3	<	n/a	n/a
Child Formula Powder	00866RF00	Total Lutein	3	<	n/a	n/a
IF Elemental	00795RF	Total Lutein	12	<	n/a	n/a
IF w FOS/GOS	50350017W1	Total Lutein	12	1.2	3.4%	4.2%
IF Milk Based	K16NTAV	Total Lutein	39	6.1	1.6%	2.3%
IF Soy Based	E10NWZC	Total Lutein	12	2.2	2.8%	3.1%
IF RTF Milk Based	EV4H2R	Total Lutein	3	0.7	16.9%	n/a
AND RTF High Protein	00730RF00	Total Lutein	12	4.9	2.9%	3.4%
AND RTF High Fat	00729RF00	Total Lutein	3	5.2	3.2%	n/a
PN AND High Lutein		Total Lutein	3	104	0.2%	n/a
PN Chocolate PND RTF		Total Lutein	3	140	1.0%	n/a
PN Infant Formula RTF		Total Lutein	3	5.5	2.0%	n/a
				Global Mean %	4.4%	3.6%

REQUIREMENTS SMPR	Conc. Level	RSDr	RSD iR
	X	≤ Z %	≤ W %
Total Lutein	1-100	8%	
Total Lutein	100-1300	5%	

Table 3.2

Precision - repeatability.

Product	SPIFAN Code	Analyte	Nr. replicates (n)	Conc. (mcg/100 g)	RSDr	RSD iR
Child Formula Placebo	00847RF00	Total Beta-Carotene	3	0.9	1.8%	n/a
Infant Elemental Placebo	00796RF	Total Beta-Carotene	3	<	n/a	n/a
AND RTF High Protein Placebo	00821RF00	Total Beta-Carotene	3	0.8	11.2%	n/a
AND RTF High Fat Placebo	00820RF00	Total Beta-Carotene	3	0.6	48.5%	n/a
IF RTF Milk Based Placebo	EV4H2Q	Total Beta-Carotene	3	0.9	2.6%	n/a
SRM 1849a	CLC10-B	Total Beta-Carotene	3	<	n/a	n/a
IF Partially Hydrolyzed Milk Based	410057652Z	Total Beta-Carotene	3	1.8	1.2%	n/a
IF Partially Hydrolyzed Soy Based	410057651Z	Total Beta-Carotene	3	1.3	3.9%	n/a
Toddler Formula Milk Based	4052755861	Total Beta-Carotene	12	13.9	0.8%	2.2%
IF Milk Based	4044755861	Total Beta-Carotene	12	14.4	1.5%	2.4%
AND Powder	00859RF00	Total Beta-Carotene	3	0.3	5.9%	n/a
Child Formula Powder	00866RF00	Total Beta-Carotene	3	0.7	5.4%	n/a
IF Elemental	00795RF	Total Beta-Carotene	12	22.7	0.5%	0.6%
IF w FOS/GOS	50350017W1	Total Beta-Carotene	12	4.8	2.3%	3.8%
IF Milk Based	K16NTAV	Total Beta-Carotene	39	19.2	0.6%	1.7%
IF Soy Based	E10NWZC	Total Beta-Carotene	12	17.5	2.2%	1.9%
IF RTF Milk Based	EV4H2R	Total Beta-Carotene	3	1.0	4.6%	n/a
AND RTF High Protein	00730RF00	Total Beta-Carotene	12	799	1.6%	2.4%
AND RTF High Fat	00729RF00	Total Beta-Carotene	3	0.4	5.9%	n/a
PN AND High Lutein		Total Beta-Carotene	3	0.5	18.3%	n/a
PN Chocolate PND RTF		Total Beta-Carotene	3	<	n/a	n/a
PN Infant Formula RTF		Total Beta-Carotene	3	25.5	0.4%	n/a
				Global Mean %	6.3%	2.1%

REQUIREMENTS SMPR	Conc. Level	RSDr	RSD iR
	X	≤ Z %	≤ W %
Total Beta-Carotene	1-100	8%	
Total Beta-Carotene	100-1300	5%	

Table 4

Precision - reproducibility

Product	SPIFAN Code	Analyte	Nr. replicates (n)	Conc. Level	RSD_R
				Global Mean %	#DIV/0!

REQUIREMENTS SMPR	Conc. Level	RSD_R
	X	≤ Z %
Analyte 1		
Analyte 2		
Analyte 3		
Analyte 4		
Analyte 5		
Analyte 1		
Analyte 2		
Analyte 3		
Analyte 4		
Analyte 5		
Analyte 1		
Analyte 2		
Analyte 3		
Analyte 4		
Analyte 5		
Analyte 1		
Analyte 2		
Analyte 3		
Analyte 4		
Analyte 5		

AOAC SMPR 2014.002

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

Intended Use: Reference Method for Dispute Resolution

1 Applicability

Determination of fructans in all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates).

2 Analytical Technique

Any analytical technique that meets the following method performance requirements is acceptable.

3 Definitions

Adult/pediatric formula.—Nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment [AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN); 2010], made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.

Fructans.—Fructans including oligofructose, fructooligosaccharides, and inulin. General formulae are shown in Figure 1.

Infant formula.—Breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding (Codex Standard 72-1981) made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.

Limit of detection (LOD).—The minimum concentration or mass of analyte that can be detected in a given matrix with no greater than 5% false-positive risk and 5% false-negative risk.

GF$_n$ Type: (ß-D-Fru*f*-(2→1))$_n$-ß-D-Fru*f*-(2↔1)-α-D-Glc*p*

F$_m$ Type: (ß-D-Fru*f*-(2→1))$_n$-(2 →1)-D-Fru*f*

where n≥1

Figure 1. General formulae for the two major inulin-type fructans. GF$_n$ type do not have a reducing end and contain a terminal glucose, F$_m$ type have a reducing end and do not have a terminal glucose. Fru*f* = fructofuranose; Glc*p* = glucopyranose.

Table 1. Method performance requirements[a]

Analytical range	0.03–5.0[b]
Limit of quantitation (LOQ)	≤0.03[b]
Repeatability (RSD$_r$)	≤6%
Recovery	90 to 110% of mean spiked recovery over the range of the assay
Reproducibility (RSD$_R$)	≤12%

[a] Concentrations apply to (a) "ready-to-feed" liquids "as is"; (b) reconstituted powders (25 g into 200 g of water); and (c) liquid concentrates diluted 1:1 by weight.

[b] g/100 g reconstituted final product.

Limit of quantitation (LOQ).—The minimum concentration or mass of analyte in a given matrix that can be reported as a quantitative result.

Repeatability.—Variation arising when all efforts are made to keep conditions constant by using the same instrument and operator, and repeating during a short time period. Expressed as the repeatability standard deviation (SD$_r$); or % repeatability relative standard deviation (%RSD$_r$).

Reproducibility.—The standard deviation or relative standard deviation calculated from among-laboratory data. Expressed as the reproducibility relative standard deviation (SD$_R$); or % reproducibility relative standard deviation (%RSD$_R$).

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

4 Method Performance Requirements

See Table 1.

5 System Suitability Tests and/or Analytical Quality Control

Suitable methods will include blank check samples, and check standards at the lowest point and midrange point of the analytical range.

6 Reference Material(s)

No National Institute of Standards and Technology (NIST) Standard Reference Material® (SRM) 1849a Infant/Adult Nutritional Formula or equivalent is available.

7 Validation Guidance

Recommended level of validation: *Official Methods of Analysis*^{SM}.

8 Maximum Time-to-Result

No maximum time.

Approved by AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). Final Version Date: March 18, 2014.

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To: Delia Boyd (dboyd@aoac.org)

YOUR REF.
OUR REF. NRC/AS sau/cit
Sean Austin
LAUSANNE, July 25, 2016

Dear Delia

On behalf of Eurofins Carbohydrate Competence Centre (The Netherlands) and Nestlé Research Centre (Switzerland), we are pleased to send you herewith a copy of our SLV report entitled:

Dual Lab Validation of a Method for the Determination of Fructans in Infant Formula & Adult Nutritionals

By Sean Austin, Peter Sanders, Véronique Spichtig, Veronica Ernste-Nota, Kimberley Iwanoff and Kommer Brunt

This document is confidential and should not be copied or disclosed to others than to the Expert Review Panel without proper authorization.

We will be happy to provide you with any further information you may require.

Yours sincerely

Sean Austin

NESTEC LTD. 1800 VEVEY

R&D Report

Dual Lab Validation of a Method for the Determination of Fructans in Infant Formula & Adult Nutritionals

Sean Austin[1], Peter Sanders[2], Véronique Spichtig[1], Veronica Ernste-Nota[2], Kimberley Iwanoff[3], Paulina Zyskowska[1,4,5], Jeroen Van Soest[2], Paul Kong Thoo Lin[4], Kommer Brunt[6]

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Date of issue	25 Jul 2016
Project	DNUT-100472 / SPIFAN

SUMMARY

A method has been co-developed at Nestlé Research Centre (NRC), Lausanne Switzerland and Eurofins Carbohydrate Competence Centre (CCC), Heerenveen, The Netherlands. For analysis, samples are reconstituted in water and further diluted to a suitable concentration. The diluted sample is treated with a mixture of sucrase and α-glucanases to hydrolyse sucrose and alpha-glucans to their constituent monosaccharides. The sample is passed through a solid phase extraction cartridge packed with graphitized carbon. Salts and monosaccharides pass through and are washed away, while the fructans are retained. Fructans are released from the column using an acetonitrile solution. The released fructans are hydrolysed with an inulinase mixture, and the released glucose and fructose are analysed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The fructan content is calculated by summing the glucose and 0.9 × fructose content. The main advantages of the method over existing methods are (1) sucrose and monosaccharides are eliminated, avoiding their interference in the analysis, (2) all components of the fructan are measured, thus there is no need for ingredient-specific correction factors, (3) the method is sufficiently sensitive to detect low amounts of fructan (0.03 g/100g in reconstituted products).

Some minor differences in the application of the method exist in the two labs:-

(1) Just prior to SPE, the CCC applied a Carrez clean-up step. This was not performed at NRC
(2) During SPE, CCC used 1 × 400 µL then 2 × 800 µL of NaCl (1M) while NRC used 2 × 1000 µL of NaCl (1M)
(3) For SPE elution CCC used 5 × 400 µL elute solution, NRC used 3 × 400 µL elute solution
(4) Chromatographic conditions are different since CCC employ a Carbopac PA1 column (2 × 250 mm) and NRC employ a Carbopac PA20 (3 × 150 mm).

The method has been validated independently in both laboratories using the SPIFAN material test kit. Of the 19 matrices in the SPIFAN kit, 6 were found to contain fructans.

At NRC the 6 fructan-containing SPIFAN samples and 2 Nestlé materials were analysed on 6 different days in duplicate to determine the precision of the method. Of the 13 fructan-free samples, 6 were selected for use in spike/recovery experiments. The 6 matrices were spiked with 3 different fructan ingredients at three different levels. CCC followed the same SLV procedure using the 6 fructan-containing SPIFAN samples.

The results of the two SLVs are summarized in the table below. In most cases the data are better than required by the SMPR. However, out of the 18 experiments carried out at NRC for the spike-recovery experiment, 3 resulted in recoveries slightly outside the SMPR guidelines. The results outside the SMPR targets were at the extremes of the working range (i.e. at 0.03 g/100g and at 5 g/100g) and the results on the other matrices tested at the same levels were within the target range of the SMPR. Likewise at Eurofins, out of the 12 spike-recovery experiments, only one result was outside of the SMPR at 89%. The method precision was well within the limits for all samples at NRC,

and for all but one sample at CCC. The single sample with poorer precision characteristics at CCC was the sample with the lowest fructan content (0.036 g/100g) which is close to the method LoQ.

The measured fructan content of the 6 fructan-contining SPIFAN matrices is in good agreement between the two laboratories and also with the results obtained by the current first action method for fructans.

The good agreement of results between the two laboratories also indicates that the method is sufficiently robust to resist the minor changes in protocols between the two labs.

Parameter	SMPR	NRC SLV Results	CCC SLV Results	Meets SMPR? NRC / CCC
Analytical Range	0.03 – 5.0 g/100 g	0.03 – 5.0 g/100 g	0.03 – 5.0 g/100 g	Y / Y
LoQ	≤ 0.03 g/100 g	0.018 g/100 g	0.016 – 0.030	Y / Y
RSD$_{(r)}$	≤ 6 %	0.78 – 3.2 %	1.1 – 8.9	Y / N[a]
RSD$_{(iR)}$	No target	1.1 – 4.2 %	4.6 – 14 %[b]	n/a
RSD$_{(R)}$	≤ 12 %	n/a	n/a	Cannot test in SLV
Recovery	90 – 110 % of mean spiked recovery over the range of the assay	86 – 119 %	89 - 102	N[c] / N[d]

[a] RSD$_{(r)}$ for all samples except 1 was between 1.1 – 2.6 %, high RSD of 8.9 % at the lowest level (0.036 g/100g) only.

[b] RSD$_{(iR)}$ was between 4.6 – 7.4 % in all samples except at the lowest concentration (0.036 g/100g) where it reached 14%.

[c] For 2 out of 6 matrices spiked at 0.03 g/100g recoveries were above 110% (114% and 119%). For 1 matrix out of 6 spiked at 5g/100g recovery was below 90% (86%). Excluding those 3 results, recoveries were in the range 92 – 105%.

[d] Spike-recoveries were between 91 – 102% for all samples except 1, which had a spike recovery of 89%.

Full SLV Report from Nestle Research Centre

Véronique Spichtig[1], Kimberley Iwanoff[2], Sean Austin[1].

1. Nestlé Research Centre, Vers-Chez-Les-Blanc, Lausanne, Switzerland
2. Nestlé Quality Assurance Centre, York, UK.

TABLE OF CONTENTS

1 INTRODUCTION

After opening a call for methods to determine fructans in infant formula and adult nutritionals, the AOAC received 4 method proposals. So far two have been considered by the expert review panel (ERP), one has been rejected and the other has been accepted as first action.

The method currently having first action status requires that a pre-analysis is performed on the sample to assess the fructan type used in the product. This information is necessary due to the use of fructan-dependant correction factors. The method described in this report is designed to determine the fructan content without the need for correction factors thus avoiding the need for pre-analysis (or knowledge of the fructan type in the product).

The method has been developed in partnership between Nestlé Research Centre (Lausanne, Switzerland) and the Carbohydrate Competence Centre of Eurofins (Heerenveen, The Netherlands). Single lab validations have been performed in both centres, this report covers the SLV performed at Nestlé Research Centre.

2.1. Samples

A SPIFAN single laboratory validation material test kit was used. The kit contains representative products covering the scope of the project as well as a standard reference material (SRM 1849a). The kit contains 5 products described as placebo products and 14 products described as fortified products. Five of the fortified products were found to contain FOS. It was also found that one of the placebo products contained FOS. In addition to the SPIFAN matrices, two Nestlé reference samples were analysed. The Nestlé samples were analysed without prior reconstitution.

Table 1: Samples Used In This Study

Product Description	Lot No	NRC Reference
Placebo Products		
1. Child Formula Powder	00847RF00	KBI-00020
2. Infant Elemental Powder	00796RF	KBI-00028
3. Adult Nutritional RTF, High Protein	00821RF00	KBI-00029
4. Adult Nutritional RTF, High Fat	00820RF00	KBI-00030
5. Infant Formula RTF, Milk Based	EV4H2Q	KBI-00031
Fortified Products		
1. SRM 1849a	CLC10-b	KBI-00032
2. Infant Formula Powder Partially Hydrolyzed Milk Based	410057652Z	KBI-00033
3. Infant Formula Powder Partially Hydrolyzed Soy Based	410457651Z	KBI-00034
4. Toddler Formula Powder Milk Based	4052755861	KBI-00035
5. Infant Formula Powder Milk Based	4044755861	KBI-00044
6. Adult Nutritional Powder Low Fat	00859RF00	KBI-00045
7. Child Formula Powder	00866RF00	KBI-00046
8. Infant Elemental Powder	00795RF	KBI-00047
9. Infant Formula Powder FOS/GOS Based	50350017W1	KBI-00053
10. Infant Formula Powder Milk Based	K16NTAV	KBI-00048
11. Infant Formula Powder Soy Based	E10NWZC	KBI-00052
12. Infant Formula RTF Milk Based	EV4H2R	KBI-00049
13. Adult Nutritional RTF High Protein	00730RF00	KBI-00050
14. Adult Nutritional RTF High Fat	00729RF00	KBI-00051
Nestlé Products		
1. Milk Powder With Prebiotics	Ref. 40C	Ref. 40C
2. Infant Formula DDP 3/2016	Ref. 64A	Ref. 64A

2.2. Method

2.2.1 Principle

Samples are reconstituted in water and further diluted until the concentration of fructan in solution is such that after hydrolysis the fructose and glucose concentration will be within the range covered by the standard curve. The diluted sample is treated with a mixture of sucrase and α-glucanases to hydrolyse sucrose and alpha-glucooligosaccharides to their constituent monosaccharides. The sample is passed through a solid phase extraction cartridge packed with graphitized carbon. Salts and monosaccharides pass through and are washed away, while the fructans are retained. Fructans are released from the column using an acetonitrile solution. The released fructans are hydrolysed with an inulinase mixture, and the released glucose and fructose are analysed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The fructan content is calculated by summing the glucose and 0.9 × fructose content measured.

2.2.2 Equipment

- pH-meter, reading 0.1 pH
- Microcentrifuge tubes 2 mL & 1.5 mL
- Water bath at 80°C ± 1 °C
- Water bath at 40°C ± 1 °C
- Centrifuge for 2-mL microtubes able to operate at 10000 g
- Micropipettes with tips (0.1 to 1) mL
- Vortex mixer
- Single-use plastic pipettes 5 mL & 10 mL
- Single-use syringes 2 mL
- Graphitized carbon (100-200 mg) SPE columns
- Membrane filter nylon, 0.2 µm, Ø 4.7 cm
- Analytical column CarboPac PA20, 250 x 4 mm, 10 µm
- Metal free liquid chromatograph consisting of
    - Gradient pump
    - Autosampler
    - Column compartment (able to maintain a temperature of 30°C)
    - Electrochemical detector working in pulsed amperometric detection mode
    - Isocratic pump for post-column delivery

2.2.3 Chemicals & Reagents

- Maleic acid, puriss., >99 %,
- Acetonitrile gradient grade for liquid chromatography
- Acetic acid glacial 100 % anhydrous, GR for analysis
- Trifluoroacetic acid
- Sodium hydroxide 50 % (w/w)
- D-(-)-Fructose, >99 %,
- D-(+)-Glucose, ≥99.5 %
- Chitobiose
- Milli-Q purified water or equivalent

- Mix of highly purified sucrase , β-amylase, pullulanase and maltase (from Fructan Assay Kit, K-FRUC from Megazyme International Ireland Ltd)

- Mix of highly purified exo-inulinase and endo-inulinase
(from Fructan Assay Kit, K-FRUC from Megazyme International Ireland Ltd)

Sodium maleate buffer (100 mM, pH 6.5): Into a large beaker (>1000 mL) weigh 11.6 g of maleic acid and dissolve with 900 mL of water(using magnetic stirrer). Adjust the pH to 6.5 with sodium hydroxide solution 1 M. Transfer the solution to a 1000 mL volumetric flask and make up to the mark with water.

Sodium acetate buffer (100 mM, pH 4.5): Into a large beaker (>1000 mL) containing 900 mL of demineralised water, pipette 5.8 mL of glacial acetic acid. Adjust to pH 4.5 with sodium hydroxide solution 1 M. Transfer the solution to a 1000 mL volumetric flask and make up to the mark with water.

Chitobiose solution (600 µg / mL): Into a 25 mL volumetric flask weigh 15 mg of chitobiose and make up to the mark with water.

Glucose stock solution (5 mg/mL): Into a 25 mL volumetric flask weigh 125 mg of glucose and make up to the mark with water.

Fructose stock solution (10 mg/mL): Into a 25 mL volumetric flask weigh 250 mg of fructose and make up to the mark with water.

Sucrase / β-amylase / pullulanase / maltase: Dissolve the contents of the vial containing powdered sucrase , β-amylase , pullulanase and maltase in 22.0 mL of sodium maleate buffer (100mM, pH 6.5). Mix well and divide into aliquots of 2.0 mL each and store frozen at -20°C in polypropylene tubes until use.
NOTE: For the development and validation of this method, the pre-prepared enzyme mixture available in the Megazyme kit, K-FRUC, was used. When enzymes from another source are used it is imperative to ensure the enzyme mixture will completely hydrolyse any sucrose in the product without hydrolysing the fructan. This can be checked by performing an analysis with sucrose as an analyte and with a pure fructan as an analyte. No fructan should be measured when sucrose is analysed, and > 90% recovery should be achieved when a pure fructan is analysed.

Fructanase (exo-inulinase + endo-inulinase): Dissolve the contents of the vial containing powdered exo-inlulinase and endo-inulinase in 22.0 mL of sodium acetate buffer (100 mM, pH 4.5). Mix well and divide into aliquots of 2.0 mL each and store frozen at -20°C in polypropylene tubes until use.
NOTE: For the development and validation of this method, the pre-prepared enzyme mixture available in the Megazyme kit, K-FRUC, was used. When enzymes from another source are used it is imperative to ensure the enzyme mixture employed will completely hydrolyse the fructan without hydrolysing any other glucose or fructose containing oligo- or polysaccharide that may be present after treatment with the sucrase mixture above.

Wash solution for graphitized carbon column, TFA 0.1 % in acetonitrile 80 % (v/v): Into a 100 mL volumetric flask, add 80 mL of acetonitrile (HPLC grade) and 100 µL of TFA. Make up to the mark with water.

Sodium chloride solution (1M) for graphitized carbon column: Into a 100 mL volumetric flask weigh 5.8 g of sodium chloride and dissolve with 90 mL of demineralised water. Make up to the mark with water.

Elute solution for graphitized carbon column, TFA 0.05 % in acetonitrile 25 % (v/v): Into a 100 mL volumetric flask, add 25 mL of acetonitrile (HPLC grade) and 50 µL of TFA. Make up to the mark with water.

LC eluent A, sodium hydroxide solution (300 mM): Into a HPLC bottle, introduce 985 mL water and degas for 20 min. Add 15.6 mL of the NaOH 50 % solution (using a single-use plastic pipette). Degas with helium for 20 min and keep under a blanket of helium until, and during, use.

LC eluent B, Milli-Q water: Into a HPLC bottle, introduce 2000 mL water and degas with helium for 20 min. Thereafter keep under a blanket of helium until, and during, use.

LC eluent C, sodium acetate (500mM) and sodium hydroxide (150 mM) solution: Into a 1000 mL volumetric flask, weigh 41.0 g of anhydrous sodium acetate and dissolve with 800 mL of water by mixing. Make up to the mark with water and filter on a 0.20 µm nylon membrane filter into an HPLC bottle. Degas with helium for 20 min then add (using a single-use plastic pipette) 7.8 mL of the 50 % (w/w) NaOH solution. Swirl gently to mix and sparge with helium for another 15 min. Thereafter keep under a blanket of helium until, and during, use.

LC post-column addition reagent, sodium hydroxide (300 mM): Into a HPLC bottle, introduce 985 mL water and add 15.6 mL of the NaOH 50 % solution (using a single-use plastic pipette) swirl the solution gently to mix.

2.2.4 Sample Preparation

For analysis of products on a ready to feed basis:
Reconstitute powder or liquid concentrates according to instructions. For SPIFAN samples; powders (25 g) were weighed in to a bottle and water (200 g) was added. The mixture was mixed well at room temperature, and the pH was confirmed to be between 5 – 9. Samples were then placed in a water bath at 80°C with constant agitation for 20 min.

Reconstituted products (as prepared above), or products which are sold as ready to feed (RTF), were weighed (9 g, m) in to a 50 mL (V) volumetric flask and made up to the mark with water (this is Solution A).

For analysis of powder products without prior reconstitution:
Weigh 1 g (m) of powder in to a 50 mL (V) volumetric flask. Add water (30 mL) and heat at 80 °C with constant agitation for 20 min. Cool to room temperature and complete to the mark with water (this is Solution A). NOTE: For the SLV, SRM 1849a was analysed as a powder using this procedure.

Dilution

The solutions prepared above were further diluted depending on the expected fructan content following the guidelines in the table below and the resulting solution is “Solution B”.

Table 2: Scheme for Sample Dilution Depending on Expected Fructan Content

Expected fructan content (g/100g)		Dilution		Dilution factor (D)
Powder	Ready-to-feed	Volume of Solution A (mL)	Final Volume (mL)	
< 4.5	< 0.5	No dilution	No dilution	1
4.5 - 9	0.5 - 1.0	5	10	2
9 - 27	1.0 – 3.0	5	25	5
27 - 36	3.0 – 4.0	5	50	10
36 - 45	4.0 – 5.0	5	100	20

Hydrolysis of sucrose and α-glucans

Transfer 200 µL of Solution B in to a 1.5 mL microtube and add 100 µL of chitobiose solution (600 µg/mL) and 200 µL of the sucrose, maltase, amylase, pullulanase enzyme mixture. Mix well and incubate at 40°C for 90 min.

Removal of monosaccharides

Prepare the graphitized carbon SPE column as follows:-

- Flush with 3 × 400 µL of wash solution
- Flush with 3 × 400 µL of water

Then perform the following steps under gravity (i.e without applying vacuum or positive pressure),

- Apply 400 µL of the enzyme treated solution.
- Wash with 2 × 1000 µL of sodium chloride solution (1 M)
- Wash with 4 × 1000 µL of water
- Elute the fructans in to a 2 mL microtube using 3 × 400 µL of elute solution
- Apply a little positive pressure to eliminate all solution from the column

Hydrolysis of Fructans (samples containing ≤ 0.15 g/100 g fructan on a ready-to-feed basis)

Take the eluate from the SPE column and add 300 µL of sodium acetate buffer (100 mM, pH 4.5). Transfer 700 µL of the solution in to one microtube (blank) and 700 µL of the solution in to another microtube (sample). To the blank tube add 100 µL of sodium acetate buffer (100 mM, pH 4.5). To the sample tube add 100 µL of the inulinase enzyme mixture. Mix the tubes well and incubate both at 40°C for 40 min. After cooling centrifuge the tube at 10000 × g. Transfer 700 µL of the supernatant in to a vial suitable for the instrument autosampler.

Hydrolysis of Fructans (samples containing > 0.15 g/100 g fructan on a ready-to-feed basis)
Take the eluate from the SPE column and add 300 µL of sodium acetate buffer (100 mM, pH 4.5) and 200 µL of the inulinase enzyme mixture. Mix well and incubate at 40°C for 40 min. After cooling centrifuge the tube at 10000 × g. Transfer 1000 µL of the supernatant in to a vial suitable for the instrument autosampler.

2.2.5 Preparation of Standard Curve

Prepare the solutions for the 6-level standard curve by diluting the glucose stock solution (5 mg/mL) and the fructose stock solution (10 mg/mL) as described in the following table:

Table 3: Dilution Scheme for the Preparation of the Standard Curve

Standard	Volume of fructose stock solution (µL)	Volume of glucose stock solution (µL)	Final Volume (mL)	Fructose Conc. (µg/mL)	Glucose Conc (µg/mL)
Level 1	200	40	100	20	2
Level 2	400	200	20	200	50
Level 3	800	400	20	400	100
Level 4	1200	600	20	600	150
Level 5	1600	800	20	800	200
Level 6	2000	1000	20	1000	250

Take the six solutions of standards and treat each one as follows: Into a microtube transfer 200 µL of standard solution and add 200 µL of water and 100 µL of chitobiose internal standard solution. Then transfer 400 µL of this solution to another tube and add 800 µL of SPE elute solution and 500 µL of sodium acetate buffer. Mix well then centrifuge at 10000 × g. Transfer 1400 µL of the supernatant in to a vial suitable for the instrument autosampler.

2.2.6 Chromatographic Conditions

Analyse the prepared samples, standards (and blanks if required) on an HPAEC-PAD system under the following conditions:
Column: CarboPac PA20 (3 × 150 mm, 6.5 µm), or equivalent (strong anion-exchange resin composed of ethylvinylbenzene 55% crosslinked with divinylbenzene agglomerated with 130 nm beads containing a difunctional quaternary ammonium ion (5% crosslinked)). The complete column has an anion exchange capacity of approximately 65 µeq.
Column temperature: 30°C
Injection volume: 25 µL
Post-Column Addition: Sodium Hydroxide (300 mM, 0.2 mL/min)
Flow Rate: 0.5 mL/min
Gradient:

Time (min)	Flow (mL/min)	%A (300mM NaOH)	%B (Water)	%C (500mM NaOAc + 150 mM NaOH)
0.0	0.5	2	98	0
17.0	0.5	2	98	0
17.1	0.5	0	0	100
22.0	0.5	0	0	100
22.1	0.5	100	0	0
27.0	0.5	100	0	0
27.1	0.5	2	98	0
33.0	0.5	2	98	0

PAD Waveform:

Time (s)	Voltage (V)	Gain Region
0.00	+ 0.10	Off
0.20	+ 0.10	On
0.40	+ 0.10	Off
0.41	- 2.00	Off
0.42	- 2.00	Off
0.43	+ 0.60	Off
0.44	- 0.10	Off
0.50	-0.10	Off

2.2.7 Calibration & Calculations

For each analyte (glucose and fructose) use the instrument software to plot a six point standard curve of (Instrument response for analyte / Instrument response for internal standard) against the concentration of the analyte in the standard. Fit a quadratic curve to the data without forcing through zero.

Use the calibration curve to calculate the glucose and fructose concentration in Solution B.

Calculate the concentration of fructan in the sample as follows:-

$C_G = C_{GB} \times D \times (V/m) \times 0.0001$

$C_F = C_{FB} \times D \times (V/m) \times 0.0001$

$TF = (C_F \times 0.9) + C_G$

Where,
TF = Total fructan in sample (in g/100 g)
C_G = Glucose released from fructan (in g/100 g)
C_{GB} = Glucose concentration in Solution B (in µg/mL)
C_F = Fructose released from fructan (in g/100 g)
C_{FB} = Fructose concentration in Solution B (in µg/mL)
D = Dilution factor between Solution A and solution B (from Table)
V = Total volume of Solution A (in mL)
m = Amount of sample weighed to prepare Solution A (in g)
0.001 = factor to convert analyte concentration in solution (in µg/mL) to analyte concentration in sample (in g/100 g)
0.9 = factor to correct for uptake of water during fructan hydrolysis

For samples with low fructan content requiring the blank correction adapt the above equations as follows:-

$C_G = (C_{GB} – C_{G0}) \times D \times (V/m) \times 0.0001$

$C_F = (C_{FB} – C_{F0}) \times D \times (V/m) \times 0.0001$

Where
C_{G0} = Glucose concentration in Blank Solution B (in µg/mL)
C_{F0} = Fructose concentration in Blank Solution B (in µg/mL)

2.3. Validation Design

2.3.1 Linearity

Calibration solutions of glucose (2 µg/mL – 300 µg/mL) and fructose (20 µg/mL – 1100 µg/mL) at 8 different levels, were prepared in triplicate all containing the chitobiose internal standard (at 600 µg/mL). The ratio of the peak areas (analyte / chitobiose) was plotted against the concentration of analyte (since the chitobiose concentration remained constant throughout there was no need to plot the ratio of the concentrations on the x-axis). A quadratic model was used to fit the data for calibration purposes.
The relative residuals were calculated and plotted against the analyte concentration.

2.3.2 Precision

All samples from the SPIFAN kit were analysed in duplicate on a single day, and six of the samples were found to contain fructans; The Placebo Child Formula Powder and the following fortified products: Toddler Formula Powder Milk Based, Infant Formula Powder Milk Based, Child Formula Powder, Infant Formula FOS/GOS Based, Adult Nutritional RTF High Fat, Milk Powder with Prebiotics. In addition, two Nestlé samples were also analysed giving a total of 8 matrices for the precision study.

Each of the fructan-containing samples from the SPIFAN kit were reconstituted as instructed and analysed in duplicate, on at least six different days, using 2 different instruments (same model) and 2 different columns (same stationary phase and manufacturer, but different lot). At the same time the two Nestlé samples were also analysed (without reconstitution). SD(r) and SD(iR) were calculated from the data obtained using the equations below:-

SD(r)

$$\mathrm{SD}(\mathrm{r}) = \sqrt{\frac{\sum_{i=1}^{n} \mathrm{SD}_i^2}{n}} = \sqrt{\frac{\sum_{i=1}^{n} (x_{i1} - x_{i2})^2}{2n}}$$

SD(iR)

$$\mathrm{SD}(\mathrm{iR}) = \sqrt{\mathrm{SD}^2(\mathrm{b}) + \frac{1}{2} \times \mathrm{SD}^2(\mathrm{r})}$$

Where:

n is the number of (single or duplicate) determinations
x_i is the individual result within the set of single determinations with i going from 1 to n ;
x_{i1} and x_{i2} are the two results within the set of duplicate determination with i going from 1 to n ;
SD(b) is the standard deviation between the means of duplicates

2.3.3 Accuracy

Six matrices were selected that covered both infant formula and adult nutritionals (see Table 4 for details). They were analysed in duplicate on three different days by two different operators on two different instruments without spiking (level 0) and spiked with Orafti HP, Orafti P95 or Nutraflora P95, each at one of three levels (Table 4). The fructan powders used for spiking were separately analysed using method AOAC 997.08 [1] to confirm their fructan content.

Table 4: Design of Spike-Recovery Experiment Using Three Fructan Ingredients in Six SPIFAN Matrices

Nestlé Code	**Sample**	**Level 0 (0 g/100g)**	**Level 1 (0.03 g/100g)**	**Level 2 (2 g/100g)**	**Level 3 (5.0 g/100g)**
KBI-00048	Infant Formula Powder Milk Based	none	Orafti P95	Orafti HP	NutraFlora P95
KBI-00052	Infant Formula Powder Soy Based	none	Orafti HP	NutraFlora P95	Orafti P95
KBI-00050	Adult Nutritional RTF High Protein	none	NutraFlora P95	Orafti P95	Orafti HP
KBI-00045	Adult Nutritional Powder Low Fat	none	Orafti P95	Orafti HP	NutraFlora P95
KBI-00033	Infant Formula Powder Partially Hydrolyzed Milk Based	none	Orafti HP	NutraFlora P95	Orafti P95
KBI-00047	Infant Elemantal Powder	none	NutraFlora P95	Orafti P95	Orafti HP

2.3.4 Limits of Detection and Quantification

Limits of detection and quantification were determined by analyzing a blank sample (KBI-00052, Infant formula Powder Soy Based) 14 times (7 days in duplicate). The sample has minor signals at the expected retention times of glucose and fructose. They have been determined as fructan and the data used to calculate LoD and LoQ by taking the mean fructan content determined by the 14 analyses and adding 3 times the standard deviation to estimate LoD, and adding 10 times the standard deviation to estimate LoQ.

3 RESULTS

3.1. System Calibration

A quadratic model was used to fit the data for calibration purposes. The relative residuals were calculated and plotted against the analyte concentration. For glucose the quadratic line fits the data well (Figure 1), and the predicted concentration is never more than 2.2% away from the actual concentration (Figure 2).

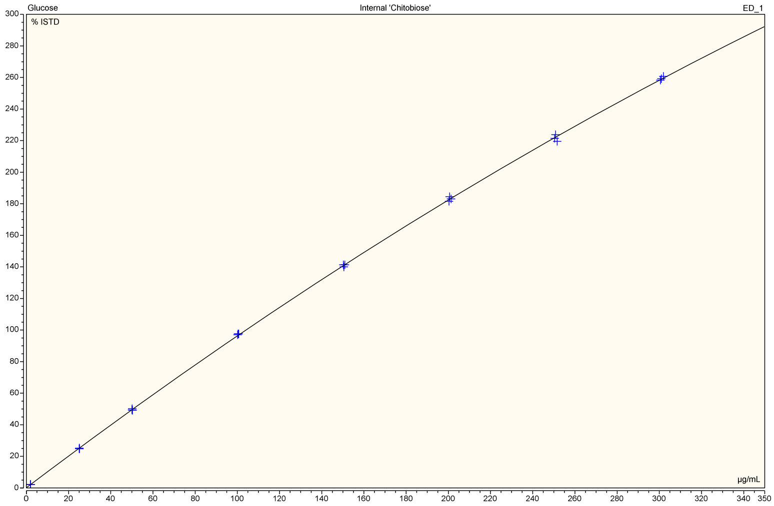

Figure 1: Glucose Calibration Curve

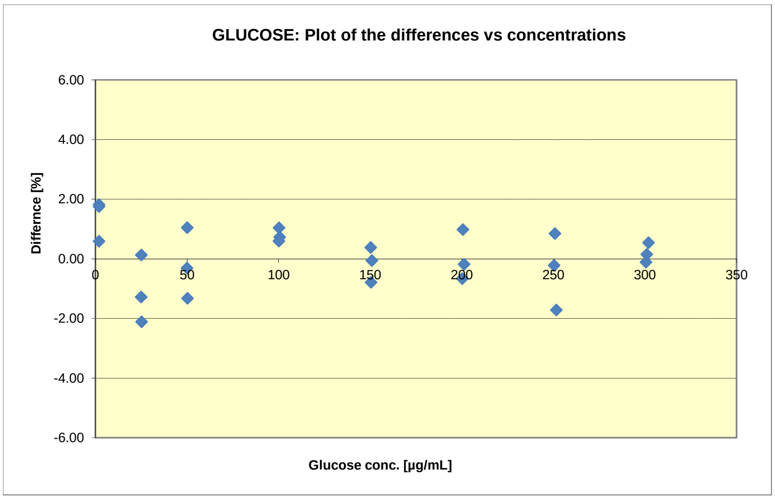

Figure 2: Plot of Relative Residuals (%) Against Glucose Concentration

For fructose, the quadratic line fits the data well (Figure 3), the predicted concentration is always within 2% of the actual concentration except at the lowest concentration (20 µg/mL) where there can be up to a 6 % difference (Figure 4).

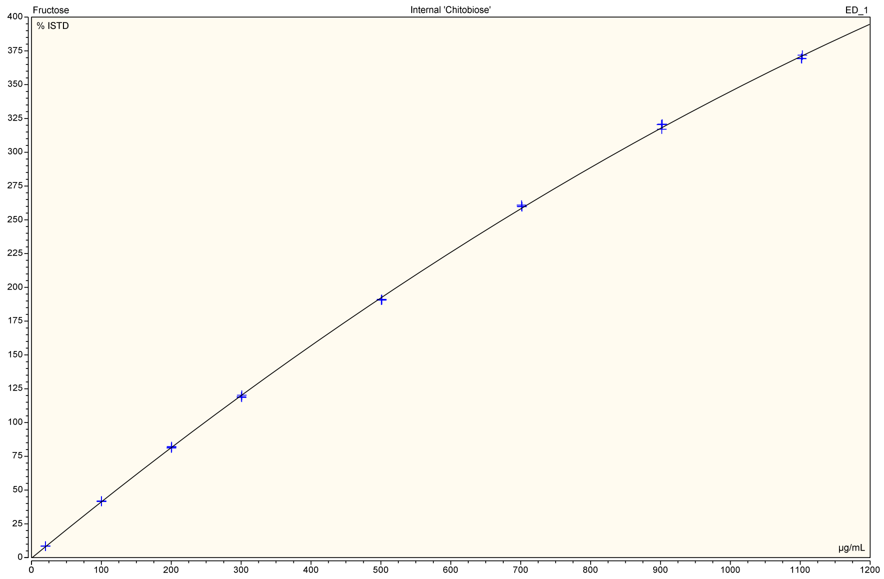

Figure 3: Fructose Calibration Curve

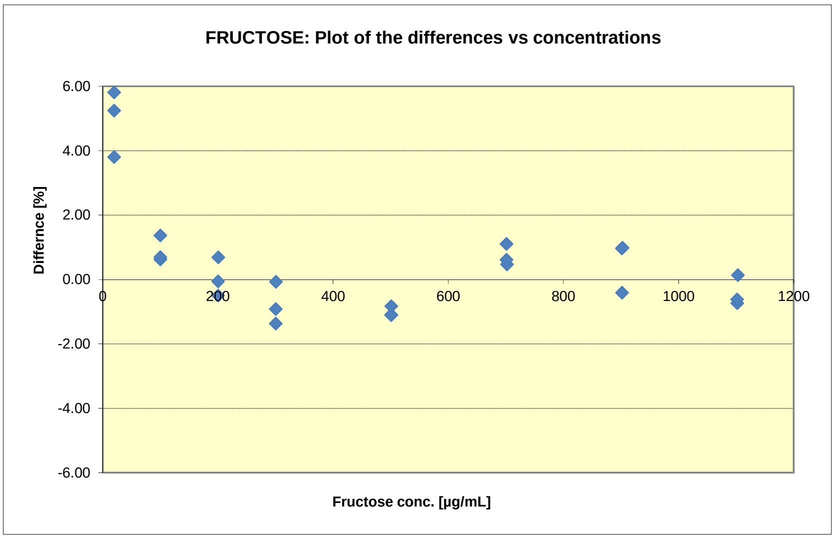

Figure 4: Plot of Relative Residuals (%) Against Fructose Concentration

3.2. Analytical Precision

(QSTAT 66616)

Method precision was assessed in a single laboratory by analyzing the samples on at least 6 days in duplicate. Analyses were performed by a single operator on two different instruments (of same model) using two different columns (same column type, different batch number), results are shown in Table 5.

The SMPR [2] requires that the RSD(r) should be less than or equal to 6%. In all cases the achieved RSD(r) was less than 3%, thus the method meets the requirements of the SMPR for RSD(r). The RSD(iR) was also less than 3% in all cases suggesting that the method should be able to achieve an RSD(R) of less than 12% during multi lab testing (as required in the SMPR).

Table 5: Analyses of Fructan-Containing Samples Under Intermediate Reproducibility Conditions

Nestle Code	Sample Description	n	Mean Conc. (g/100g)	RSD(r) (%)	RSD(iR) (%)	Target RSD(r) (%)	Meets Target (Y/N)
KBI-00020	Child Formula Powder	6×2	0.273	0.78	1.1	≤ 6	Y
KBI-00035	Toddler Formula Powder Milk Based	6×2	0.210	1.2	2.4	≤ 6	Y
KBI-00044	Infant Formula Powder Milk Based	7×2	0.280	2.5	3.0	≤ 6	Y
KBI-00046	Child Formula Powder	6×2	0.275	1.2	1.1	≤ 6	Y
KBI-00053	Infant Formula Powder FOS/GOS Based	6×2	0.033[a]	0.9	2.5	≤ 6	Y
KBI-00051	Adult Nutritional RTF High Fat	6×2	0.481	1.8	1.6	≤ 6	Y
Ref 40C	Milk Powder with Prebiotics	6×2	2.85[b]	1.1	1.8	≤ 6	Y
Ref 64A	Infant Formula	24×2	0.390[a,b]	3.2	4.2	≤ 6	Y

[a] Although it is recommended to analyse samples with fructan content below 0.15 g/100g (on a RTF basis) using the procedure including a blank subtraction, this had not been defined at the time of the precision experiment, thus the precision data on these samples do not include the blank subtraction step. All concentrations are in g/100g of ready to feed product (either reconstituted powders or the liquid products directly) except [b] in which the concentration is reported in g/100g of the non-reconstituted powder.

3.3. Method Accuracy

Six different blank matrices were spiked at three levels with three different fructan ingredients (as described in Table 4). Recoveries were calculated by comparing the spiked amount vs the measured amount (Table 6). At the 2 higher spiking levels recoveries were in general very good (92 – 104 %) with one exception, the adult nutritional RTF high protein sample, for which the average recovery was only 86 %. At the lowest spike level (0.03 g/100g) which is equivalent to the desired LoQ specified in the SMPR [2], the recoveries are less good, varying from 101 – 151 %, with three matrices achieving the SMPR requirements (recoveries between 101 – 105 %) and three matrices being outside the requirements (recoveries between 114 – 151%). Because the spike level is very low, a small amount of interference can have a significant impact on the recovery. In order to correct for this interference the method was adapted for samples containing very low fructan levels by introducing a blank subtraction step. In such a case the samples are processed as usual until the end of the SPE step. After SPE the sample is split in two. One aliquot is treated with inulinase, and the other is not. Afterwards glucose and fructose are measured as usual in both aliquots. The levels measured in the blank are subtracted from the levels measured in the inulinase treated sample to arrive at the final result (Table 7). The recoveries on the samples with low spike levels are significantly improved (95-119%) using the method employing a blank subtraction, but still do not meet the SMPR in all cases (for two matrices the recovery exceeds 110%, being 117% and 119%). This improvement implies that the blank subtraction is warranted and necessary for samples expected to contain low levels of fructans (we estimate at levels below 0.15 g/100g).

Table 6: Fructan Recoveries

Nestlé Code	**Sample**	**Recovery**								
		Level 1			**Level 2**			**Level 3**		
		Spike (g/100g)	Rec (%)	RSD (%)	Spike (g/100g)	Rec (%)	RSD (%)	Spike (g/100g)	Rec (%)	RSD (%)
KBI-00048	Infant Formula Powder Milk Based	0.031	101	5.0	2.00	99.7	2.2	5.02	98.2	2.4
KBI-00052	Infant Formula Powder Soy Based	0.030	114	3.0	2.02	104	4.2	5.02	93.6	2.8
KBI-00050	Adult Nutritional RTF High Protein	0.030	151	11	1.99	95.5	2.2	4.95	86.0	3.8
KBI-00045	Adult Nutritional Powder Low Fat	0.031	102	5.1	1.99	102	2.0	5.02	102	1.6
KBI-00033	Infant Formula Powder Partially Hydrolyzed Milk Based	0.031	122	7.3	2.00	103	2.5	5.01	92.0	2.2
KBI-00047	Infant Elemental Powder	0.030	105	5.2	2.02	95.7	1.8	5.00	95.5	6.1

Table 7: Level 1 Fructan Recoveries With Blank Subtraction

Nestlé Code	**Sample**	**Recovery**		
		Spike (g/100g)	Rec (%)	RSD (%)
KBI-00048	Infant Formula Powder Milk Based	0.031	95.4	2.6
KBI-00052	Infant Formula Powder Soy Based	0.030	104	11
KBI-00050	Adult Nutritional RTF High Protein	0.030	119*	7.1
KBI-00045	Adult Nutritional Powder Low Fat	0.031	95.6	7.4
KBI-00033	Infant Formula Powder Partially Hydrolyzed Milk Based	0.031	117	12
KBI-00047	Infant Elemantal Powder	0.030	96.0	5.6

* sample analysed on 4 days in duplicate, all others on 3 days in duplicate.

3.4. Limits of Detection & Quantification

One blank sample (Infant Formula Powder Soy Based) was analysed on seven different days in duplicate. The average fructan content (on a reconstituted basis) was determined to be 0.0025 g/100g with a standard deviation of 0.0016 g/100g. The limit of detection (LoD) and quantification (LoQ) were estimated as:-

LoD = 0.0025 + (3 × 0.0016) = 0.0073 g/100g

LoQ = 0.0025 + (10 × 0.0016) = 0.018 g/100g

This matches the requirements of the SMPR (LoQ ≤ 0.03 g/100g)

4 CONCLUSIONS

The method tested has sufficient performance to accurately determine the fructan content of most infant formulae and adult nutritional products within the concentrations typically used in such products. At the extremes of the desired working range (i.e. 0.03 g/100g and 5g/100g) the method does not always fulfill the accuracy requirements for some matrices.

The significant advantage of this method compared to previous methods is that correction factors are not required.

REFERENCES

[1] Official Methods of Analysis of AOAC International (2005), 18th Ed., AOAC International, Gaitherburg, MD, Method 997.08.

[2] J. AOAC Int. (2015) **98**: 1038. SMPR 2014.002

1. EXAMPLE CHROMATOGRAMS

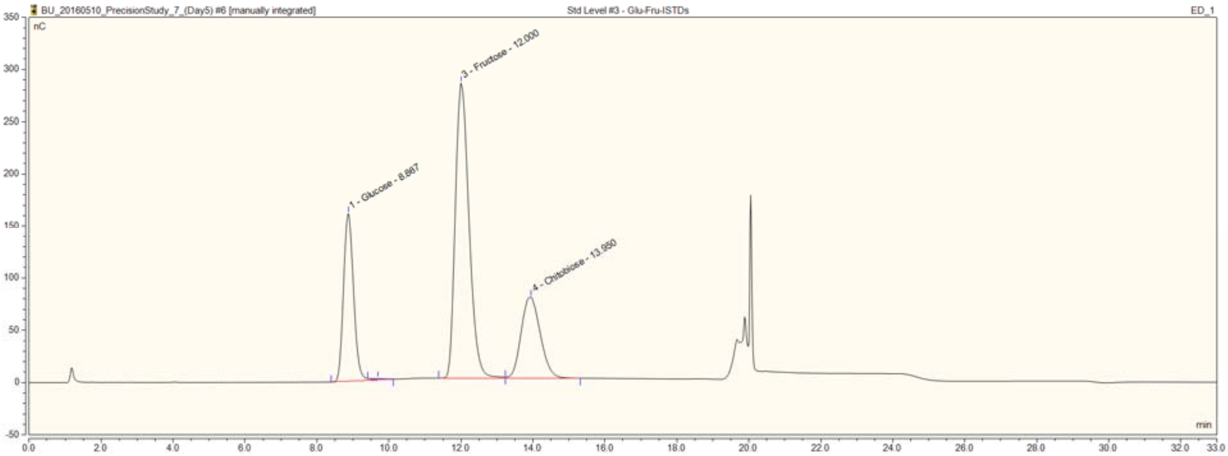

Figure 5: Chromatogram of Standards (Level 3)

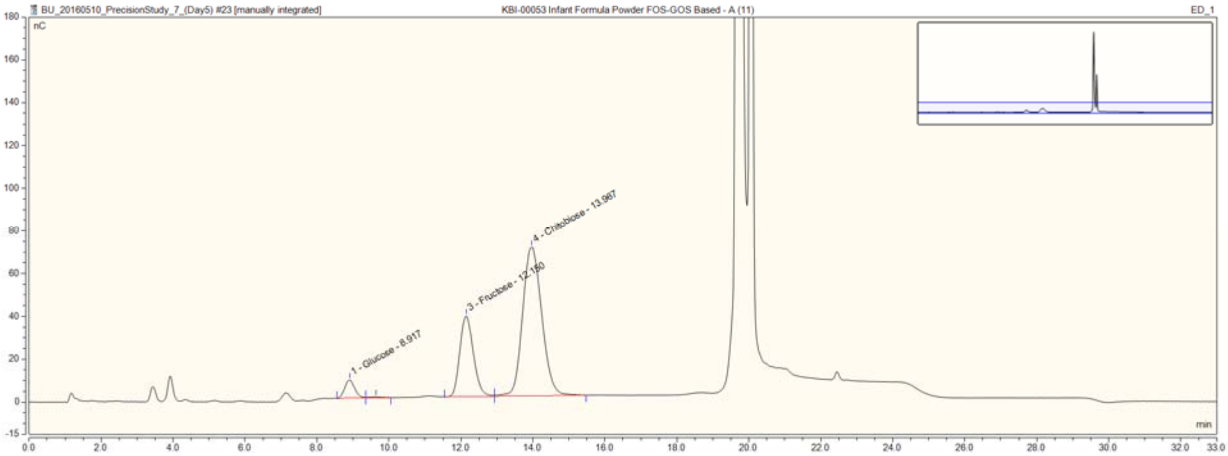

Figure 6: Chromatogram of Infant Formula Powder GOS/FOS Based containing 0.03 g/100g fructan

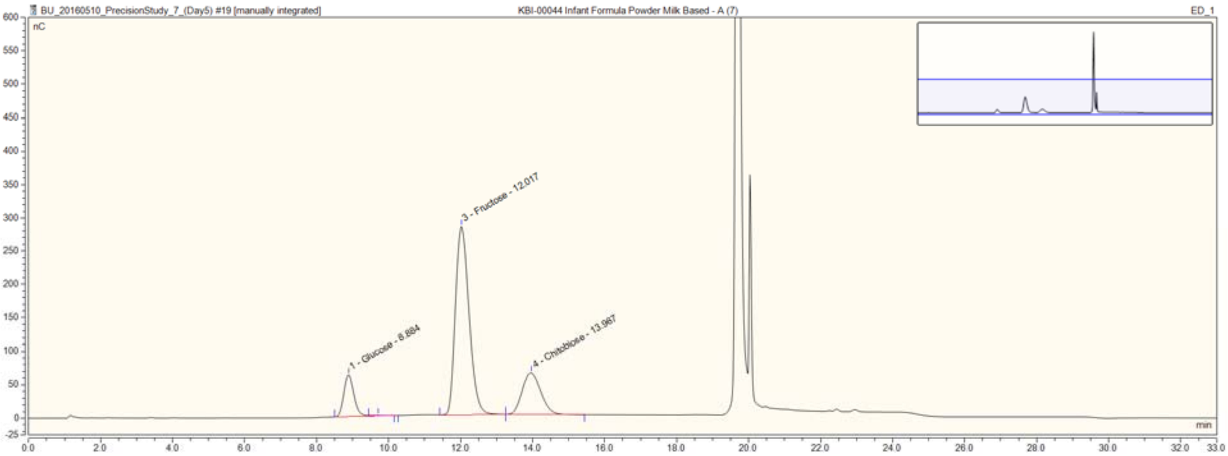

Figure 7: Chromatogram of Infant Formula Powder Milk Based containing 0.28 g/100g fructan

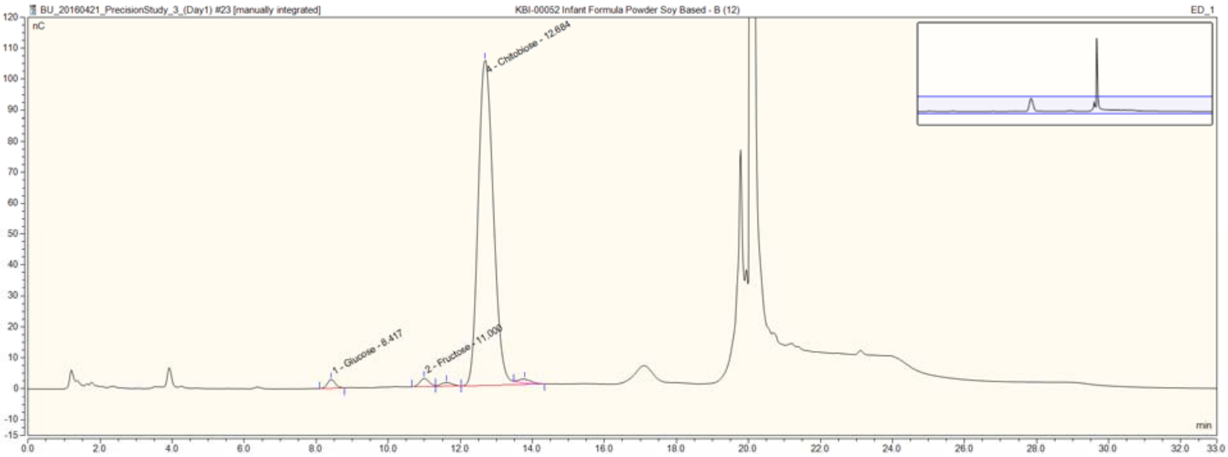

Figure 8: Chromatogram of Infant Formula Powder Soy Based Containing No Fructan (Sample Used For Determination of LoQ)

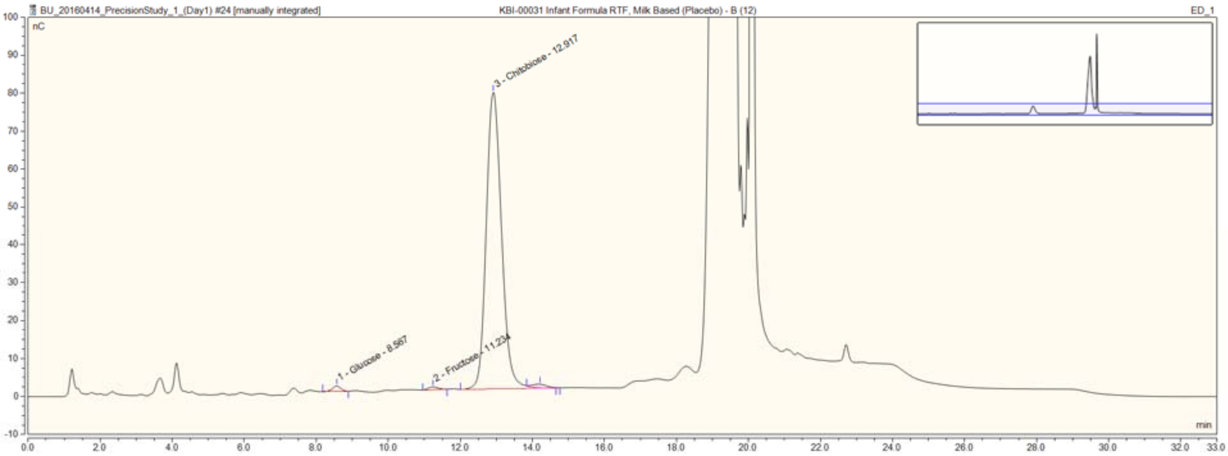

Figure 9: Chromatogram of Infant Formula RTF, Milk Based Containing No Fructan

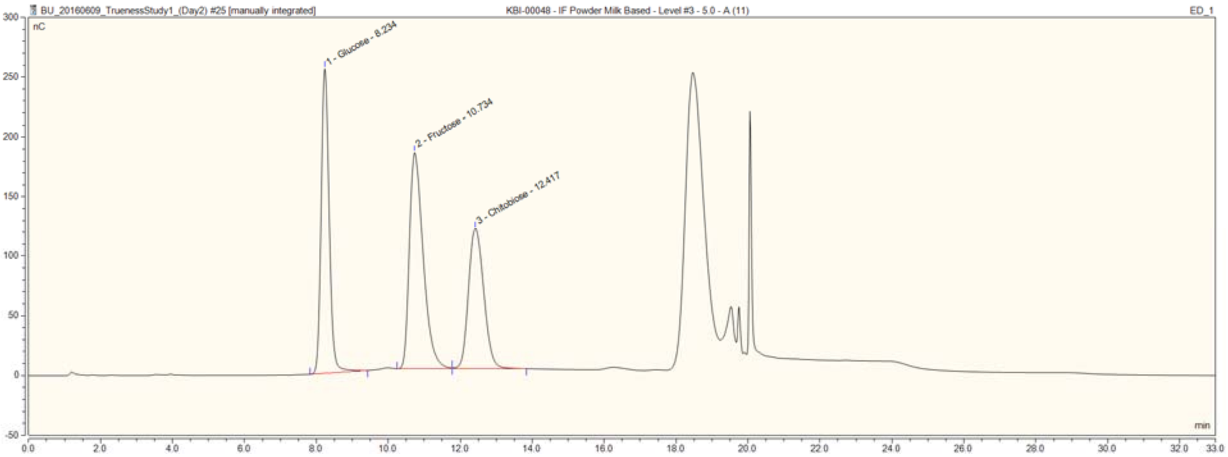

Figure 10: Chromatogram of Infant Formula Powder, Milk Based Spiked With 5.0 g/100g NutraFlora P95

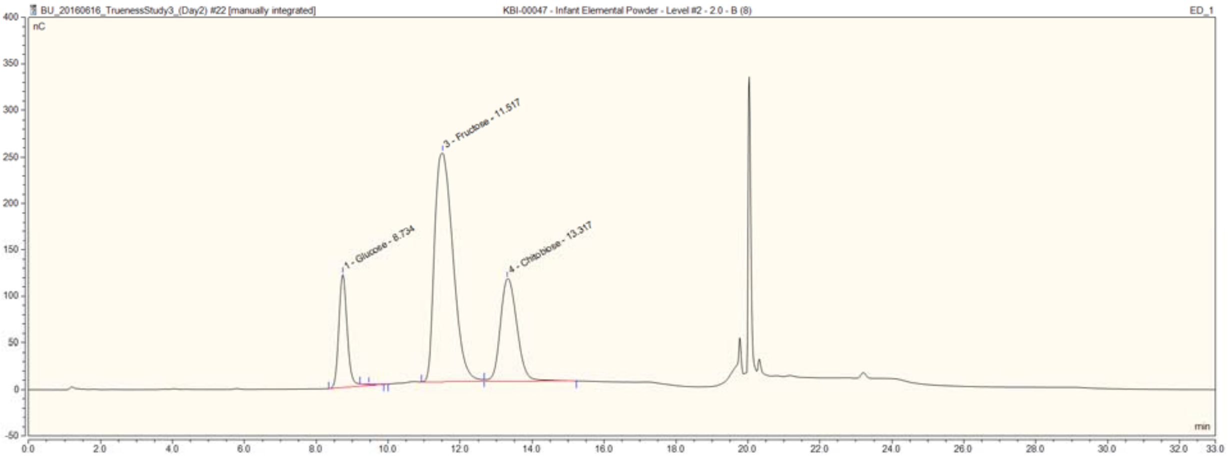

Figure 11: Chromatogram of Infant Elemental Powder Spiked With 2.0 g/100 g Orafti P95

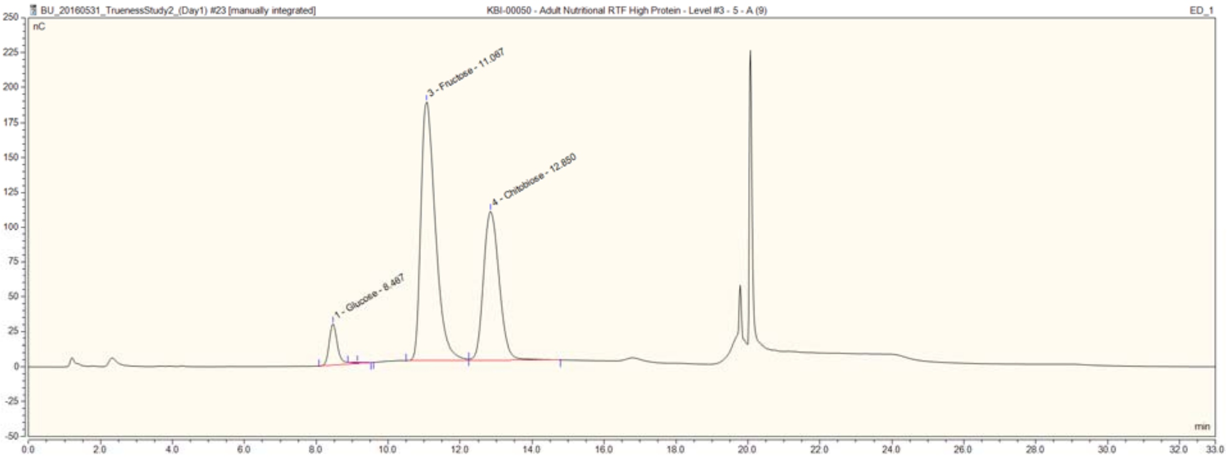

Figure 12: Chromatogram of Adult Nutritional RTF High Protein Spiked With 5.0 g/100 g Orafti HP

2. RAW DATA (PRECISION)

AOAC SPIFAN Samples (results reported on reconstituted samples)

NRC Ref	Batch	Sample Description	Date	Analyst	Inst.	Result A (g/100g)	Result B (g/100g)	Mean (g/100g)	RSD (%)
Fructan-Containing Samples									
KBI-00020	00847RF00	Child Formula Powder	14.04.2016	VG	Sys 2	0.276	0.275	**0.275**	0.1
			26.04.2016	VG	Sys 2	0.277	0.276	**0.277**	0.1
			02.05.2016	VG	Sys 2	0.276	0.272	**0.274**	1.2
			03.05.2016	VG	Sys 1	0.272	0.272	**0.272**	0.1
			10.05.2016	VG	Sys 1	0.272	0.266	**0.269**	1.8
			12.05.2016	VG	Sys 1	0.272	0.272	**0.272**	0.0
KBI-00035	4052755861	Toddler Formula Powder Milk Based	19.04.2016	VG	Sys 2	0.208	0.204	**0.206**	1.3
			26.04.2016	VG	Sys 2	0.224	0.211	**0.217**	4.0
			02.05.2016	VG	Sys 2	0.212	0.208	**0.210**	1.1
			03.05.2016	VG	Sys 1	0.211	0.209	**0.210**	0.7
			10.05.2016	VG	Sys 1	0.210	0.210	**0.210**	0.1
			12.05.2016	VG	Sys 1	0.206	0.208	**0.207**	0.7
			01.06.2016	KI	Sys 1	0.231	0.220	**0.225**	3.5
KBI-00044	4044755861	Infant Formula Powder Milk Based	19.04.2016	VG	Sys 2	0.276	0.274	**0.275**	0.5
			26.04.2016	VG	Sys 2	0.280	0.283	**0.281**	0.8
			02.05.2016	VG	Sys 2	0.270	0.280	**0.275**	2.5
			03.05.2016	VG	Sys 1	0.280	0.279	**0.279**	0.4
			10.05.2016	VG	Sys 1	0.282	0.302	**0.292**	4.8
			12.05.2016	VG	Sys 1	0.282	0.287	**0.284**	1.1
			01.06.2016	KI	Sys 1	0.267	0.278	**0.273**	2.9
KBI-00046	0086RF00	Child Formula Powder	19.04.2016	VG	Sys 2	0.274	0.275	**0.275**	0.4
			26.04.2016	VG	Sys 2	0.278	0.278	**0.278**	0.0
			02.05.2016	VG	Sys 2	0.276	0.277	**0.277**	0.3
			03.05.2016	VG	Sys 1	0.277	0.269	**0.273**	2.0
			10.05.2016	VG	Sys 1	0.276	0.270	**0.273**	1.6
			12.05.2016	VG	Sys 1	0.277	0.271	**0.274**	1.4
KBI-00053	50350017W1	Infant Formula Powder FOS/GOS Based	21.04.2016	VG	Sys 2	0.031	0.032	**0.031**	1.7
			26.04.2016	VG	Sys 2	0.033	0.033	**0.033**	0.9
			02.05.2016	VG	Sys 2	0.033	0.033	**0.033**	0.6
			03.05.2016	VG	Sys 1	0.034	0.034	**0.034**	0.4
			10.05.2016	VG	Sys 1	0.033	0.033	**0.033**	0.0
			12.05.2016	VG	Sys 1	0.033	0.033	**0.033**	0.2
KBI-00051	00729RF00	Adult Nutritional RTF High Fat	21.04.2016	VG	Sys 2	0.497	0.477	**0.487**	3.0
			26.04.2016	VG	Sys 2	0.469	0.484	**0.477**	2.2
			02.05.2016	VG	Sys 2	0.469	0.482	**0.475**	2.0
			03.05.2016	VG	Sys 1	0.479	0.486	**0.483**	1.0
			10.05.2016	VG	Sys 1	0.480	0.481	**0.480**	0.1
			12.05.2016	VG	Sys 1	0.489	0.483	**0.486**	0.8
BLANK MATRICES									
KBI-00028	00796RF	Infant Elemental Powder	14.04.2016	VG	Sys 2	nd	0.001	**0.001**	
KBI-00029	00821RF00	Adult Nutritional RTF High Protein	14.04.2016	VG	Sys 2	0.003	0.003	**0.003**	3.8
KBI-00030	00820RF00	Adult Nutritional RTF High Fat	14.04.2016	VG	Sys 2	0.005	0.005	**0.005**	3.2
KBI-00031	EV4H2Q	Infant Formula RTF Milk Based	14.04.2016	VG	Sys 2	0.002	0.001	**0.001**	38.8

KBI-00032	CLC10-b	SRM 1849a	14.04.2016	VG	Sys 2	nd	nd	**nd**	
KBI-00033	410057652Z	Infant Formula Powder Partially Hydrolyzed Milk Based	19.04.2016	VG	Sys 2	0.002	0.003	**0.003**	20.9
KBI-00034	410457651Z	Infant Formula Powder Partially Hydrolyzed Soy Based	19.04.2016	VG	Sys 2	0.005	0.004	**0.005**	0.5
KBI-00045	00859RF00	Adult Nutritional Powder Low Fat	19.04.2016	VG	Sys 2	0.003	0.004	**0.003**	19.9
KBI-00047	00795RF	Infant Elemental Powder	21.04.2016	VG	Sys 2	nd	nd	nd	
KBI-00048	K16NTAV	Infant Formula Powder Milk Based	21.04.2016	VG	Sys 2	0.001	0.001	**0.001**	9.6
KBI-00052	E10NWZC	Infant Formula Powder Soy Based	21.04.2016	VG	Sys 2	0.003	0.003	**0.003**	5.6
KBI-00049	EV4H2R	Infant Formula RTF Milk Based	21.04.2016	VG	Sys 2	0.001	0.001	**0.001**	2.5
KBI-00050	00730RF00	Adult Nutritional RTF High Protein	21.04.2016	VG	Sys 2	0.008	0.008	**0.008**	1.7
KBI-00049	EV4H2R	Infant Formula RTF Milk Based	02.05.2016	VG	Sys 2	nd	nd	nd	
KBI-00028	00796RF	Infant Elemental Powder	03.05.2016	VG	Sys 1	nd	nd	nd	
KBI-00034	410457651Z	Infant Formula Powder Partially Hydrolyzed Soy Based	10.05.2016	VG	Sys 1	0.006	0.006	**0.006**	2.3
KBI-00033	410057652Z	Infant Formula Powder Partially Hydrolyzed Milk Based	12.05.2016	VG	Sys 1	nd	nd	nd	

Nestlé Reference Samples (NOT Reconstituted)

NRC Ref	Batch	Sample Description	Date	Analyst	Inst.	Result A (g/100g)	Result B (g/100g)	Mean (g/100g)	RSD (%)
40 C		Milk Powder	14.04.2016	VG	Sys 2	2.909	2.871	**2.890**	0.9
			19.04.2016	VG	Sys 2	2.852	2.846	**2.849**	0.1
			21.04.2016	VG	Sys 2	2.774	2.786	**2.780**	0.3
			26.04.2016	VG	Sys 2	2.775	2.853	**2.814**	1.9
			02.05.2016	VG	Sys 2	2.909	2.853	**2.881**	1.4
			03.05.2016	VG	Sys 1	2.898	2.900	**2.899**	0.0
64A	DDP 3/2016	Infant Formula	14.04.2016	VG	Sys 2	0.378	0.389	**0.383**	1.9
			19.04.2016	VG	Sys 2	0.376	0.369	**0.372**	1.3
			21.04.2016	VG	Sys 2	0.370	0.364	**0.367**	1.2
			26.04.2016	VG	Sys2	0.389	0.394	**0.391**	1.0
			02.05.2016	VG	Sys 2	0.374	0.372	**0.373**	0.3
			03.05.2016	VG	Sys 1	0.396	0.383	**0.390**	2.4
			10.05.2016	VG	Sys 1	0.397	0.398	**0.398**	0.3
			12.05.2016	VG	Sys 1	0.386	0.393	**0.389**	1.3
			24.05.2016	VG	Sys 2	0.398	0.384	**0.387**	1.0
			31.05.2016	VG	Sys 2	0.385	0.411	**0.398**	4.6
			01.06.2016	KI	Sys 1	0.417	0.420	**0.418**	0.5
			02.06.2016	VG	Sys 2	0.407	0.387	**0.397**	3.5
			03.06.2016	KI	Sys 1	0.409	0.409	**0.409**	0.0
			06.06.2016	KI	Sys 1	0.376	0.405	**0.390**	5.4
			07.06.2016	VG	Sys 2	0.381	0.387	**0.384**	1.1
			08.06.2016	KI	Sys 1	0.390	0.397	**0.394**	1.4
			09.06.2016	VG	Sys 2	0.364	0.390	**0.377**	5.0
			13.06.2016	KI	Sys 1	0.404	0.426	**0.415**	3.8
			14.06.2016	VG	Sys 1	0.393	0.399	**0.396**	1.0
			15.06.2016	KI	Sys 2	0.410	0.404	**0.407**	1.0
			16.06.2016	VG	Sys 1	0.412	0.352	**0.382**	11.1
			17.06.2016	KI	Sys 2	0.379	0.383	**0.381**	0.7
			22.06.2014	KI	Sys 2	0.395	0.390	**0.392**	0.9
			23.06.2014	VG	Sys 1	0.365	0.365	**0.365**	0.1

3. RAW DATA (TRUENESS)

KBI-00050: Adult Nutritional RTF High Protein

FOS Type	Orafti P95	Orafti HP	NutraFlora P95
Spike Level (g/100g)	0.031	1.995	5.022
Day 1	0.029	1.940	5.060
	0.029	1.960	4.970
Day 2	0.029	1.980	5.020
	0.030	1.970	4.970
Day 3	0.029	2.020	4.790
	0.030	2.060	4.790
Mean Recovery (%)	**95.4**	**99.7**	**98.2**

KBI-00052: Infant Formula Powder Soy Based

FOS Type	Orafti HP	NutraFlora P95	Orafti P95
Spike Level (g/100g)	0.030	2.023	5.019
Day 1	0.033	2.060	4.610
	0.034	2.010	4.660
Day 2	0.034	2.050	4.590
	0.033	2.040	4.620
Day 3	0.028	2.240	4.810
	0.026	2.160	4.910
Mean Recovery (%)	**104**	**104**	**93.6**

KBI-00050: Adult Nutritional RTF High Protein

FOS Type	NutraFlora P95	Orafti P95	Orafti HP
Spike Level (g/100g)	0.030	1.985	4.954
Day 1	0.036	1.850	4.250
	0.033	1.840	4.190
Day 2	0.038	1.920	4.250
	0.032	1.940	4.040
Day 3	0.039	1.914	4.294
	0.036	1.918	4.536
Day 4	0.033		
	0.035		
Mean Recovery (%)	**119**	**95.6**	**86.0**

KBI-00045: Adult Nutritional Powder Low Fat			
FOS Type	Orafti P95	Orafti HP	NutraFlora P95
Spike Level (g/100g)	0.031	1.992	5.022
Day 1	0.027	1.990	5.070
	0.032	1.970	5.150
Day 2	0.028	2.040	5.010
	0.028	2.020	5.030
Day 3	0.029	2.080	5.120
	0.032	2.050	5.230
Mean Recovery (%)	**95.6**	**102**	**102**

KBI-00033: Infant Formula Powder Partially Hydrolyzed Milk Based			
FOS Type	Orafti HP	NutraFlora P95	Orafti P95
Spike Level (g/100g)	0.031	1.997	5.013
Day 1	0.029	2.060	4.650
	0.039	2.000	4.670
Day 2	0.039	2.052	4.431
	0.036	2.120	4.560
Day 3	0.040	2.021	4.641
	0.032	2.128	4.717
Mean Recovery (%)	**117**	**103**	**92.0**

KBI-00047: Infant Elemental Powder			
FOS Type	NutraFlora P95	Orafti P95	Orafti HP
Spike Level (g/100g)	0.030	2.015	4.996
Day 1	0.029	1.880	4.820
	0.030	1.900	4.980
Day 2	0.030	1.930	4.620
	0.030	1.950	4.320
Day 3	0.028	1.930	5.160
	0.026	1.980	4.740
Mean Recovery (%)	**96.0**	**95.7**	**95.5**

Full SLV Report for the FOS determination in Infant Formula and Adult Nutrition from Eurofins Carbohydrate Competence Centre

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Method title:
Determination of total fructans (inulins and fructo-oligosaccharides) in infant and adult nutritionals applying High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

Method applicability:
determination of the total fructan (FOS) content in all forms of infant, adult and/or pediatric formula (powders, ready to feed liquids, and liquid concentrates

Content

1. Introduction

Presently 2 different official AOAC methods are available for the determination of the total fructan content in food products, being AOAC 997.08 [1] and AOAC 999.03 [2]. Both analytical methods for inulin/FOS have typical different fields of application in the analysis of (food) samples. The different underlying principles of both analytical methods are the reason that there is not one 'golden standard' analytical method for the fructan determination. It is necessary to choose the best method for different matrices.

The principles and the pro's and contra's of both fructan analysis methods are described below.

In the **AOAC 997.03 method** [1] first the free fructose and free sucrose content are quantified. In the next step after enzymatic conversion of starch and maltodextrins, the total glucose is measured. The last step includes total conversion of inulin/FOS and sucrose into glucose and fructose and the quantitative analysis of the glucose and fructose after these enzymatic conversions. After three high performance anion exchange chromatographic (HPAEC-PAD) measurements the fructan content is calculated by subtracting the glucose, sucrose and fructose contents as measured in step 1 and 2 from the total fructose and glucose content as measured in step 3.This implies that large corrections have to be made for samples containing large quantities of fructose, glucose, sucrose, maltodextrin and/or starch. Subtraction of two large values in order to calculate much lower inulin/FOS values generally results in less precise data with a large standard deviation.

The principle of the **AOAC 999.03 method** [2] differs from AOAC 997.08 in that here all monosaccharides present after the combined amylases and sucrase treatment are removed by converting them into alditols (borohydride reduction). So, also samples with high contents of monosaccharides, sucrose, maltodextrin and/or starch can be accurately measured in this procedure because subtractions are not needed. However, there is a major drawback in this method since also the reducing end groups of the oligo-fructose molecules F_m are reduced into alditol end groups and so these end groups escape the analysis. This implies that recovery of difructose (F_2) is only 50%, for F_3 67 %, for F_4 75% etcetera. GF_n molecules do not have a reducing end group, so these are recovered completely. FOS material prepared by depolymerization of inulin contains in general high amounts of F_3 and F_4, so total recovery will be about 80% or even lower for these preparations.

In the new developed method a solution has been found for both the drawback of the AOAC 997.08 method (determination by difference) and the AOAC 999.03 method (reduction of end-standing fructose unit in fructo-oligosaccharides). Following the idea of Cuany etal [3] a graphitized carbon solid phase extraction (GC-SPE) column is used to eliminate the interfering monosaccharides glucose and fructose before the enzymatic hydrolysis of the fructans.

2. Principle analytical method

The investigated fructan analysis protocol is based on an aqueous extraction of the fructan constituents, followed by enzymatic hydrolysis of possibly present maltodextrins/malto-oligosaccharides and free sucrose. Graphitized Carbon Solid Phase Extraction (GC-SPE) columns are applied to concentrate the fructan constituents and to remove the free monosaccharides. The free monosaccharides are removed from the GC-SPE column by washing with aqueous sodium chloride solution. The concentrated fructan constituents are eluted from the GC-SPE with water/acetonitrile/TFA. Thereafter the eluted fructans are hydrolyzed with fructanase into their monosaccharides glucose and fructose. The glucose and fructose contents are determined with gradient elution High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

3. Analytical method

In appendix A a detailed description of the method is presented.

4. Validation samples and pure fructan ingredients

For this single lab validation study the samples have been used which were present in the kit with the the SPIFAN selected test-samples, provide by Covance. This sample kit was received in December 2015. The test kit contains 19 different samples as listed in table 1. Not all samples contain fructan.

Table 1. Content of the SPIFAN SLV sample kit

No	Product description	Lot number		Country of origin
Placebo products				
1	Child formula powder	00847RF00	powder	United States
2	Infant element powder	00796RF	powder	United States
3	Adult Nutritional RTF, high protein	00821RF00	liquid	United States
4	Adult Nutritional RTF, high fat	00820RF00	liquid	United States
5	Infant formula RTF, milk based	EV4H2Q	liquid	United States
Fortified products				
6	SRM 1849a	CLC10-b	powder	United States
7	Infant formula powder partially hydrolyzed milk based	410057652Z	powder	United States
8	Infant formula powder partially hydrolyzed soy based	410457651Z	powder	United States
9	Toddler formula powder milk based	4052755861	powder	Ireland
10	Infant formula powder milk based	4044755861	powder	Ireland
11	Adult nutritional powder low fat	00859RF00	powder	United States
12	Child formula powder	00866RF00	powder	United States
13	Infant elemental powder	00795RF	powder	United States
14	Infant formula powder FOS/GOS based	50350017W1	powder	Switserland
15	Infant formula powder milk based	K16NTAV	powder	United States
16	Infant formula powder soy based	E10NWZC	powder	United States
17	Infant formula powder RTF milk based	EV4H2R	liquid	United States
18	Adult nutritional RTF high protein	00730RF00	liquid	United States
19	Adult nutritional RTF high fat	0729RF00	liquid	United States

The samples are stored in the original package in a dry place, protected from light till the moment of use. According to the SPIFAN SMPR instruction for the SLV kit, all powder products except SRM 1849a

are reconstituted by dissolving 25 g of powder in 200 g of water. The SRM 1849a is weighted directly or it was reconstituted by dissolving 10 g in 90 g of water.
In this validation study three different standards of pure fructan ingredients have been applied, being:

- Orafti HP, a long chain inulin ingredient
- Oraftie P95, an hydrolyzed inulin ingredient consisting of both GFn en Fm constituents
- Nutraflora P95, a short chain fructo-oligosaccharide based on enzymatic sucrose elongation, mainly constisting of GF_2, GF_3, and GF_4 constituents

The purity of these standards was established by analysis according the AOAC 997.08 method.

5. Validation design

a. Introduction

In the SMPR 2014.02 [4] the following demands for the FOS analyses are described:

Minimal requirements for the FOS determination	
analytical range	0,03 – 5,0 %
LOQ	≤ 0,03%
Repeatability (%RSD_r)	≤ 6%
Reproducibility (%RSD_R)	≤ 12%
recovery	90 – 110%

The above mentioned requirements relate to reconstituted products.
The SLV is organized in accordance with “Appendix L: AOAC Recommended Guidelines for Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Single-Laboratory Validation” [5].

b. Calibration fit

The SPIFAN SLV guidance [5] demands for the calibration fit a minimum of 6 concentration levels that span the entire working range of the calibration. A minimum of 3 independent calibrations have to be measured with independently prepared standards.
To meet these demands, 3 different, independently prepared, stock solutions of glucose and fructose are prepared. Each stock solution is used to prepare an independent set of calibration standards of 12 different concentrations. Chitobiose is applied as an internal standard (IS). The 3 sets of 12 calibration samples have analyzed in different HPAEC-PAD system. The obtained data will be used to calculate non-linear (quadratic) calibration curves and to establish the dynamic range of the calibration curve. The demand for a good calibration is that the calculated lack of fit for all data calibration points is less than 5% with the exception of the lowest calibration concentration, which is allowed to be higher than 5%.

c. LOD and LOQ

For the determination of the LOD and LOQ the SPIFAN SLV guidandance [5] demands for 10 independent analyses of a blank sample or 10 independent analysis of an at low level spiked blank sample.
In this study one no fructan containing samples will be selected from the 19 samples in the SPIFAN SLV sample kit. After reconstitution of this samples, it will be spiked with a pure fructan ingredient at a concentration level slightly higher than the required LOQ level in the SMPR 2014.02 of ≤0.03%. The fructan content in this spiked sample will be measured in 10-fold applying repeatability conditions. The average fructan content and the fructan standard deviation (SD_r) are calculated by applying the following equations:

$$SD_r = \sqrt{\sum_1 (x_i - \vec{x})^2} \, /(n-1)$$

x_i = individual result of the measured fructan content in the spiked sample

$\bar{x}$ = average fructan content

SD_r = repeatability standard deviation in the average fructan content

Then the LOD and LOQ will be calculated as:

$$LOD = 3 \times SD_r$$
$$LOQ = 10 \times SD_r$$

d. Precision

The SPIFAN SLV guidance [5] requires that "all samples selected for precision studies will be analyzed in duplicate on each of 6 days using multiple analysts and instruments as practical for the different days". Fresh reagents and working standards are used each day. The obtained results are used to calculate the repeatability and reproducibility for each sample.

About 6 different fructan containing samples will be selected for this part of the investigation.
The repeatability standard deviation (SD_r) and reproducibility (SD_R) will be calculated from the obtained data.
Of each sample the SD_r and RSD_r are calculated by pooling the six SD's and RSD's of the six duplicate analyzes of that sample.
For calculating the SD_R and RSD_R the analytical data of all six days are put together, and then the average, SD_R and RSD_R are calculated.

e. Accuracy (Trueness)

The SPIFAN demands for the determination of the accuracy or trueness the determination of the spiked recovery of an appropriate sampling of SPIFAN matrices. In this study 6 fructan containing samples (after reconstition) in the SPIFAN sample kit are selected for the accuracy study. In section 6d, results of the precision study, the overall means of the fructan content in these samples have been established already and those values will be applied in this part of the accuracy study. According to the AOAC SLV instructions, the samples will be spiked at about 50% and 150% level of the original fructan content as determined in precision study. The spiked and unspiked samples will be analyzed in duplicate on each of 3 different days.
Each of the 3 days of this accuracy study another pure fructan ingredient will be applied for spiking. The purity of the respective fructan ingredients are established by subjecting these three pure fructan ingredients to the AOAC 997.08 analysis.
And each day another HPAEC-PAD instrument will be applied for the analyses.

f. Specificity

There are no specific SPIFAN demands concerning the determination of specificity of the method. In principle all kind of glucose and/or fructose containing carbohydrates (oligo- and polysaccharides) which are susceptible for hydrolysis into their corresponding monosaccharides by (side) activities of the used fructanase in last enzymatic hydrolysis step in the protocol, are potential interfering constituents. And also disaccharides/oligosaccharides which chromatographically show coinciding peaks with fructose or glucose will interfere. Thus potentially interfering carbohydrates in the new developed fructan method are amongst others free disaccharides (e.g. sucrose, maltitol, palatinose) and oligosaccharides, (resistant) malto-oligosaccharides (e.g. polydextrose), (soluble) starch, and GOS. Therefore the specificity of the method is demonstrated by showing that the above mentioned constituents don’t interfere with the FOS quantitation.

6. Results

a. Introduction

The SPIFAN sample kit contains 19 samples. It is known that not all samples contain fructan. In order to establish which samples contain fructan and which not, the whole series of 19 samples are analyzed in duplicate, being as two independent analyses performed at two different days by two different technicians. The samples have been analyzed as received, thus no reconstitution has been applied preceding these single analyses.
The results are summarized in table 2.

Table 2. **Fructan content in % m/m in the SPIFAN SLV sample set measured in the samples as received (no reconstitution applied) as independent duplicate by two different technicians at two different days.**

No	Product description	Lot number		Fructan content in the untreated sample (% m/m)	
1	Child formula powder	00847RF00	powder	2.38	2.40
2	Infant element powder	00796RF	powder	0.01	0.02
3	Adult Nutritional RTF, high protein	00821RF00	liquid	0.01	0.03
4	Adult Nutritional RTF, high fat	00820RF00	liquid	0.02	0.08
5	Infant formula RTF, milk based	EV4H2Q	liquid	0.00	0.00
6	SRM 1849a	CLC10-b	powder	0.00	0.00
7	Infant formula powder partially hydrolyzed milk based	410057652Z	powder	0.00	0.02
8	Infant formula powder partially hydrolyzed soy based	410457651Z	powder	0.13	0.10
9	Toddler formula powder milk based	4052755861	powder	2.21	2.00
10	Infant formula powder milk based	4044755861	powder	3.18	3.00
11	Adult nutritional powder low fat	00859RF00	powder	0.04	0.02
12	Child formula powder	00866RF00	powder	2.39	2.45
13	Infant elemental powder	00795RF	powder	0.02	0.00
14	Infant formula powder FOS/GOS based	50350017W1	powder	0.33	0.31
15	Infant formula powder milk based	K16NTAV	powder	0.01	0.01
16	Infant formula powder soy based	E10NWZC	powder	0.03	0.02
17	Infant formula powder RTF milk based	EV4H2R	liquid	0.01	0.00
18	Adult nutritional RTF high protein	00730RF00	liquid	0.01	0.01
19	Adult nutritional RTF high fat	0729RF00	liquid	0.45	0.40

Based on the results in table 2, it can be concluded that just 7 of the 19 samples contain fructan at a level of more than 0.04 % before reconstitution of the powdered samples. The fructan containing samples are marked in yellow.

The purity of the applied fructan standards in this study is established by determining the fructan content in these standards with the AOAC 997.08 method. The analytical results are very well in agreement with the analytical results obtained with the presently submitted SPIFAN method as can be seen in table 3.

Table 3 Fructan content (% m/m) in the applied fructan standards (duplicate averages) measured with the AOAC 997.08 and with the submitted SPIFAN methods.

Fructan standard ingredient	AOAC 997.08 method		Submitted SPIFAN method	
	duplicates	average	duplicates	average
Oraftie HP	91.7 93.0	**92.7**	94.6 90.5	**92.6**
Oraftie P95	85.8 85.4	**85.6**	84.7 80.8	**82.8**
Nutraflora P95	86.0 86.4	**86.2**	88.6 85.6	**87.1**

The duplicate averages as measured with the AOAC 997.08 method are used in the standard addition experiments for calculating the exact amount of added pure fructan.

b. Calibration fit

In this method quadratic calibration models are used for quantifying the glucose and the fructose contents. Glucose is the minor constituent in fructans. Therefore the working range for glucose is at lower levels than for fructose. In the standard daily routine just 5 calibration samples for glucose and fructose are applied, ranging for glucose from 0.05– 20 mg/l and for fructose from 0.90 – 90 mg/l. For testing the calibration models 12 calibration samples are used. Two times with the ISC5000_E instrument and one time with the ICS3000_D instrument the calibration models were established for this validation study. The results are summarized in table 4.

Table 4 Quadratic calibration models for the HPAEC-PAD quantification of glucose and fructose

Constituent	Concentration range (mg/l) injected analyte	HPAEC-PAD instrument	Calibration model	Correlation coefficient
24 March 2016				
Glucose	0.054 – 21.78	ICS5000_E	$y = 0.052 x^2 + 6.539 x +0.006$	$R^2 = 0.9999$
Fructose	0.887 – 177.4	ICS5000_E	$y = 0.409 x^2 + 17,534 + 0.070$	$R^2 = 0.9999$
10 May 2016				
Glucose	0.054 – 21.78	ICS5000_E	$y = 0.058 x^2 + 5.680 x +0.005$	$R^2 = 0.9999$
Fructose	0.887 – 177.4	ICS5000_E	$y = 0.1199 x^2 + 12.574 + 0.037$	$R^2 = 0.9999$
21 June 2016				
Glucose	0.051 – 20.49	ICS3000_D	$y = 0.044 x^2 + 5.754 x +0.003$	$R^2 = 1.0000$
Fructose	0.894 – 178.7	ICS3000_D	$y = 0.811 x^2 + 18.129 + 0.251$	$R^2 = 0.9998$

In which:
Y = calculated sugar (glucose or fructose) content in the injected 20 µl sample
X = peak area of the sugar (glucose or fructose) in the chromatogram
The relative residuals were calculated of the predicted concentration and the actual concentration was, with exception of the lowest standard sample, always less than 5% relative. For the lowest calibration standards in these extended quadratic calibration models the lack of fit at most about 15% relative.
In annex B the raw data of the calibration models and the calculated lack of fit of the individual calibration standards are given.

c. LOD and LOQ

The no fructan containing sample 7 (Infant formula powder partially hydrolyzed milk based (lot number 410057652Z)) was selected for the determination of the LOD and LOQ by standard addition experiments. The measurements have been carried out at two different days, being February 28th, 2016 and May 23rd, 2016. The standard addition has been done in the reconstituted sample. The Orafti P95 fructan was applied for the spiking. For the standard addition 100 µl of an fresh prepared aqueous Orafti P95 solution with a fructan content of 942,6 mg/50ml (Febr. 28) respectively 941.4 mg/50ml (May 23) was added to 4.00 ml of the reconstituted sample. With this addition the fructan concentration in the reconstituted sample was adjusted to 0.046 % which is slightly higher than the LOQ target of 0.03 %.
The results are summarized in table 4.
All the experimental results concerning the determination of the LOQ are presented in Annex C

Table 4 Summarizing results of the LOD and LOQ determination in the reconstituted sample no 7, Infant formula powder partially hydrolyzed milk based.

	February 28th, 2016		May 23rd, 2016	
	Fructan (%m/m)	Meet requirements	Fructan (%m/m)	Meet requirements
$\bar{x}$	0.044		0.048	
SD_r	0.002		0.003	
LOD = 3 x SD	0.005	Yes	0.009	Yes
LOQ = 10 x SD_r	0.016	yes	0.030	yes

In which:

x_i = individual result of the measured fructan content in the spiked sample
$\bar{x}$ = average fructan content
SD_r = repeatability standard deviation in the average fructan content

Moreover these data can also be applied to calculate the average recovery of the spiked amounts fructan in both series. The fructan addition was 0.046 % and the average fructan content in the tenfold analyses was 0.044 (Febr. 28) respectively 0.048 % (May 23), corresponding with average recoveries of 95.6 and 104.3% of the added amount fructan. This is very well within the target range of 90 – 110 %.

d. Precision

Based on the analytical results reported in table 2, 6 of the 7 fructan containing not-reconstituted samples (marked in yellow in table 2) are selected for this precision study. Sample 8 was excluded. The fructan content in the reconstituted sample 8 will be less than the LOQ. And after spiking this sample with 50% and 150% of its original fructan content, the spiked fructan content is still less than the LOQ meaning that this experiment will not result in reliable data.

The analyses for this precision study have been done at six different days in the period March – June 2016 by 2 different technicians and applying three different HPAEC-PAD instrument (one ICS3000 instrument and two different ICS5000 instruments) and each instrument was equipped with its own chromatographic column (Carbopack PA-1).

The results of the precision study are summarized in table 5. All experimental results of this precision study are given in Annex D

Table 5 Summarizing results of the repeatability and reproducibility investigations in the reconstituted samples.

sample no	sample description	number of analyses	average content % m/m	SD_r % m/m	RSD_r %	meet target	SD_R % m/m	RSD_R %	meet target
1	Child formula powder	5 x 2 + 1 x 1	0,287	0,004	1,4	yes	0,021	7,4	yes
9	Toddler formula powder milk based	6 x 2	0,223	0,003	1,6	yes	0,013	6,3	yes
10	infant formula powder milk based	6 x 2	0,263	0,003	1,1	yes	0,017	6,5	yes
12	child formula powder	6 x 2	0,282	0,003	1,1	yes	0,016	6,3	yes
14	infant formula powder FOS/GOS based	6 x 2	0,036	0,004	8,9	no	0,005	13,8	no
19	Adult nutritional RTF high fat	6 x 2	0,507	0,013	2,6	yes	0,021	4,6	yes

As can been seen in the experimental data in Annex C, there was an outlier value measured in the duplicate at day 1 in sample 1. This outlier was detected with Grubb’s outlier test. In the summarizing statistical evaluation in table 5, this outlying value was rejected. Therefore the number analyses in sample 1 is just 5 x 2 + 1 x1 instead of 6 x 2. This means that the SD_r and RSD_r are based on 5 duplicates instead of 6 duplicates. And the SD_R and RSD_R are based on 11 values instead of 12 measured fructan concentrations.
The RSD_r and RSD_R of sample no 14 “infant formula powder FOS/GOS based” don’t meet the target values of respectively of 6 and 12%. However this is not surprisingly because the fructan content in this sample is very low, in the range of the LOQ of the method. And for that reason the RSD_r and RSD_R are somewhat higher than the target values in the SMPR 2014.02.

e. Accuracy

The same 6 FOS containing samples (after reconstition) in the SPIFAN sample kit as selected for the precision study have been applied for the accuracy study. The average fructan content in these samples was already determined in the precision study. Those date are used in the accuracy study as the fructan content before spiking the samples with a pure fructan ingredient.
The established purity of the fructan ingredients Orafti HP, Orafti P95 and Nutraflora P95, applied for the standard additions, are given already in table 3.
The addition solutions are prepared by dissolving about 1.1 gram (weighted with an accuracy of 1 mg) in 100 ml water. For all samples except sample 14, the same amounts of fructan have been added in the addition experiments. The fructan content in sample 14 (infant formula powder FOS/GOS based) was very low, near the LOQ value. Therefore in case of this sample a much lower addition level was applied.
For the samples 1, 9, 10, 12 and 19 the addition at low level a volume of 0.70 ml was added to 4.00 ml of the reconstituted sample and for addition at the high level a volume of 2.00 ml was added to 4.00 ml of the reconstituted sample. For sample 14 the added volume of the standard fructan solutions was

reduced to 0,07 respectively 0,20 ml. In table 6 the exact added quantities of three different fructan standards Orafti HP, Orafi P95 and Nutraflora P95 are summarized.

Table 6 The fructan solutions prepared for the standard addition experiments.

fructan ingredient	**Orafti HP**	**Orafti P95**	**Nutraflora P95**
purity (AOAC 997.08)	92,70%	85,60%	86,20%
ingredient concentration	1,152 g/100ml	1,1504 g/100ml	1,1494 g/100ml
fructan concentration	10,63 mg/ml	9,878 mg/ml	9,908 mg/ml
spiked level at 0.07 ml addition	0,018 %m/m	0,017 % m/m	0,017 % m/m
spiked level at 0.20 ml addition	0,051 (% m/m	0,047 % m/m	0,047 % m/m
spiked level at 0,70 ml addition	0,158 % m/m	0,171 % m/m	0,148 % m/m
spiked level at 2,00 ml addition	0,354 % m/m	0,329 % m/m	0,330 % m/m

The results of this accuracy study are summarized in table 7. All the obtained experimental results are presented in Annex E.

Table 7 Summarizing results of the accuracy study in the reconstituted samples.

sample	addition level	average recovery (%)	SD_R
Sample 1, Child formula powder	low(50%)	90,8	6,3
	high (150%)	95,7	3,8
Sample 9, Toddler formula powder milk based	low(50%)	89,0	4,7
	high (150%)	93,2	1,2
Sample 10, Infant formula powder milk based	low(50%)	94,1	2,1
	high (150%)	94,6	2,9
Sample 12, Child formula powder	low(50%)	91,0	4,9
	high (150%)	101,5	7,1
Sample 14, Infant formula powder FOS/GOS based	low(50%)	92,8*)	6,3
	high (150%)	92,2	5,5
Sample 19, Adult nutritional RTF high fat	low(50%)	94,8	9,2
	high (150%)	95,3	4,5
average recovery		**93,7**	

Marked in yellow does not meet the target range 90 - 110%

*) one (Grubb's) outlier recovery value rejected (see Annex E)

The average recovery values in table 6 indicates that low addition levels tends to result in a slightly lower value for the fructan recovery. With the exception of the low addition level in sample 9, Toddler formula powder milk based, all average recovery values meet the target range 90 – 100% recovery.

With respect to sample 14, it should be noted that the low level fructan addition results in an very small increase in the fructan concentration which is less than the LOQ value of the method.
In general it can be concluded that the established recoveries are within the SMPR target range.

f. Specificity

In this specificity study different pure carbohydrates (disaccharides, oligosaccharides and starch) have been subjected to the fructan analytical protocol. The aim of these analyzes is to assess whether, and if so, to what extent, these non-fructan carbohydrates contribute to falsely too high measured fructan levels. To this end, a number of pure carbohydrate components with a test portion of 0.5 gram, has been analyzed by using the fructan analysis method.
The analytical results are expressed in different ways.
Firstly it is was calculated as a 4 gram liquid or reconstituted sample was analyzed. In that way the above applied test portion of 0.5 gram pure carbohydrate components simulates the presence of this constituent of 0.5/4 x 100% = 12.5 % level in a 4 gram liquid (reconstituted) test sample.
Secondly the result was expressed as the fructan content in the dry product, thus in 100% investigated matrix.
Moreover two different procedures have been followed for measuring and quantifying the fructan content. The standard procedure, as given in the protocol, is the *"no A0 correction"* columns. In the other procedure, *"with A0 correction"*, a blank subtraction step was introduced. In that case the analytical protocol has been extended with a blanc measurement before incubating the processed sample with the enzyme fructanase. This blanc measurement is done directly after the graphitized carbon solid phase extraction (GC-SPE) procedure. After the GC-SPE the sample is split in two aliquots. One aliquot is treated with the fructanase exactly conform the protocol and analyzed. In the other aliquot no fructanase hydrolysis is carried out but is directly analyzed with the HPAEC-PAD as a blank measurement. Then final fructan content is calculated by subtracting the blank result from the result obtained with the standard procedure. The difference is the amount of fructose and glucose which are set free by the fructanase hydrolysis. In that way easily can be corrected amongst others for chromatographic peaks which coincide with either fructose and glucose without being fructose or glucose.
The results are presented in table 8.
Looking to the results in the column "expressed in liquid sample" it can be concluded that, with the exception of Litesse II polydextrose, applying the standard protocol without blank correction ("no A0 correction") of all potential interferences (at the high 12,5 % level in the sample to analyze!) the erroneously measured fructan contents are less than the LOQ and therefore of no concern. For the Litesse II polydextrose the erroneously measured fructan content is at the level of the LOQ.
In the table for the constituents Vivinal GOS and Litesse II polydextrose, the worst case scenario's are presented. The chromatograms (figure 1 and 2) of these two constituents shows chromatographic signals near the fructose and glucose peaks with retention times which differs somewhat of the calibration standards. However in case the GOS and polydextrose constituents are present (at these high concentration levels!) in fructan containing samples, it is very likeable that these peaks cannot been distinguished from the fructose and glucose peaks coming from the hydrolyzed fructans.

In the column “expressed in dry product” with “no A0 Correction” the erroneously measured fructan content is for all constituents, except Vivinal GOS and Litesse II polydextrose, at the LOQ level or slightly higher. In this case a blank correction gives a significant improvement., especially for Vivinal GOS and Litesse II polydextrose. However it is an unrealistic situation that in the tested pure constituents low contents of fructan should be analyzed. Moreover those pure ingredients are certainly not included in the scope of this method
Thus it can be concluded that the specificity of this new method is good.

Table 8 Results of the specificity experiments

500 mg test portion of the compound investigated	fructan content in % m/m			
	expressed in liquid sample with 12.5% of the compound added		expressed in dry product	
	no A0 correction	with A0 correction	no A0 correction	with A0 correction
Fibersol resistant maltodextrin	0,004	0,002	0,035	0,014
Soluble starch	0,010	0,009	0,077	0,069
Palatinose	0,005	0,004	0,037	0,033
Maltitol	0,002	0,001	0,015	0,007
Sucrose (250mg)	0,006	0,005	0,048	0,039
Sucrose (500 mg)	0,006	0,005	0,049	0,041
Sucrose (1000 mg)	0,004	0,003	0,029	0,023
Vivinal GOS	0,023	0,002	0,182	0,017
Litesse II polydextrose	0,034	0,007	0,271	0,058

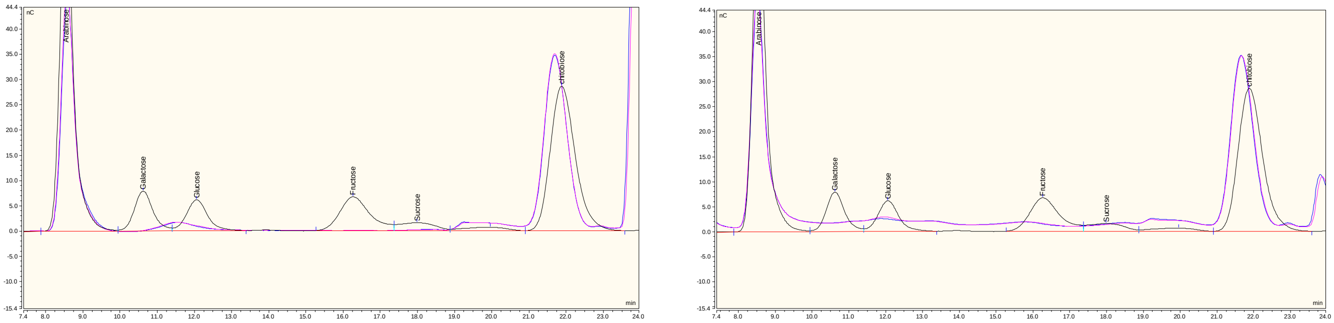


Figure 1
Chromatograms of Vivinal GOS (left) and Litesse II polydextrose (right).
In blue the chromatogram with no A0 correction, in pink the chromatogram with A0 correction and in black the standards arabinose (alternative internal standard), galactose, glucose, fructose, sucrose and chitobiose (internal standard)).

7. Conclusions

The submitted new analytical method for the determination of the fructan content in infant formula and aldult/pediatric nutritional formula meets the requirements as given in SMPR 2001.002.
The established LOQ is equal or less than the target value of 0.03%. With the exception of the tested sample with a fructan content at LOQ level, the repeatability is much better than the target of 6% and the reproducibility is much better than the target of 12%. The average accuracy is of the method is 94%. This is within the SMPR target range 90 – 110%. However the range in the 48 accuracy experiments was 85 – 114%, just a little bit wider than the target range. The specificity of the method is good.
Last but not least, in this new method no correction whatever has to be made for the type of fructans. Reducing and/or non-reducing fructan end-groups don't affect the analytical result. This is a great advantage over the AOAC 999.03 method. And just one chromatographic run is needed instead of three runs in case of the AOAC 997.08 method

8. Literature

1. AOAC official method 997.08. Fructans in food products, ion exchange chromatographic method, first action 1997, final action 1999.

2. AOAC official method 997.08. Measurement of fructan in food, enzymatic/spectrophotometric method, first action 1999, final action 2005.

3. Dennis Cuany, Thierry Bénet, Sean Austin. Development and single-laboratory validation of a method for the determination of total fructans in infant formula. Journal AOAC Int. 2010 **93(1)** 202-212

4. AOAC SMPR 2014.002, Standard method performance requirements for fructans in infant formula and adult/pediatric nutritional formula, Approved by AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). Final Version Date: March 18, 2014.

5. Appendix L: AOAC Recommended Guidelines for Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Single-Laboratory Validation, approved by the AOAC Expert Review Panel on Infant Formula and Adult Nutritionals in September 2011.

ANNEX A Detailed method description

A-1 Scope of the method

Determination of the fructan content in all forms of infant, adult and/or pediatric formula (powder, ready-to-feed liquids, and liquid concentrates.

A-2 Principle of the method

The fructan analysis protocol is based on an aqueous extraction of the FOS constituents, followed by enzymatic hydrolysis of possibly present maltodextrins/malto-oligosaccharides and free sucrose. Graphitized Carbon Solid Phase Extraction (GC-SPE) columns are applied to concentrate the FOS constituents and to remove the free monosaccharides. The free monosaccharides are removed from the GC-SPE column by washing with aqueous sodium chloride solution. The concentrated fructan constituents are eluted from the GC-SPE with water/acetonitrile/TFA. Thereafter the eluted fructans are hydrolyzed with fructanase into their monosaccharides glucose and fructose. The glucose and fructose contents are determined with gradient elution High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

A-3 Apparatus

Metal free alkaline resistant anion exchange chromatograph with post column derivatization unit, consisting of
- gradient pump
- autosampler with 20 µl injector
- column oven adjusted at 20°C
- CarboPak PA-1 guard column 2 x 50 mm
- CarboPak PA-1 analytical column 2 x 250 mm
- post column pump with pulse damping restriction
- mixing coil
- pulsed amperometric detector
- integration software

Analytical balance, accurately weighting to 0.1 mg
pH-meter
Micro centrifuge tubes 2 ml
Centrifuge for 2 ml micro tubes
Waterbath at 80 ± 2°C
Waterbath at 40 ± 2°C
Ultraturrax
black band filter paper
Multipipet
Graphitized carbon solid phase extraction columns (GC-SPE)
Vacuum manifold applicable for the GC-SPE
Single –use syringes
Syringe filters 0.2 µl
Screw cap closed 15 ml polypropylene tubes

A-4 Chemicals

Water, Millipore Q quality
Acetonitril, HPLC-grade
Maleic acid, purity ≥ 99%
Acetic acid glacial, 100% anhydrous
Trifluoroacetic acid
Hydrochloric acid (36.5 – 38%)
Sodium hydroxide 33 % m/m
Sodium hydroxide 50 % m/m
Potassium cyanohexaferrate(II)trihydrate
Zinc acetate
Sodium chloride
Sodium azide
Mix of pure enzymes sucrase + β-amylase + pullulanase + maltase from Megazyme fructan assay kit (K-FRUC)
D-(+)-glucose, purity ≥ 99%
D-(+)- fructose purity ≥ 99%
Chitobiose

A-5 Reagents

Sodium azide 0.5%, dissolve 1 g sodium azide in 200 ml demineralized water

Liquid chromatographic eluent A, *1M sodium acetate solution*, dissolve in the eluent bottle 136 ± 1 g sodium acetate trihydrate in 1000 ml carbonate free Millipore-Q water and purge directly with helium. Keep the eluent continuously under a blanket of helium

Liquid chromatographic eluent B, 0.2 M Sodium hydroxide solution, weight 3846 ± 5 g carbonate free Millipore-Q water in the eluent bottle and purge with helium for at least 15 min. Pipet carefully 40 ml 50% sodium hydroxide in the water in the flask and purge with helium for another 15 min. Keep the eluent continuously under a blanket of helium.

Liquid chromatographic eluent C, *Millipore Q water with sodium azide*, fill a 4 l eluent bottle with 3900 ml carbonate free Millipore-Q water. Add with a graduated glass 100 ml 0.5% sodium azide solution and purge with helium for 15 min. Keep the eluent continuously under a blanket of helium.

Post column eluent, *0.3M sodium hydroxide solution*, liquid chromatographic eluent, weight 3762 ± 5 g carbonate free Millipore-Q water in the eluent bottle and purge with helium for at least 15 min. Pipet carefully 60 ml 50% sodium hydroxide in the water in the flask and purge with helium for another 15 min. Keep the eluent continuously under a blanket of helium.

Sodium maleate buffer, 0.1M pH = 6.5: dissolve In a 1000 ml volumetric flask 11.6 maleic acid in 900 ml water. Adjust to pH = 6.5 ± 0.05 with 33 % sodium hydroxide solution. Make up with demineralized water to the mark and homogenize.

1M sodium hydroxide: dissolve 40 ± 1 g in 500 ml water in a 1000 ml volumetric flask. After cooling down to room temperature, make up with demineralized water to the mark and homogenize.

Sodium acetate buffer, 0.1M pH = 4.5, dissolve in a1000 ml volumetric flask 5,8 ml glacial acetic acid in 900 ml water. Adjust to pH = 4.5 ± 0.05 with 1M sodium hydroxide solution. Make up with demineralized water to the mark and homogenize.

1M hydrochloric acid: add in a 1000 ml volumetric flask 500 ml water. Then add with a graduated glass 83 ml hydrochloric acid (36.5 – 38%). Make up with demineralized water to the mark and homogenize.
80% Acetonitrile + 0.1% TFA, wash solution GC-SPE: add in a 200 ml volumetric flask 160 ml acetonitrile and 100 µl trifluoroacetic acid. Make up with demineralized water to the mark and homogenize.
25% acetonitrile + 0.05% TFA: add in a 1000 ml volumetric flask 250 ml acetonitrile. Add 100 µl trifluoroacetic acid. Make up with demineralized water to the mark and homogenize.
Carrez I solution: dissolve 106 g potassium hexacyanoferrate (II) trihydrate in 1000 ml demineralized water and store in a brown bottle.
Carrez II solution: Dissolve in a 1000 ml volumetric flask 220 g zinc acetate in 900 ml demineralized wateradd 29 ml glacial acetic acid. Make up with demineralized water to the mark and homogenize.
1M sodium chloride: dissolve in a 1000 ml volumetric flask 58.5 ± 0.1 g sodium chloride in 500 ml demineralized water. Dissolve and homogenize. Make up with demineralized water to the mark and homogenize.
Enzyme mix A (Sucrase/β-amylase/pullulanase/malase): dissolve the content of bottle 1 in the Megazyme fructan assay kit (K-FRUC) in 22 ml 0.1M sodium maleate buffer pH = 6.5. Mix well and distribute the obtained solution in 4 ml portions in 15 ml screw cap closed polypropylene tubes. Store the tubes in the freezer at – 20°C until use.
Enzyme mix B (fructanase): dissolve the content of bottle 2 in the Megazyme fructan assay kit (K-FRUC) in 22 ml 0.1M sodium acetate buffer pH = 4.5. Mix well and distribute the obtained solution in 4 ml portions in 15 ml screw cap closed polypropylene tubes. Store the tubes in the freezer at – 20°C until use.

A-6 Sample preparation

Reconstitution of (milk) powder products. Dissolve 25.0 ± 0.1 g powder sample in 200 ± 0.2 g demineralized water in an erlemeyer flask. The mixture is well homogenized at ambient temperature. The reconstitution factor F can easily be calculated with the following formula:

$F = (w_1 + w_2) / w_1$ In which:

F = factor reconstitution
w_1 = weight powder original
w_2 = weight added amount of water

The weight of the test sample depends on the expected fructan content in the sample to analyze. The scheme of the weight of the test portion is given table 1.

Table 1 Scheme of the weight of the testportion dependent on the expected fructan content

Expected fructan content in the sample	Required test portion of the sample (g)
< 1% m/m	4 ± 0.1
reconstituted samples	4 ± 0.1
> 1% m/m	1 ± 0.1

Put the required test portion weight, accurately weighted at 0.1 mg, in an erlemeyer flask and disperse in 40 ml demineralized water. In case of lumps, use an ultraturrax to disperse to sample.
Check the pH and, if necessary, adjust the pH within the range 5.0 – 9.0 with respectively 1M hydrochloric acid or 1M sodium hydroxide solutiom.
Heat the sample solutions during 20 min. in a waterbath adjusted to 80 ± 2 °C
Cool down to room temperature in a cold water or icebath.
Transfer the sample quantitatively in a 100 ml volumetric flask. Make up with demineralized water to the mark and homogenize.
Filter the sample solution over black band filter paper.
Diluted the filtered sample solution with demineralized water according to the dilution scheme in table 2

Table 2 Dilution scheme

Expected fructan content (%)	Dilution factor	ml prepared sample solution	ml demineralized water
0 - 5	1	1	0
5 - 10	2	1.00	1.00
10 – 20	5	1.00	4.00
20 - 100	20	0.25	4.75

Pipet 0.2 ml of the according table 2 diluted sample solution in a 2 ml micro centrifuge tube
Add 0.10 ml chitobiose internal standard solution
Add 0.20 ml enzyme mix A and homogenize
Heat the obtained solution in the micro centrifuge tubes during 90 min. in a waterbath adjusted to 40 ± 2 °C
Add 10 µl Carrez I solution and homogenize
Add 10 µl Carrez II solution and homogenize
Centrifuge the prepared samples in the micro centrifuge tubes at 14000 rpm during 10 min..

Optional determination of the free sugars

Mix 400 µl of the centrfugated sample with 400 µl 0.1M sodium acetate buffer pH = 4.5.
Filter the mixture with a 0.2 µm syringe filter in a chromatographic vial and close the vial with a screw cap.
The sample is now ready for the chromatographic analysis of the free sugars.

A-7 GC-SPE removal of monosaccharides

A-7-a Actrivation of the GC-SPE columns

Mount the required number of GC-SPE column on the vacuum manifold.
Flush the GC-SPE 4 times with 400 µl 80% acetonitrile + 0.1% TFA followed by three times flushing with 400 µl demineralized water

NOTE Perform all flushing steps at gravity conditions (without applying vacuum or positive pressure) and take care that at each flushing step the entire flushing solution passes the GS-SPE

A-7-b SPE clean-up

Pipet 400 µl of the centrifuged sample at the top of the GC-SPE column and flush carefully over the activated GC-SPE column.
Flush (at gravity conditions) with successively
- 1 x 400 µl 1M sodium chloride solution
- 2 x 800 µl 1M sodium chloride solution
- 5 x 800 µl demineralized water till just dry

Collect the following 5 respective flushing's with 400 µl of 25% acetonitrile + 0.05 TFA solution. Suck the GC-SPE columns just dry. And homogenize the collected eluates.

A-8 Hydrolysis of the fructans

Pipet 1.0 ml of the collected GC-SPE eluate in a 2 ml micro centrifuge tube.
Add 350 µl 0.1M sodium acetate buffer ph = 4.5.
Pipet 0.1 ml enzyme mix B (fructanase) and homogenize.
Incubate the solution during 40 min. in a waterbath adjusted to 40 ± 2 °C
Filter the hydrolysate with a 0.2 µm syringe filter in a chromatographic vial and close the vial with a screw cap.
The sample is now ready for the chromatographic analysis

A-9 Preparation of the calibration standard solutions

Primary calibration standard: dissolve 100mg glucose and 400 mg fructose, accurately weighted at 0.1 mg, in a 500 ml volumetric flask in 100 ml demineralized water. Add 25 ml acetonitrile, make up with demineralized water to the mark and homogenize. The concentration of the stcock standard solution is calculated as:

$$C_{stock} = w \times z / V$$

In which
C_{stock} = concentration standard in mg/ml
w = weight of the dissolved standard in mg
z = purity of the standard
V = volume of the volumetric flask in ml

In total 5 calibration standards are applied, S1, S2, S3, S4, and S5
The calibration standards S1, S2 and S3 are prepared by two successive dilutions as specified in table 3. Pipet the specified volume of the primary standard in a 15 ml polypropylene tube. Pipet also the specified amount of demineralized water in the polypropylene tube and homogenize.

Table 3 First dilution of the primary standard for preparing the calibration standards S1, S2 and S3

Dilution for preparing intermediate calibration standard	Volume primary calibration standard (ml)	Volume demineralized water (ml)	
(S1)	0.1	4.9	
(S2)	0.5	4.5	
(S3)	2.5	2.5	

Compose the calibration standards by pipetting according to the scheme in table 4

Table 4 Pipetting scheme for preparation of the calibration standards

Calibration standard	Volume intermediate calibration standard (ml)	Volume primary calibration standard (ml)	Volume chitobiose stock solution (ml)	Volume demineralized water (ml)	Volume 20% acetonitrile solution (ml)	Total volume (ml)
S1	0.8	0	0.4	0.1	13.2	14.5
S2	0.8	0	0.4	0.1	13.2	14.5
S3	0.8	0	0.4	0.1	13.2	14.5
S4	0	0.8	0.4	0.1	13.2	14.5
S5	0	1.6	0.4	0.1	12.4	14.5

The concentrations of the sugars in the intermediate calibration standards (S1), (S2) and (S3) are calculated with the formula:

$$C_{interm} = (V_{stock} \times C_{stock})/5$$

The concentration of the secondary standards S1, S2 and S3 is calculated with the formula:

$$C_{stand} = (V_{interm} \times C_{interm})/14.5$$

The concentrations of the secondary calibration standards S4 and S5 are calculated with the formula:

$$C_{stand} = (V_{stock} \times C_{stock})/14.5$$

In which:
C_{stock} = concentration sugar (mg/l)
C_{interm} = concentration intermediate calibration standard (mg/l)
C_{stand} = concentration secondary calibration standard (mg/l)
V_{stock} = volume stock solution in intermediate solution (ml)
V_{interm} = volume intermediate solution in secondary standard solution.
5.0 = volume intermediate solution (ml)
14.5 = total volume secondary calibration standard (ml)

A-10 Chromatographic conditions

The analysis of the prepared samples , standards(and blanks if required)are performed on the HPAEC-PAD system under the following conditions:
- flow mobile phase 0.25 ml/min for a 2 mm ID column
- temperature column oven 20 °C
- injection volume 20 µl
- linear gradient elution profile according to the scheme in table 5

Table 5 Linear gradient elution profile for the chromatographic separation of the sugars

Time (min.)	% Eluent A 1M CH_3COONa	% Eluent B 0.2M NaOH	% Eluent C H_2O
0	0.0	7.5	92.5
13.0	00.0	7.5	92.5
14.0	00.0	25.0	75.0
20.0	00.0	25.0	75.0
21.0	30.0	40.0	30.0
28.0	30.0	40.0	30.0
30	0.0	4.0	60.0
31	0.0	7.5	92.5
43	0.0	7.5	92.5

Post column pump:
- flow post column sodium hydroxide solution 0.13 ml/min
- minimal back pressure 500 psi

Detector adjustment:

The time program of the PAD waveform is specified in table 6

Table 6 Time programming of the detector potential settings of the gold working electrode

Step	Time (sec)	Potential gold electrode$^{**)}$	Integration detector signal
1	0.00	0.10	
2	0.20	0.10	start
3	0.40	0.10	end
4	0.41	-2.00	
5	0.42	-2.00	
6	0.43	0.60	
7	0.44	-0.10	
8	0.50	-0.10	

$^{**)}$ potential vs Ag/AgCl reference electrode

Integration window 0.20 – 0.40 s

A-11 calibration and calculations

For each analyte (glucose and fructose) use the instrument software to plot a five point standard curve of (instrument response for the analyte/instrument response for the internal standard chitobiose) against the concentration of the analyte. Fit a quadratic curve to the data without forcing through zero ($y = ax^2 + bx + c$). Apply the peak area as instrumental response.

The analyses are done with bracketed calibration standards. First the five secondary calibration standards (S1 –S5) are analyzed. Then 12 samples are analyzed followed by the five secondary calibration samples. Thus alternately be analyzed 5 standards and 12 samples. The calibration curve is calculated using both the 5 standards before the series of 12 samples and the 5 standards measured after the series with 12 samples.

Use the calibration curve to calculate the glucose and fructose content in the injected samples.

The total fructan content in the analyzed ample is calculated by the following formula:

$$C_{fructan} = C_{glucose} + (C_{fructose} \times 0.91)$$

In which:

$C_{fructan}$ = concentration fructan in sample (%m/m)

$C_{glucose}$ = concentation glucose in sample (%m/m)set free by the fructanase

$C_{fructose}$ = concentration fructose in sample (%m/m) set free by the fructanase

0.91 = anhydro-factor for oligomers based on an average chain length of 10 monomers

For reconstituted samples the fructan content in the original non-reconstituted sample can be calculated from the established fructan content in the reconstituted sample by multiplying with the reconstitution factor:

$$C_{fructan,os} = C_{fructan,rs} \times F$$

In which:

$C_{fructan,\ os}$ = fructan content original (not reconstituted sample) in % m/m

$C_{fructan,\ rs}$ = fructan content in the reconstituted sample

F = factor reconstitution (see A-6 sample preparation)

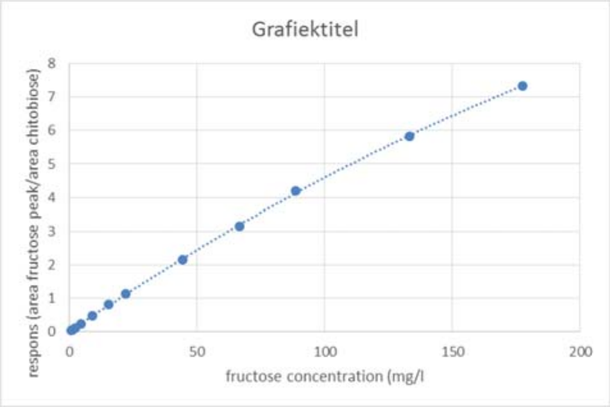
ANNEX B Experimental data lack of fit quadratic calibration curves

Results calibration test 24 March 2016

Fructose calibration ISC 5000 E instrument

Fructose range: 0.887 – 177.4 mg/l $y = 0.409 x^2 + 17,534 x + 0.070$ $R^2 = 0.9999$

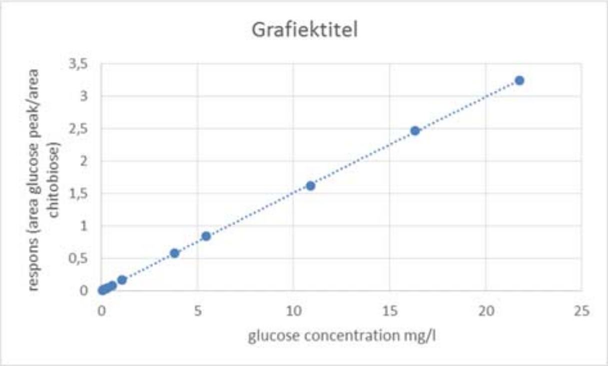
fructose respons t.o.v. IS	fructose conc mg/l	fructose calculated mg/l	lack of fit	
			absolute	%
0,0426	0,887	1,026	-0,139	-15,6
0,0619	1,331	1,376	-0,045	-3,4
0,114	2,218	2,327	-0,109	-4,9
0,235	4,436	4,549	-0,113	-2,5
0,473	8,873	9,017	-0,144	-1,6
0,816	15,53	15,59	-0,062	-0,4
1,15	22,18	22,19	-0,010	0,0
2,16	44,36	43,22	1,14	2,6
3,15	66,55	65,45	1,10	1,7
4,20	88,73	90,62	-1,89	-2,1
5,82	133,1	133,2	-0,1	-0,1
7,34	177,5	177,1	0,4	0,2



Glucose calibration ISC 5000 E instrument

Glucose range 0.054 – 21.78 mg/l $y = 0.052 x^2 + 6.539 x + 0.006$ $R^2 = 0.9999$

fructose respons t.o.v. IS	fructose conc mg/l	fructose calculated mg/l	lack of fit	
			absolute	%
0,0426	0,887	1,026	-0,139	-15,6
0,0619	1,331	1,376	-0,045	-3,4
0,114	2,218	2,327	-0,109	-4,9
0,235	4,436	4,549	-0,113	-2,5
0,473	8,873	9,017	-0,144	-1,6
0,816	15,53	15,59	-0,062	-0,4
1,15	22,18	22,19	-0,010	0,0
2,16	44,36	43,22	1,14	2,6
3,15	66,55	65,45	1,10	1,7
4,20	88,73	90,62	-1,89	-2,1
5,82	133,1	133,2	-0,1	-0,1
7,34	177,5	177,1	0,4	0,2

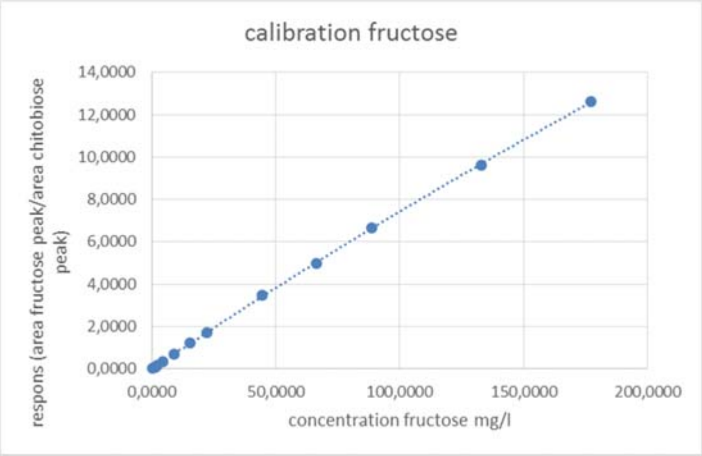


Results calibration test 10 May 2016

Fructose calibration ISC 5000 E instrument

Fructose range: 0.887 – 177.4 mg/l $y = 0.1199\,x^2 + 13.574x + 0.03780$ $R^2 = 0.9999$

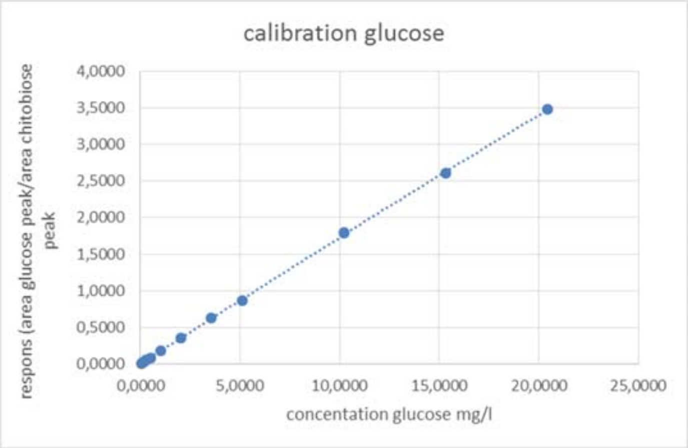
respons Fructose/chitobiose tov IS	fructose conc mg/ml	fructose calculated	lack of fit absolute	%
0,0159	0,2215	0,2373	-0,016	-7,1
0,0966	1,329	1,253	0,076	5,7
0,162	2,215	2,074	0,141	6,3
0,341	4,430	4,345	0,085	1,9
0,684	8,860	8,694	0,166	1,9
1,23	15,50	15,72	-0,22	-1,4
1,72	22,15	21,99	0,16	0,7
3,49	44,30	45,33	-1,03	-2,3
4,99	66,45	65,82	0,63	0,9
6,66	88,60	89,07	-0,48	-0,5
9,61	132,9	132,0	0,9	0,7
12,6	177,2	177,6	-0,4	-0,2



Glucose calibration ISC 5000 E instrument

Glucose range 0.054 – 21.78 mg/l $y = 0.052\,x^2 + 6.539\,x + 0.006$ $R^2 = 0.9999$

respons glucose/chitobiose	glucose conc mg/l	fructose calculated mg/ml	lack of fit absolute	%
0,0080	0,0511	0,0504	0,0006	1,3
0,0168	0,1022	0,1006	0,0016	1,5
0,0347	0,2043	0,2019	0,0024	1,2
0,0572	0,3065	0,3300	-0,0235	-7,7
0,0853	0,5108	0,4898	0,0210	4,1
0,182	1,022	1,040	-0,018	-1,8
0,358	2,043	2,046	-0,003	-0,1
0,635	3,576	3,633	-0,057	-1,6
0,863	5,108	4,953	0,155	3,0
1,795	10,22	10,39	-0,17	-1,7
2,606	15,32	15,20	0,13	0,8
3,479	20,43	20,46	-0,03	-0,2

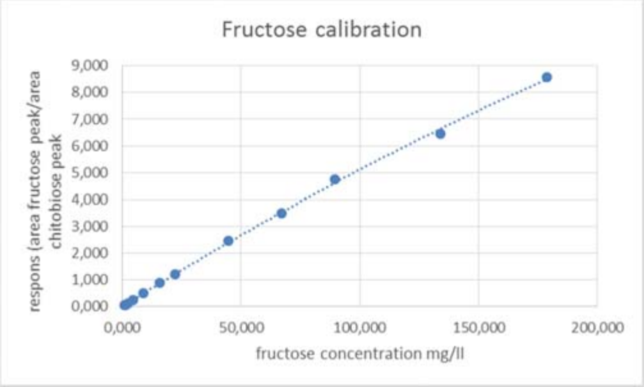


<u>Results calibration test 21 June 2016</u>

<u>Fructose calibration ISC 3000 D instrument</u>

Fructose range: 0.894 – 178.7 mg/l $y = 0.811 x^2 + 18.129 x + 0.251$ $R^2 = 0.9998$

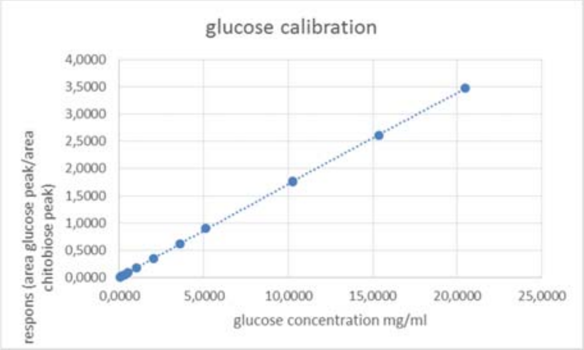
respons Fructose/chitobiose	fructose conc mg/l	fructose calculated mg/l	lack of fit	
			abs	%
0,050	0,894	0,954	-0,060	-6,7
0,075	1,341	1,387	-0,046	-3,5
0,126	2,234	2,277	-0,043	-1,9
0,253	4,469	4,525	-0,056	-1,3
0,501	8,937	8,951	-0,014	-0,2
0,886	15,64	15,93	-0,29	-1,9
1,22	22,34	22,16	0,18	0,8
2,47	44,69	45,90	-1,22	-2,7
3,49	67,03	66,22	0,81	1,2
4,74	89,37	92,43	-3,06	-3,4
6,47	134,1	130,5	3,5	2,6
8,56	178,7	180,1	-1,4	-0,8



<u>Glucose calibration ISC 3000 D instrument</u>

Glucose range 0.051 – 20.49 mg/l $y = 0.044 x^2 + 5.754 x + 0.003$ $R^2 = 1.000$

respons Glucose/chitobise	glucose conc mg/l	glucose calculated mg/l	lack of fit	
			absolute	%
0,0093	0,0512	0,05622	-0,0050	-9,7
0,0182	0,1025	0,10763	-0,0052	-5,0
0,0350	0,2049	0,20406	0,0009	0,4
0,0519	0,3074	0,30118	0,0062	2,0
0,0881	0,5123	0,51022	0,0021	0,4
0,171	1,025	0,989	0,035	3,5
0,354	2,049	2,043	0,006	0,3
0,615	3,586	3,560	0,026	0,7
0,900	5,123	5,220	-0,097	-1,9
1,761	10,25	10,27	-0,03	-0,3
2,601	15,37	15,27	0,10	0,7
3,475	20,49	20,54	-0,04	-0,2



ANNEX C Experimental data LOD and LOQ

Experimental data of the LOD and LOQ determination in the fructan spiked reconstituted sample no 7, Infant formula powder partially hydrolyzed milk based.

repetition no	date	
	28 Februari 2016	23 May 2016
	Fructan concentration (%)	Fructan concentration (%)
1	0,044	0,047
2	0,044	0,044
3	0,043	0,045
4	0,042	0,046
5	0,042	0,045
6	0,045	0,051
7	0,044	0,049
8	0,047	0,050
9	0,045	0,049
10	0,046	0,053
average	0,044	0,048
SD_r	0,002	0,003
LOD	0,005	0,009
LOQ	0,016	0,030

ANNEX D Experimental data precision study.

Sample 1 **Child formula powder, lot no 00847RF00**

	Fructan concentration (%m/m)					
day	repetition 1	repetition 2		Daily average	Daily SD_r	Daily RSD_r (%)
1	0,278	**0,375**		0,327	**0,069**	**21,0**
2	0,306	0,304		0,305	0,001	0,5
4	0,294	0,290		0,292	0,003	1,0
5	0,255	0,246		0,251	0,006	2,5
7	0,302	0,307		0,305	0,004	1,2
8	0,287	0,283		0,285	0,003	1,0
Total average	0,287	0,301			**Pooled RSD_r**	**8.8**
SD_R	0,019	0,042			**F-test outlier**	
RSD_R	6.5	13.9	**Excluding the outlier at day 1**			
Total average	**0,294**		**Total average**		**0,287**	
SD_R total	**0,032**		**SD_R total**		**0,021**	
RSD_R total	**14,1**		**RSD_R total**		**7,4**	

Grubb'stest outlier

Ex outlier
Pooled RSD_r
1.4

monster 9 **Toddler formula powder milk based**

	Fructan concentration (%m/m)					
day	repetition 1	repetition 2		Daily average	Daily SD_r	Daily RSD_r (%)
1	0,230	0,228		0,229	0,001	0,6
2	0,232	0,228		0,230	0,003	1,2
4	0,217	0,221		0,219	0,003	1,3
5	0,200	0,197		0,199	0,002	1,1
7	0,221	0,231		0,226	0,007	3,1
8	0,236	0,237		0,237	0,001	0,3
Total average	0,223	0,224			**Pooled RSD_r**	**1.6**
SD_R	0,013	0,014				
RSD_R	0,013	0,014				
Total average	**0,223**					
SD_R total	**0,013**					
RSD_R total	**6,3**					

sample 10 **Infant formulapowder milk based**

	Fructan concentration (%m/m)					
day	repetition 1	repetition 2		Daily average	Daily SD$_r$	Daily RSD$_r$ (%)
1	0,236	0,239		0,238	0,002	0,9
2	0,266	0,273		0,270	0,005	1,8
4	0,270	0,274		0,272	0,003	1,0
5	0,241	0,245		0,243	0,003	1,2
7	0,275	0,273		0,274	0,001	0,5
8	0,283	0,279		0,281	0,003	1,0
Total average	0,262	0,264			**Pooled RSD$_r$**	**1.1**
SD$_R$	0,019	0,017				
RSD$_R$	7.3	6.4				
Total average	**0,263**					
SD$_R$ total	**0,017**					
RSD$_R$ total	**6,5**					

sample 12 **Child formula powder**

	Fructan concentration (%m/m)					
day	repetition 1	repetition 2		Daily average	Daily SD$_r$	Daily RSD$_r$ (%)
1	0,283	0,279		0,281	0,003	1,0
2	0,299	0,293		0,296	0,004	1,4
4	0,279	0,279		0,279	0,000	0,0
5	0,254	0,247		0,251	0,005	2,0
7	0,297	0,295		0,296	0,001	0,5
8	0,289	0,290		0,290	0,001	0,2
Total average	0,284	0,281			**Pooled RSD$_r$**	**1.1**
SD$_R$	0,016	0,018				
RSD$_R$	5.6	6.4				
Total average	**0,282**					
SD$_R$ total	**0,016**					
RSD$_R$ total	**6,3**					

sample 14

Fructan concentration (%m/m)

day	repetition 1	repetition 2		Daily average	Daily SD_r	Daily RSD_r (%)
1	0,036	0,047		0,042	**0,008**	**18,7**
2	0,037	0,039		0,038	0,001	3,7
4	0,037	0,041		0,039	0,003	7,3
5	0,027	0,030		**0,029**	0,002	7,4
7	0,040	0,040		0,040	0,000	0,0
8	0,041	0,040		0,041	0,001	1,7
gem	0,036	0,040			**Pooled RSD_r**	**8.9**
std	0,005	0,005				
gem totaal	**0,038**					
std totaal	**0,005**					
VC(%) totaal	**13,8**					

sample 19

Fructan concentration (%m/m)

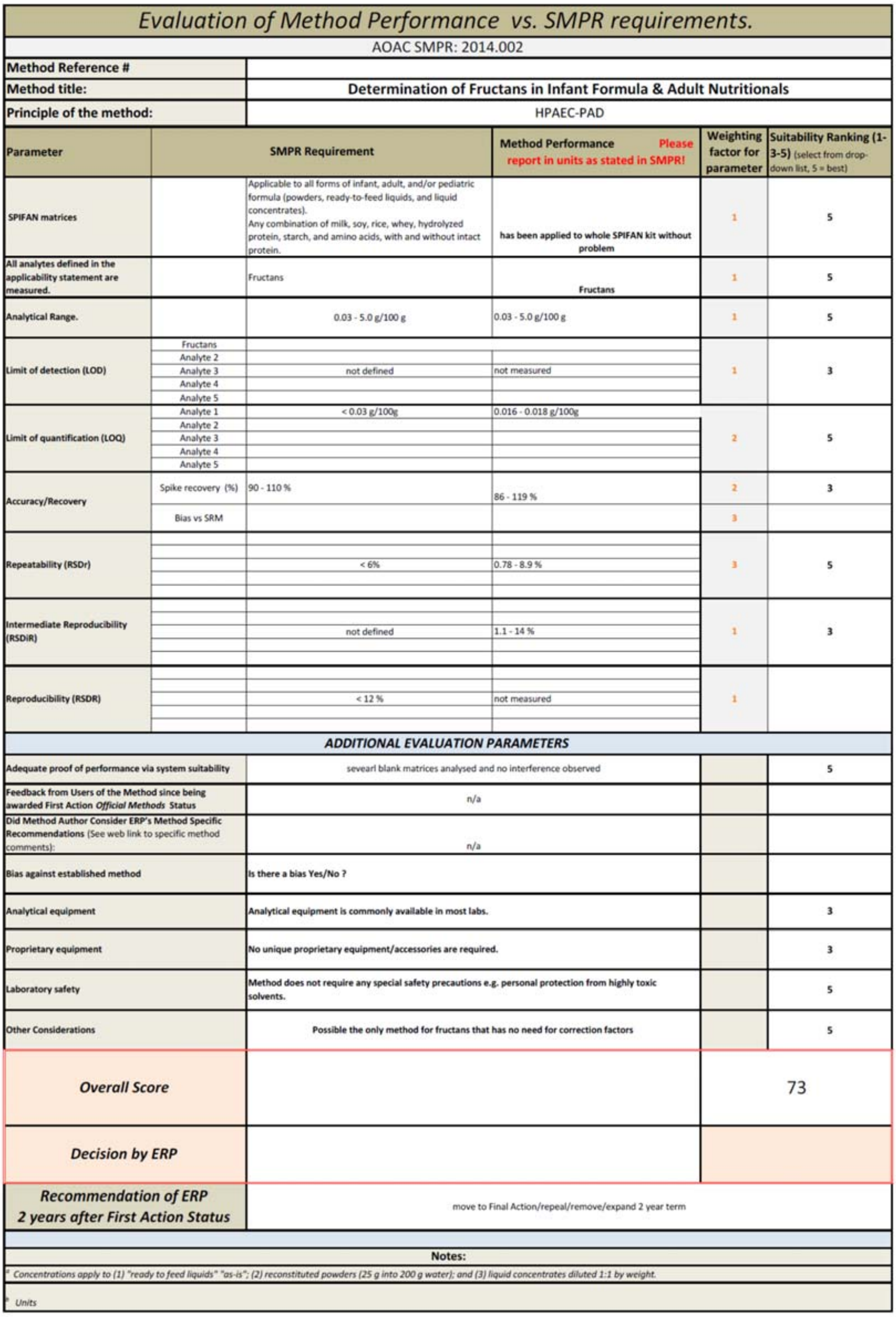
day	repetition 1	repetition 2		Daily average	Daily SD_r	Daily RSD_r (%)
1	0,504	0,516		0,510	0,008	1,7
2	0,518	0,503		0,511	0,011	2,1
4	0,509	0,508		0,509	0,001	0,1
5	0,474	0,474		0,474	0,000	0,0
7	0,535	0,526		0,531	0,006	1,2
8	0,502	0,543		0,523	0,029	5,5
gem	0,507	0,512			**Pooled RSD_r**	**2.6**
std	0,020	0,023				
gem totaal	**0,509**					
std totaal	**0,021**					
VC(%) totaal	**4,6**					

ANNEX E Experimental data accuracy study

		recoveries (%)						
		day 1	day 6	day 10				
spiked with		Oraftie P95	Nutraflora P95	Oraftie HP	average recovery (%)	SD_R	average recovery (%)	SD_R
applied HPAEC-PAD		ICS5000_E	ICS5000_F	ICS3000_D				
sample no	addition level							
Sample 1 Child formula powder	low(50%)	88,5	89,3	96,4	90,8	6,3	93,2	5,6
		90,9	80,8	98,7				
	high (150%)	95,0	91,3	97,0	95,7	3,8		
		97,5	91,9	101,6				
Sample 9 Toddler formula powder milk based	low(50%)	89,0	84,1	95,0	89,0	4,7	91,1	3,9
		87,9	84,0	93,9				
	high (150%)	93,3	91,3	93,6	93,2	1,2		
		94,9	93,4	92,9				
Sample 10 Infant formula powder milk based	low(50%)	93,9	96,5	96,7	94,1	2,1	94,3	2,5
		92,2	91,7	93,3				
	high (150%)	91,3	93,5	94,7	94,6	2,9		
		93,1	95,3	99,9				
Sample 12 Child formula powder	low(50%)	87,3	91,3	94,0	91,0	4,9	96,2	8,0
		85,4	89,2	98,9				
	high (150%)	96,0	114,8	103,1	101,5	7,1		
		97,6	96,5	100,8				
Sample 14 Infant formula powder FOS/GOS based	low(50%)	56,8	91,5	97,5	92,8	16,0	94,4	11,7
		83,6	95,0	99,8				
	high (150%)	85,4	96,9	95,1	92,2	5,5		
		85,7	97,7	92,5				
Sample 19 Adult nutritional RTF high fat	low(50%)	92,2	83,6	105,7	94,8	9,2	95,0	6,9
		87,7	93,9	105,7				
	high (150%)	95,8	87,5	95,8	95,3	4,5		
		96,1	94,8	101,6				
average recovery		**91,3**	**92,3**	**97,7**	**93,7**		**94,1**	

🟥 recovery	Grubb's outlier, result rejected in the statistical evaluation
🟨 recovery	does not meet the target range 90 - 110%
🟩 recovery	meets the target range 90 - 110%

ANNEX F Method Evaluation Form (Combined CCC/NRC Data)



Evaluation of Method Performance vs. SMPR requirements.

AOAC SMPR: 2014.002

Method Reference #					
Method title:	**Determination of Fructans in Infant Formula & Adult Nutritionals**				
Principle of the method:	HPAEC-PAD				
Parameter		**SMPR Requirement**	**Method Performance** **Please report in units as stated in SMPR!**	**Weighting factor for parameter**	**Suitability Ranking (1-3-5)** (select from drop-down list, 5 = best)
SPIFAN matrices		Applicable to all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates). Any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.	**has been applied to whole SPIFAN kit without problem**	1	**5**
All analytes defined in the applicability statement are measured.		Fructans	**Fructans**	1	**5**
Analytical Range.		0.03 - 5.0 g/100 g	0.03 - 5.0 g/100 g	1	**5**
Limit of detection (LOD)	Fructans			1	**3**
	Analyte 2				
	Analyte 3	not defined	not measured		
	Analyte 4				
	Analyte 5				
Limit of quantification (LOQ)	Analyte 1	< 0.03 g/100g	0.016 - 0.018 g/100g	2	**5**
	Analyte 2				
	Analyte 3				
	Analyte 4				
	Analyte 5				
Accuracy/Recovery	Spike recovery (%)	90 - 110 %	86 - 119 %	2	**3**
	Bias vs SRM			3	
Repeatability (RSDr)		< 6%	0.78 - 8.9 %	3	**5**
Intermediate Reproducibility (RSDiR)		not defined	1.1 - 14 %	1	**3**
Reproducibility (RSDR)		< 12 %	not measured	1	
ADDITIONAL EVALUATION PARAMETERS					
Adequate proof of performance via system suitability	sevearl blank matrices analysed and no interference observed				**5**
Feedback from Users of the Method since being awarded First Action *Official Methods* Status	n/a				
Did Method Author Consider ERP's Method Specific Recommendations (See web link to specific method comments):	n/a				
Bias against established method	**Is there a bias Yes/No ?**				
Analytical equipment	**Analytical equipment is commonly available in most labs.**				**3**
Proprietary equipment	**No unique proprietary equipment/accessories are required.**				**3**
Laboratory safety	**Method does not require any special safety precautions e.g. personal protection from highly toxic solvents.**				**5**
Other Considerations	**Possible the only method for fructans that has no need for correction factors**				**5**
Overall Score				73	
Decision by ERP					
Recommendation of ERP 2 years after First Action Status	move to Final Action/repeal/remove/expand 2 year term				

Notes:

[a] *Concentrations apply to (1) "ready to feed liquids" "as-is"; (2) reconstituted powders (25 g into 200 g water); and (3) liquid concentrates diluted 1:1 by weight.*

[b] *Units*

AOAC SMPR 2014.003

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

Intended Use: Reference Method for Dispute Resolution

1 Applicability

Determination of galactooligosaccharides (GOS) in all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates).

2 Analytical Technique

Any analytical technique that meets the following method performance requirements is acceptable.

3 Definitions

Adult/pediatric formula.—Nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment [AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN); 2010], made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.

Galactooligosaccharides (GOS).—Also known as oligogalactosyllactose, oligogalactose, or transgalactooligosaccharides (TOS) produced by transgalactosylation of lactose. General formulae are shown in Figure 1.

Infant formula.—Breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding (Codex Standard 72-1981) made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.

$$(\text{ß-D-Gal}p\text{-}(1\rightarrow\text{B}))_n\text{-ß-D-Gal}p\text{-}(1\rightarrow\text{A})\text{-D-Glc}p$$

$$(\text{ß-D-Gal}p\text{-}(1\rightarrow\text{B}))_m\text{-D-Gal}p$$

where n = 0-8; m = 1-9
B = 2,3,4 or 6
If n>0, A=1,2,3,4 or 6
If n= 0, A=1,2,3 or 6

Figure 1. General formulae for galactooligosaccharides which may or may not contain a terminal glucose. Although not obvious from this generalized scheme, branched structures may also exist. Gal*p* = galactopyranose; Glc*p* = glucopyranose.

Table 1. Method performance requirements[a]

Analytical range	0.2–5.0[b]
Limit of quantitation (LOQ)	≤0.2[b]
Repeatability (RSD_r)	≤6%
Recovery	90 to 110% of mean spiked recovery over the range of the assay
Reproducibility (RSD_R)	≤12%

[a] Concentrations apply to (a) “ready-to-feed” liquids “as is”; (b) reconstituted powders (25 g into 200 g of water); and (c) liquid concentrates diluted 1:1 by weight.

[b] g/100 g reconstituted final product.

Limit of detection (LOD).—The minimum concentration or mass of analyte that can be detected in a given matrix with no greater than 5% false-positive risk and 5% false-negative risk.

Limit of quantitation (LOQ).—The minimum concentration or mass of analyte in a given matrix that can be reported as a quantitative result.

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

Reproducibility.—The standard deviation or relative standard deviation calculated from among-laboratory data. Expressed as the reproducibility relative standard deviation (SD_R); or % reproducibility relative standard deviation (%RSD_R).

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

4 Method Performance Requirements

See Table 1.

5 System Suitability Tests and/or Analytical Quality Control

Suitable methods will include blank check samples, and check standards at the lowest point and midrange point of the analytical range.

6 Reference Material(s)

No National Institute of Standards and Technology (NIST) Standard Reference Material® (SRM) 1849a Infant/Adult Nutritional Formula or equivalent is available.

7 Validation Guidance

Recommended level of validation: *Official Methods of Analysis*^{SM}.

8 Maximum Time-to-Result

No maximum time.

Approved by AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). Final Version Date: March 18, 2014.

Determination of β-Galactooligosaccharides in Infant Formula & Adult Nutritionals

Sean Austin, Denis Cuany, Catherine Murset-Mounoud
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1. PRINCIPLE

Samples are reconstituted in water and further diluted until the concentration of GOS in solution is appropriate such that the chromatographic signals will be within the range covered by the standard curve. An aliquot of the sample solution is taken and an internal standard is added, this is then split into two aliquots. One aliquot is treated with a β-galactosidase the other is not. Then both aliquots are labelled with a fluorescent tag, 2-aminobenzamide (2AB). After labelling both aliquots are treated with amyloglucosidase to remove maltodextrins, then they are analysed by hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection (FLD). Since it is the 2AB label that is detected, each oligosaccharide has an equivalent molar response in the detector. It is thus necessary to know the molecular weight of each signal in the chromatogram to convert the molar quantities analysed to mass quantities. This can be done by coupling a mass spectrometer (but once a GOS ingredient profile has been characterized by HILIC-FLD-MS, future samples can be analysed without the MS).

2. EQUIPMENT

- 2-mL microtubes safe lock or screw cap
- 5-mL plastic tubes with caps
- Floating rack for microtubes
- Centrifuge for 2-mL microtubes able to operate at 10000 *g*
- Water bath(s) for 2-mL tubes able to operate at 65 °C and 50 °C ± 1.0 °C
- Vortex mixer
- Micropipettes with tips (0.1 to 1) mL (e.g. Eppendorf Multipipette plus)
- Analytical balance reading down to 0.1 mg (e.g. Mettler AT200)
- Ultrasonic bath
- UHPLC column: BEH-Glycan (2.1 × 150 mm, 1.7 µm) or equivalent.
- UHPLC guard column: BEH-Amide (2.1 × 5 mm, 1.7 µm) or equivalent
- Ultra high performance liquid chromatograph (UHPLC) equipped with the following modules:-
  - Gradient pump
  - On-line degasser
  - Autosampler with a cooled sample compartment (8 - 10°C)
  - Temperature controlled column compartment
  - Fluorescence detector
  - Ultra High Pressure switching valve (2-way 6-port, 2-way 10-port, or column selection valve)

3. CHEMICALS & REAGENTS

- Dimethylsulfoxide, puriss p.a.
- Anthranilic acid amide, purum (2-aminobenzamide, 2AB)
- Ammonium acetate, p.a.

- Sodium cyanoborohydride
- Formic acid, GR for analysis
- Acetic Acid anhydrous, GR for analysis
- Acetonitrile, Gradient grade for LC
- Ammonium hydroxide solution (25%), GR for analysis
- Di-potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Hydrochloric acid solution (1 mol/L)
- Sodium hydroxide solution (1 mol/L)
- Bovine Serum Albumin (BSA)
- Laboratory water Type I
- Laminaritriose, >90 %
- Maltotriose min. 95 %
- Amyloglucosidase (AMG enzyme) from aspergilus niger (Roche #11202367001)
- High purity β-Galactosidase (4000 U/mL) from aspergilus niger (Megazyme E-BGLAN)

Maltotriose stock solution (4 µmol/mL): Into a 50-mL volumetric flask, weigh 100 mg ± 5 mg of maltotriose standard and dissolve with 30 mL water, then complete to the mark with water. (This can be divided into 5 mL aliquots, and stored frozen for up to 1 year).

Laminaritriose stock solution (2 µmol/mL): Weigh 50 mg of Laminaritriose into a 50-mL volumetric flask, dissolve in 30 mL water, then complete to the mark with water. (This can be divided into 4 mL aliquots, and stored frozen for up to 1 year).

Laminaritriose working solution (0.3 µmol/mL): Using a pipette transfer 3.8 mL of Laminaritriose stock solution to a 20-mL volumetric flask and complete to the mark phosphate buffer. (This can be divided into 5 mL aliquots, and stored frozen for up to 1 year).

Ammonium acetate buffer (0.1 mol/L, pH 5.5): In a 100-mL beaker, dissolve 0.771 g ± 0.005 g (10 mmol) of anhydrous ammonium acetate with 80 mL ± 5 mL of water and adjust pH to 5.50 ± 0.05 with acetic acid. Transfer quantitatively to a 100-mL volumetric flask and complete to the mark with water.

Potassium phosphate buffer (0.2 mol/L, pH 6.0): Into a 1000-mL beaker dissolve 22.0 g potassium dihydrogen phosphate KH_2PO_4 and 4.59 g di-potassium hydrogen phosphate K_2HPO_4 in 600 mL of water (using a magnetic stirrer). Adjust the pH to 6.0 with hydrochloric acid (1 mol/L) or sodium hydroxide (1 mol/L). Transfer the solution to a 1000-mL volumetric flask and make up to the mark with water.

BSA stock solution (15 mg/mL): Weigh 30 mg of bovine serum albumin (BSA) in to a 5-ml plastic tube. Add 2.0 mL of potassium phosphate buffer (0.2 mol/L, pH 6.0) and mix well.

BSA working solution (0.5 mg/mL): Into a 5-mL plastic tube, pipette 50 µL of BSA stock solution. Add 1450 µL of phosphate buffer pH 6.0 and mix well.

B-Galactosidase solution (2000 U/mL with BSA (0.25 mg/mL)): In a 1.5-mL microtube mix 300 µL of β-galactosidase (4000 U/mL) with 300 µL of BSA working solution and mix well.
NOTE: This method has been validated with β-galactosidase from Megazyme (# E-BGLAN). Other sources of β-galactosidase must be evaluated to ensure that there are no side activities on other oligosaccharides which may be

present in the sample (including the internal standard) and that under the conditions described complete hydrolysis of the GOS is achieved (if a significant lactose peak is still apparent in the chromatogram this indicates that there may have been insufficient β-galactosidase activity).

Amyloglucosidase solution (60 U/mL in 0.1 mol/L ammonium acetate pH 5.5): Weigh the amount of amyloglucosidase corresponding to 180 Units ± 20 U and dissolve with 3.0 mL of ammonium acetate buffer (0.1 mol/L pH 5.5).

NOTE: This method has been validated with amyloglucosidase from Roche (#11202367001). Other sources of amyloglucosidase must be evaluated in terms of baseline interference and maltodextrin hydrolysis efficiency. The evaluation is performed by running a blank with amyloglucosidase only, and a test with a pure maltodextrin in place of the sample.

LC Eluent B: Ammonium formate (0.1 mol/L, pH 4.4): Add 4.6 g ± 0.1 g (3.78 mL) of formic acid (100 %) in a beaker containing 800 mL of water. Adjust the pH to 4.40 ± 0.05 with ammonium hydroxide (25 %). Transfer quantitatively to a 1000-mL volumetric flask, and dilute to the volume with water.

2AB labelling reagent: 2AB (0.35 mol/L) - $NaBH_3CN$ (1.0 mol/L) in DMSO - acetic acid (30 %) solution. Depending on the number of samples to be labelled, pipette the appropriate volume of DMSO and acetic acid in to a 10-mL glass tube (see Table 1 below for quantities). Mix the solution using a vortex mixer. In a second 10-mL glass tube weigh the amount of anthranilamide (2AB) and sodium cyanoborohydride ($NaBH_3CN$) indicated in Table 1 below, then add the corresponding volume of 30 % acetic acid in DMSO. Mix using a vortex mixer, and if necessary use an ultrasonic bath to achieve complete dissolution of the solids (about 10 min).

Table 1: Examples of quantities for 2AB reagent preparation.

Max. number of tests	30 % Acetic acid in DMSO		2AB (0.35 mol/L), $NaBH_3CN$(1 mol/L) in 30 % Acetic acid/DMSO		
	DMSO (mL)	Acetic acid 100 % (mL)	30 % Acetic Acid in DMSO (mL)	2AB (mg)	$NaBH_3CN$ (mg)
11	2.10	0.90	2.50	118 ± 5	157 ± 5
22	4.20	1.80	5.00	236 ± 10	314 ± 10
35	6.30	2.70	7.50	354 ± 10	471 ± 10
47	7.70	3.30	10.00	708 ± 10	942 ± 10

4. SAMPLE PREPARATION

For analysis of products on a ready to feed basis:

Reconstitute powder or liquid concentrates according to instructions (for SPIFAN samples powder samples (25 g) were weighed in to a bottle and water (200 g) was added. For SRM 1849a, the powder (10 g) was weighed in to a bottle and water (100 g) was added). The mixture was placed in a water bath at 70°C with constant agitation for 20-25 min then cooled to room temperature.

Weigh 10 g (m) of the reconstituted sample in to a 50-mL (V) volumetric flask and adjust to the volume with phosphate buffer (0.2 mol/L, pH 6.0). Depending on GOS concentration and oligosaccharide profile the weight (m) can be adjusted between 0.5 – 15 g to optimise the chromatography signals.

For analysis of ready-to-feed products

Into a 50-mL (V) volumetric flask, weigh 10 g (m) of liquid sample and add a magnetic stirring bar and 20 mL of phosphate buffer (0.2 mol/L, pH 6.0.). Place the sample in a water bath at 70 °C for 20-25 min with constant stirring. Cool the solution to the room temperature, remove the magnetic stirring bar and adjust to the final volume with phosphate buffer (0.2 mol/L, pH 6.0). Depending on GOS concentration and oligosaccharide profile the weight (m) can be adjusted between 0.5 – 15 g to optimise the chromatography signals.

For analysis of powder products without prior reconstitution:

Weigh 1.5 g (m) of powder in to a 50 mL (V) volumetric flask. Add a magnetic stirring bar and 35 mL of phosphate buffer (0.2 mol/L, pH 6.0.). Place the sample in a water bath at 70 °C for 20-25 min with constant stirring. Cool the solution to the room temperature, remove the magnetic stirring bar, and adjust to the final volume with phosphate buffer (0.2 mol/L, pH 6.0). Depending on GOS concentration and oligosaccharide profile the weight (m) can be adjusted between 0.2 – 3 g to optimise the chromatography signals.

Addition of Internal Standard

Transfer 1000 µL of diluted sample into a 2-mL microtube. Add 400 µL of laminaritriose (0.3 µmol/mL) and mix well. Take an aliquot of 500 µL and transfer to a microtube for the "non-treated procedure". Take a 2nd aliquot of 500 µL and transfer to a microtube for the "enzyme-treated procedure".

Non-treated Procedure

To the microtube containing 500 µL of sample solution add 25 µL of phosphate buffer (0.2 mol/L, pH 6.0). Mix well and place in a water bath at 60°C for 60 min. Cool sample to room temperature and continue with 2AB Labelling.

Enzyme-treated Procedure

To the microtube containing 500 µL of sample solution add 25 µL of β-galactosidase solution. Mix well and place in a water bath at 60°C for 60 min. Cool sample to room temperature and continue with 2AB Labelling.

2AB Labelling

Transfer 20 µL of sample to a 2-mL microtube. Add 200 µL of 2AB labelling reagent and mix well. Place the sample in a water bath at 65 °C for 2 hours. After incubation, mix well then cool sample at 4°C for 10 min.

Maltodextrin Hydrolysis

To each tube add 1 mL of ammonium acetate buffer and mix well. Transfer 500 µL of this solution to a 2-mL microtube and add 200 µL of AMG solution (60 U/mL). Mix well and incubate in a water bath at 50°C for 30 min. After incubation, cool sample to room temperature and prepare sample for chromatography.

Preparation for Chromatography

Add 700 µL of acetonitrile to the sample or standard and mix well. Centrifuge this solution at 10000 × g for 5 min, then transfer 1 mL of the supernatant to a vial suitable for the LC autosampler.

5. PREPARATION OF STANDARD CURVE

Prepare the solutions for the 6-level standard curve by diluting the maltotriose stock solution (4 µmol/mL) as described in the following table:

Table 2: Dilution Scheme for the Preparation of the Standard Curve

Calibration Curve Dilutions in PHOSPHATE BUFFER (0.2 mol/L, pH 6.0)				
Carbohydrate	**# Std**	**Maltotriose Stock volume [µL]**	**Final Volume [mL]**	**Standard Concentration [µmol/mL]**
Maltotriose	Level #1	50	20	**0.0100**
	Level #2	250	20	**0.0500**
	Level #3	750	20	**0.1500**
	Level #4	1120	10	**0.4480**
	Level #5	1750	10	**0.7000**
	Level #6	2250	10	**0.9000**

Preparation Procedure for Standards:

For each standard, transfer 1000 µL of solution to a 2-mL microtube, add 400 µL of laminaritriose solution (0.3 µmol/mL) then take a 500 µL aliquot and follow the non-treated procedure but stop before the maltodextrin hydrolysis. Follow the maltodextrin hydrolysis procedure but add 200 µL of ammonium acetate buffer (0.1 mol/L, pH 5.5) instead of AMG solution (i.e. no hydrolysis should occur). Then continue with the preparation for chromatography.

6. CHROMATOGRAPHIC CONDITIONS

Analyse the prepared samples and standards on a UHPLC system equipped with a FLD under the following conditions:

FLD: Excitation λ: 330 nm Emission λ: 420 nm

Trap Column: Acquity BEH Amide (2.1 mm x 5 mm; 1.7 µm) or equivalent (see Figure 1 for plumbing)

Column: Acquity UPLC BEH Glycan (2.1 mm x 150 mm; 1.7 µm) or equivalent

Column temperature: 25°C ± 1 °C

Injection volume: 3 µL

Eluent A: Acetonitrile

Eluent B: Ammonium formate 0.1 mol/L, pH 4.4

Gradient:

Time (min)	Flow (mL/min)	%A (Acetonitrile)	%B (Formate)	Switching Valve	Comment
0	0.6	97	3	6-1	Inject sample on trap column
5.0	0.6	97	3	6-1	End of sample wash, start acquisition
5.5	0.6	88	12	1-2	Switch valve, start analytical gradient
12.5	0.6	88	12	1-2	
22.5	0.6	83	17	1-2	
26.5	0.6	83	17	1-2	
45.0	0.6	68	32	1-2	End of analytical gradient
45.1	0.2	68	32	1-2	Reduce flow
45.5	0.2	30	70	1-2	Start column wash
55.5	0.2	30	70	1-2	End column wash
57.0	0.2	88	12	1-2	Start analytical column equilibration
62.0	0.2	88	12	1-2	
72.0	0.6	88	12	1-2	End analytical column equilibration
72.5	0.6	97	3	6-1	Switch valve, equilibrate trap column
73.5	0.6	97	3	6-1	End

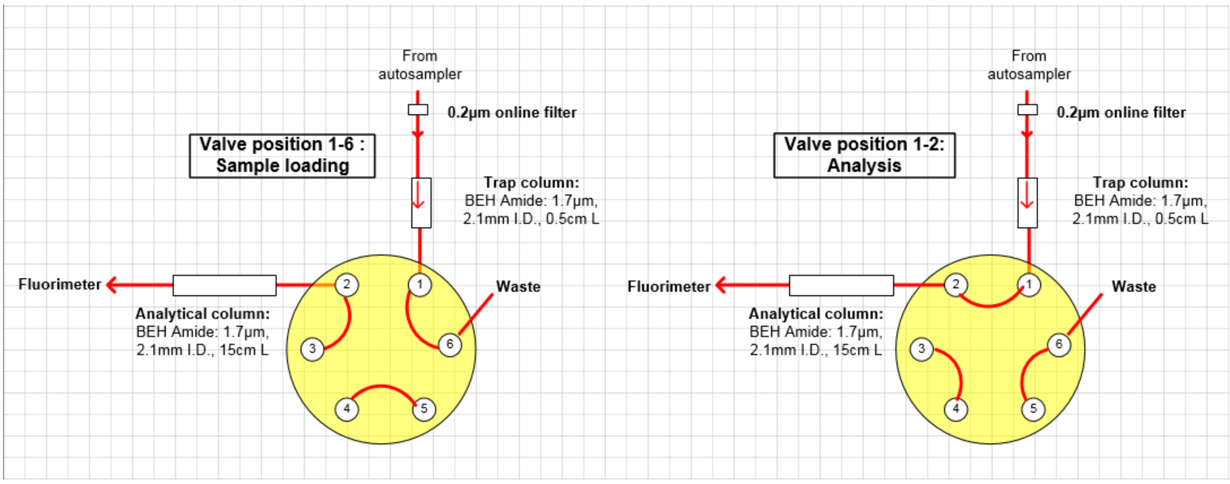

Figure 1: Configuration of 2-way 6-port switching valve

7. CALIBRATION & CALCULATIONS

Use the instrument software to plot a six point standard curve of “Instrument response for maltotriose / Instrument response for laminaritriose” against the “concentration of maltotriose” in the standard (in µmol/mL). Fit a linear model to the data without forcing through zero.

Use the standard curve to calculate the molar concentration (in µmol/mL) of each oligosaccharide in the chromatogram (C_m) without enzyme treatment, and calculate the total oligosaccharides in that sample as follows:-

$$C_{TOS} = \sum (C_m \times MW) \times \frac{V}{m} \times 0.0001$$

Use the standard curve to calculate the molar concentration (in µmol/mL) of each oligosaccharide in the chromatogram (C_m) with enzyme treatment, and calculate the total oligosaccharides in that sample as follows:-

$$C_B = \sum (C_m \times MW) \times \frac{V}{m} \times 0.0001$$

Then calculate the GOS content of sample as follows:-

$$C_{GOS} = C_{TOS} - C_B$$

Where,

C_{TOS} = Total concentration of oligosaccharides in the untreated sample (in g/100 g)
C_B = Total concentration of oligosaccharides in the enzyme-treated sample (in g/100 g)
C_{GOS} = Total concentration of GOS in the sample (in g/100 g)
C_m = molar concentration of each individual oligosaccharide in the sample (in µmol/mL)
MW = molecular weight of each individual oligosaccharide in the sample
V = volume to which the original sample weight was diluted (in mL)
m = weight of sample diluted to volume (V) (in g)
0.0001 = factor to convert result from µg/g to g/100 g.

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To: Delia Boyd (dboyd@aoac.org)

YOUR REF.

OUR REF.
NRC/AS sau/cit
Sean Austin

LAUSANNE,
July 25, 2016

Dear Delia

We are pleased to send you herewith a copy of our SLV report entitled:

Single Lab Validation of a Method for the Determination of β-Galactooligosaccharides in Infant Formula & Adult Nutritionals

By Sean Austin, Denis Cuany and Catherine Murset-Mounoud (NRC, Lausanne).

This document is confidential and should not be copied or disclosed to others than to the Expert Review Panel without proper authorization.

We will be happy to provide you with any further information you may require.

Yours sincerely

Sean Aut

Sean Austin

Nestlé Research Center
Lausanne

R&D Memo

Single Lab Validation of a Method for the Determination of β-Galactooligosaccharides in Infant Formula & Adult Nutritionals

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Objective Question	Determine if the performance of a method to determine β-galactooligosaccharides (GOS) in infant formula and adult nutritionals meets the requirements set by the AOAC-SPIFAN working group
Authors People to ask	Sean Austin (NRC, Lausanne), Denis Cuany (NRC, Lausanne), Catherine Murset-Mounoud (NRC, Lausanne)
Summary Answer	The method developed meets most of the standard method performance requirements that can be tested in a single lab. Unfortunately the trueness of the method is, in general, slightly below the target of 90-110%, but the method is currently the best solution available for the determination of GOS in lactose-containing matrices.
Date of issue	25 Jul 2016
Project	DNUT-100472 / SPIFAN

SUMMARY

Methodology and Trials

The SLV was performed on the SPIFAN matrix kit containing 19 different matrices, two products purchased in a Swiss supermarket, and a Nestlé reference sample. Of the 19 matrices in the SPIFAN kit, 5 were found to contain GOS. Those 5 and the other 3 materials were analysed on 6 different days in duplicate to determine the precision of the method. Of the 14 GOS-free samples, 4 were selected for use in spike/recovery experiments. The four matrices were spiked with 2 different GOS ingredients at four different levels.

Results and Conclusions

The results of the SLV are summarized in the table below. The single lab precision data are better than required by the SMPR. However, the spike recoveries are in general slightly below the SMPR guidelines (with one result being high). However, the method is currently the only solution available for determination of GOS in lactose-containing matrices.

Parameter	SMPR	SLV Results	Meets SMPR?
Analytical Range	0.2 – 5.0 g/100 g	0.2 – 5.0	Y
LoQ	≤ 0.2 g/100 g	≤ 0.15 g/100 g[a]	Y
$RSD_{(r)}$	≤ 6 %	1.0 -3.6 %	Y
$RSD_{(iR)}$	No target	1.7 – 7.9 %	n/a
$RSD_{(R)}$	≤ 12 %	n/a	Cannot test in SLV
Recovery	90 – 110 % of mean spiked recovery over the range of the assay	85 – 114 %	N

[a] The method LoQ depends strongly on the GOS oligosaccharide profile. The value of 0.15 g/100g has been estimated from the spike/recovery and precision data recorded during this SLV.

1 INTRODUCTION

After opening a call for methods to determine β-galactooligosaccharides (GOS) in infant formula and adult nutritionals, the AOAC received 2 method proposals. Both were considered by the expert review panel (ERP), and both were rejected. One of the two previous methods was rejected due to a lack of specificity for GOS. That method has since been modified to improve specificity and this report described the single lab validation (SLV) of that method.

2.1. Samples

A SPIFAN single laboratory validation material test kit was used. The kit contains representative products covering the scope of the project as well as a standard reference material (SRM 1849a). The kit contains 5 products described as placebo products and 14 products described as fortified products. Four of the fortified products were found to contain GOS. It was also found that one of the placebo products contained GOS. In addition to the SPIFAN matrices, one Nestlé reference sample was analysed, and GOS-containing products from two other companies were purchased in the supermarket to extend the total number of samples. The Nestlé reference sample was analysed without prior reconstitution.

Table 1: Samples Used In This Study

Product Description	Lot No	NRC Reference
Placebo Products		
1. Child Formula Powder	00847RF00	KBI-00020
2. Infant Elemental Powder	00796RF	KBI-00028
3. Adult Nutritional RTF, High Protein	00821RF00	KBI-00029
4. Adult Nutritional RTF, High Fat	00820RF00	KBI-00030
5. Infant Formula RTF, Milk Based	EV4H2Q	KBI-00031
Fortified Products		
1. SRM 1849a	CLC10-b	KBI-00032
2. Infant Formula Powder Partially Hydrolyzed Milk Based	410057652Z	KBI-00033
3. Infant Formula Powder Partially Hydrolyzed Soy Based	410457651Z	KBI-00034
4. Toddler Formula Powder Milk Based	4052755861	KBI-00035
5. Infant Formula Powder Milk Based	4044755861	KBI-00044
6. Adult Nutritional Powder Low Fat	00859RF00	KBI-00045
7. Child Formula Powder	00866RF00	KBI-00046
8. Infant Elemental Powder	00795RF	KBI-00047
9. Infant Formula Powder FOS/GOS Based	50350017W1	KBI-00053
10. Infant Formula Powder Milk Based	K16NTAV	KBI-00048
11. Infant Formula Powder Soy Based	E10NWZC	KBI-00052
12. Infant Formula RTF Milk Based	EV4H2R	KBI-00049
13. Adult Nutritional RTF High Protein	00730RF00	KBI-00050 / L3B-00037
14. Adult Nutritional RTF High Fat	00729RF00	KBI-00051
Nestlé Products		
1. Infant formula powder with GOS/FOS	Ref 56A	Ref 56A
Additional Products		
1. Infant formula with GOS	L15515	L4B-00006
2. Infant formula with GOS/FOS	F 0135742031 2040 006394	L4B-00007

2.2. Method

2.2.1 Principle

Samples are reconstituted in water and further diluted until the concentration of GOS in solution is appropriate such that the chromatographic signals will be within the range covered by the standard curve. An aliquot of the sample solution is taken and an internal standard is added, this is then split into two aliquots. One aliquot is treated with a β-galactosidase the other is not. Then both aliquots are labelled with a fluorescent tag, 2-aminobenzamide (2AB). After labelling both aliquots are treated with amyloglucosidase to remove maltodextrins, then they are analysed by hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection (FLD). Since it is the 2AB label that is detected, each oligosaccharide has an equivalent molar response in the detector. It is thus necessary to know the molecular weight of each signal in the chromatogram to convert the molar quantities analysed to mass quantities. This can be done by coupling a mass spectrometer (but once a GOS ingredient profile has been characterized by HILIC-FLD-MS, future samples can be analysed without the MS).

2.2.2 Equipment

- 2-mL microtubes safe lock or screw cap
- 5-mL plastic tubes with caps
- Floating rack for microtubes
- Centrifuge for 2-mL microtubes able to operate at 10000 *g*
- Water bath(s) for 2-mL tubes able to operate at 65 °C and 50 °C ± 1.0 °C
- Vortex mixer
- Micropipettes with tips (0.1 to 1) mL (e.g. Eppendorf Multipipette plus)
- Analytical balance reading down to 0.1 mg (e.g. Mettler AT200)
- Ultrasonic bath
- UHPLC column: BEH-Glycan (2.1 × 150 mm, 1.7 µm) or equivalent.
- UHPLC guard column: BEH-Amide (2.1 × 5 mm, 1.7 µm) or equivalent
- Ultra high performance liquid chromatograph (UHPLC) equipped with the following modules:-
  - Gradient pump
  - On-line degasser
  - Autosampler with a cooled sample compartment (8 - 10°C)
  - Temperature controlled column compartment
  - Fluorescence detector
  - Ultra High Pressure switching valve (2-way 6-port, 2-way 10-port, or column selection valve)

2.2.3 Chemicals & Reagents

- Dimethylsulfoxide, puriss p.a.
- Anthranilic acid amide, purum (2-aminobenzamide, 2AB)
- Ammonium acetate, p.a.
- Sodium cyanoborohydride
- Formic acid, GR for analysis
- Acetic Acid anhydrous, GR for analysis

- Acetonitrile, Gradient grade for LC
- Ammonium hydroxide solution (25%), GR for analysis
- Di-potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Hydrochloric acid solution (1 mol/L)
- Sodium hydroxide solution (1 mol/L)
- Bovine Serum Albumin (BSA)
- Laboratory water Type I
- Laminaritriose, >90 %
- Maltotriose min. 95 %
- Amyloglucosidase (AMG enzyme) from aspergilus niger (Roche #11202367001)
- High purity β-Galactosidase (4000 U/mL) from aspergilus niger (Megazyme E-BGLAN)

Maltotriose stock solution (4 µmol/mL): Into a 50-mL volumetric flask, weigh 100 mg ± 5 mg of maltotriose standard and dissolve with 30 mL water, then complete to the mark with water. (This can be divided into 5 mL aliquots, and stored frozen for up to 1 year).

Laminaritriose stock solution (2 µmol/mL): Weigh 50 mg of Laminaritriose into a 50-mL volumetric flask, dissolve in 30 mL water, then complete to the mark with water. (This can be divided into 4 mL aliquots, and stored frozen for up to 1 year).

Laminaritriose working solution (0.3 µmol/mL): Using a pipette transfer 3.8 mL of Laminaritriose stock solution to a 20-mL volumetric flask and complete to the mark phosphate buffer. (This can be divided into 5 mL aliquots, and stored frozen for up to 1 year).

Ammonium acetate buffer (0.1 mol/L, pH 5.5): In a 100-mL beaker, dissolve 0.771 g ± 0.005 g (10 mmol) of anhydrous ammonium acetate with 80 mL ± 5 mL of water and adjust pH to 5.50 ± 0.05 with acetic acid. Transfer quantitatively to a 100-mL volumetric flask and complete to the mark with water.

Potassium phosphate buffer (0.2 mol/L, pH 6.0): Into a 1000-mL beaker dissolve 22.0 g potassium dihydrogen phosphate KH_2PO_4 and 4.59 g di-potassium hydrogen phosphate K_2HPO_4 in 600 mL of water (using a magnetic stirrer). Adjust the pH to 6.0 with hydrochloric acid (1 mol/L) or sodium hydroxide (1 mol/L). Transfer the solution to a 1000-mL volumetric flask and make up to the mark with water.

BSA stock solution (15 mg/mL): Weigh 30 mg of bovine serum albumin (BSA) in to a 5-ml plastic tube. Add 2.0 mL of potassium phosphate buffer (0.2 mol/L, pH 6.0) and mix well.

BSA working solution (0.5 mg/mL): Into a 5-mL plastic tube, pipette 50 µL of BSA stock solution. Add 1450 µL of phosphate buffer pH 6.0 and mix well.

B-Galactosidase solution (2000 U/mL with BSA (0.25 mg/mL)): In a 1.5-mL microtube mix 300 µL of β-galactosidase (4000 U/mL) with 300 µL of BSA working solution and mix well.

NOTE: This method has been validated with β-galactosidase from Megazyme (# E-BGLAN). Other sources of β-galactosidase must be evaluated to ensure that there are no side activities on other oligosaccharides which may be present in the sample (including the internal standard) and that under the conditions described complete hydrolysis of the GOS is achieved (if a significant lactose peak is still apparent in the chromatogram this indicates that there may have been insufficient β-galactosidase activity).

Amyloglucosidase solution (60 U/mL in 0.1 mol/L ammonium acetate pH 5.5): Weigh the amount of amyloglucosidase corresponding to 180 Units ± 20 U and dissolve with 3.0 mL of ammonium acetate buffer (0.1 mol/L pH 5.5).
NOTE: This method has been validated with amyloglucosidase from Roche (#11202367001). Other sources of amyloglucosidase must be evaluated in terms of baseline interference and maltodextrin hydrolysis efficiency. The evaluation is performed by running a blank with amyloglucosidase only, and a test with a pure maltodextrin in place of the sample.

LC Eluent B: Ammonium formate (0.1 mol/L, pH 4.4): Add 4.6 g ± 0.1 g (3.78 mL) of formic acid (100 %) in a beaker containing 800 mL of water. Adjust the pH to 4.40 ± 0.05 with ammonium hydroxide (25 %). Transfer quantitatively to a 1000-mL volumetric flask, and dilute to the volume with water.

2AB labelling reagent: 2AB (0.35 mol/L) - $NaBH_3CN$ (1.0 mol/L) in DMSO - acetic acid (30 %) solution. Depending on the number of samples to be labelled, pipette the appropriate volume of DMSO and acetic acid in to a 10-mL glass tube (see Table 2 below for quantities). Mix the solution using a vortex mixer. In a second 10-mL glass tube weigh the amount of anthranilamide (2AB) and sodium cyanoborohydride ($NaBH_3CN$) indicated in Table 2 below, then add the corresponding volume of 30 % acetic acid in DMSO. Mix using a vortex mixer, and if necessary use an ultrasonic bath to achieve complete dissolution of the solids (about 10 min).

Table 2: Examples of quantities for 2AB reagent preparation.

Max. number of tests	30 % Acetic acid in DMSO		2AB (0.35 mol/L), $NaBH_3CN$(1 mol/L) in 30 % Acetic acid/DMSO		
	DMSO (mL)	Acetic acid 100 % (mL)	30 % Acetic Acid in DMSO (mL)	2AB (mg)	$NaBH_3CN$ (mg)
11	2.10	0.90	2.50	118 ± 5	157 ± 5
22	4.20	1.80	5.00	236 ± 10	314 ± 10
35	6.30	2.70	7.50	354 ± 10	471 ± 10
47	7.70	3.30	10.00	708 ± 10	942 ± 10

2.2.4 Sample Preparation

For analysis of products on a ready to feed basis:
Reconstitute powder or liquid concentrates according to instructions (for SPIFAN samples powder samples (25 g) were weighed in to a bottle and water (200 g) was added. For SRM 1849a, the powder (10 g) was weighed in to a bottle and water (100 g) was added). The mixture was placed in a water bath at 70°C with constant agitation for 20-25 min then cooled to room temperature. Weigh 10 g (m) of the reconstituted sample in to a 50-mL (V) volumetric flask and adjust to the volume with phosphate buffer (0.2 mol/L, pH 6.0). Depending on GOS concentration and oligosaccharide profile the weight (m) can be adjusted between 0.5 – 15 g to optimise the chromatography signals.

For analysis of ready-to-feed products
Into a 50-mL (V) volumetric flask, weigh 10 g (m) of liquid sample and add a magnetic stirring bar and 20 mL of phosphate buffer (0.2 mol/L, pH 6.0.). Place the sample in a water bath at 70 °C for 20-25 min with constant stirring. Cool the solution to the room temperature, remove the magnetic stirring bar and adjust to the final volume with phosphate buffer (0.2 mol/L, pH 6.0). Depending on GOS concentration and oligosaccharide profile the weight (m) can be adjusted between 0.5 – 15 g to optimise the chromatography signals.

For analysis of powder products without prior reconstitution:
Weigh 1.5 g (m) of powder in to a 50 mL (V) volumetric flask. Add a magnetic stirring bar and 35 mL of phosphate buffer (0.2 mol/L, pH 6.0.). Place the sample in a water bath at 70 °C for 20-25 min with constant stirring. Cool the solution to the room temperature, remove the magnetic stirring bar, and adjust to the final volume with phosphate buffer (0.2 mol/L, pH 6.0). Depending on GOS concentration and oligosaccharide profile the weight (m) can be adjusted between 0.2 – 3 g to optimise the chromatography signals.

Addition of Internal Standard
Transfer 1000 µL of diluted sample into a 2-mL microtube. Add 400 µL of laminaritriose (0.3 µmol/mL) and mix well. Take an aliquot of 500 µL and transfer to a microtube for the “non-treated procedure”. Take a 2nd aliquot of 500 µL and transfer to a microtube for the “enzyme-treated procedure”.

Non-treated Procedure
To the microtube containing 500 µL of sample solution add 25 µL of phosphate buffer (0.2 mol/L, pH 6.0). Mix well and place in a water bath at 60°C for 60 min. Cool sample to room temperature and continue with 2AB Labelling.

Enzyme-treated Procedure
To the microtube containing 500 µL of sample solution add 25 µL of β-galactosidase solution. Mix well and place in a water bath at 60°C for 60 min. Cool sample to room temperature and continue with 2AB Labelling.

2AB Labelling
Transfer 20 µL of sample to a 2-mL microtube. Add 200 µL of 2AB labelling reagent and mix well. Place the sample in a water bath at 65 °C for 2 hours. After incubation, mix well then cool sample at 4°C for 10 min.

Maltodextrin Hydrolysis
To each tube add 1 mL of ammonium acetate buffer and mix well. Transfer 500 µL of this solution to a 2-mL microtube and add 200 µL of AMG solution (60 U/mL). Mix well and incubate in a water bath at 50°C for 30 min. After incubation, cool sample to room temperature and prepare sample for chromatography.

Preparation for Chromatography
Add 700 µL of acetonitrile to the sample or standard and mix well. Centrifuge this solution at 10000 × g for 5 min, then transfer 1 mL of the supernatant to a vial suitable for the LC autosampler.

2.2.5 Preparation of Standard Curve

Prepare the solutions for the 6-level standard curve by diluting the maltotriose stock solution (4 µmol/mL) as described in the following table:

Table 3: Dilution Scheme for the Preparation of the Standard Curve

Calibration Curve Dilutions in PHOSPHATE BUFFER (0.2 mol/L, pH 6.0)				
Carbohydrate	**# Std**	**Maltotriose Stock volume [µL]**	**Final Volume [mL]**	**Standard Concentration [µmol/mL]**
Maltotriose	Level #1	50	20	**0.0100**
	Level #2	250	20	**0.0500**
	Level #3	750	20	**0.1500**
	Level #4	1120	10	**0.4480**
	Level #5	1750	10	**0.7000**
	Level #6	2250	10	**0.9000**

Preparation Procedure for Standards:
For each standard, transfer 1000 µL of solution to a 2-mL microtube, add 400 µL of laminaritriose solution (0.3 µmol/mL) then take a 500 µL aliquot and follow the non-treated procedure but stop before the maltodextrin hydrolysis. Follow the maltodextrin hydrolysis procedure but add 200 µL of ammonium acetate buffer (0.1 mol/L, pH 5.5) instead of AMG solution (i.e. no hydrolysis should occur). Then continue with the preparation for chromatography.

2.2.6 Chromatographic Conditions

Analyse the prepared samples and standards on a UHPLC system equipped with a FLD under the following conditions:
FLD: Excitation λ: 330 nm   Emission λ: 420 nm
Trap Column: Acquity BEH Amide (2.1 mm x 5 mm; 1.7 µm) or equivalent (see Figure 1 for plumbing)
Column: Acquity UPLC BEH Glycan (2.1 mm x 150 mm; 1.7 µm) or equivalent
Column temperature: 25°C ± 1 °C
Injection volume: 3 µL
Eluent A: Acetonitrile
Eluent B: Ammonium formate 0.1 mol/L, pH 4.4
Gradient:

Time (min)	Flow (mL/min)	%A (Acetonitrile)	%B (Formate)	Switching Valve	Comment
0	0.6	97	3	6-1	Inject sample on trap column
5.0	0.6	97	3	6-1	End of sample wash, start acquisition
5.5	0.6	88	12	1-2	Switch valve, start analytical gradient
12.5	0.6	88	12	1-2	
22.5	0.6	83	17	1-2	
26.5	0.6	83	17	1-2	
45.0	0.6	68	32	1-2	End of analytical gradient
45.1	0.2	68	32	1-2	Reduce flow
45.5	0.2	30	70	1-2	Start column wash
55.5	0.2	30	70	1-2	End column wash
57.0	0.2	88	12	1-2	Start analytical column equilibration
62.0	0.2	88	12	1-2	
72.0	0.6	88	12	1-2	End analytical column equilibration
72.5	0.6	97	3	6-1	Switch valve, equilibrate trap column
73.5	0.6	97	3	6-1	End

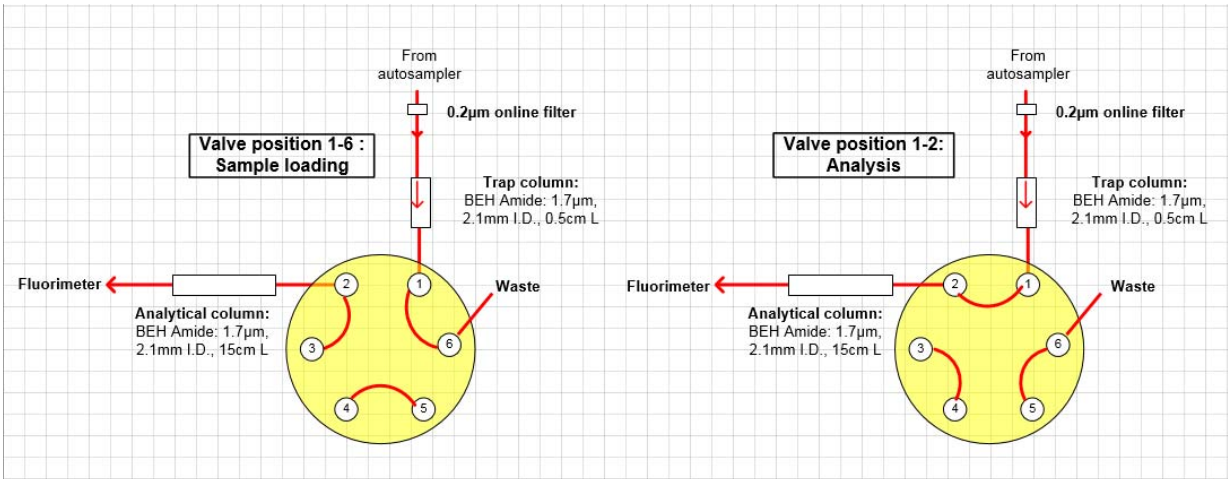


Figure 1: Configuration of 2-way 6-port switching valve

2.2.7 Calibration & Calculations

Use the instrument software to plot a six point standard curve of "Instrument response for maltotriose / Instrument response for laminaritriose" against the "concentration of maltotriose" in the standard (in µmol/mL). Fit a linear model to the data without forcing through zero.

Use the standard curve to calculate the molar concentration (in µmol/mL) of each oligosaccharide in the chromatogram (C_m) without enzyme treatment, and calculate the total oligosaccharides in that sample as follows:-

$$C_{TOS} = \sum (C_m \times MW) \times \frac{V}{m} \times 0.0001$$

Use the standard curve to calculate the molar concentration (in µmol/mL) of each oligosaccharide in the chromatogram (C_m) with enzyme treatment, and calculate the total oligosaccharides in that sample as follows:-

$$C_B = \sum (C_m \times MW) \times \frac{V}{m} \times 0.0001$$

Then calculate the GOS content of sample as follows:-

$$C_{GOS} = C_{TOS} - C_B$$

Where,

C_{TOS} = Total concentration of oligosaccharides in the untreated sample (in g/100 g)
C_B = Total concentration of oligosaccharides in the enzyme-treated sample (in g/100 g)
C_{GOS} = Total concentration of GOS in the sample (in g/100 g)
C_m = molar concentration of each individual oligosaccharide in the sample (in µmol/mL)
MW = molecular weight of each individual oligosaccharide in the sample
V = volume to which the original sample weight was diluted (in mL)
m = weight of sample diluted to volume (V) (in g)
0.0001 = factor to convert result from µg/g to g/100 g.

2.3. Validation Design

2.3.1 Linearity

Calibration solutions of maltotriose (0.005 nmol/mL – 0.9 nmol/mL) at 11 different levels, were prepared in triplicate all containing the laminaritriose internal standard (at 0.304 nmol/mL). The ratio of the peak areas (maltotriose / laminaritriose) was plotted against the concentration of maltotriose (since the laminaritriose concentration remained constant throughout there was no need to plot the ratio of the concentrations on the x-axis). A linear model was used to fit the data for calibration purposes.
The relative residuals were calculated and plotted against the analyte concentration.

2.3.2 Precision

All samples from the SPIFAN kit were analysed in duplicate on a single day, and five of the samples were found to contain GOS; The Placebo Infant Formula RTF, Milk Based and the following fortified products: Infant Formula Powder Partially Hydrolysed, Milk Based, Infant Formula Powder Milk Based, Infant Formula Powder FOS/GOS Based, Infant Formula RTF, Milk Based. In addition, infant formula products from other companies and a Nestlé reference sample were also analysed giving a total of 8 matrices for the precision study.

Each of the GOS-containing samples from the SPIFAN kit, and the two samples purchased from the supermarket, were reconstituted as instructed in the SPIFAN guideline and analysed in duplicate, on at least six different days, using 2 different instruments (same model) and 2 different columns (same stationary phase and manufacturer, but different lot) by 2 different analysts. At the same time the Nestlé reference sample was also analysed (without reconstitution). SD(r) and SD(iR) were calculated from the data obtained using the equations below:-

SD(r)

$$\mathrm{SD}(\mathrm{r}) = \sqrt{\frac{\sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{SD}_{\mathrm{i}}^{2}}{\mathrm{n}}} = \sqrt{\frac{\sum_{\mathrm{i}=1}^{\mathrm{n}} (\mathrm{x}_{\mathrm{i}1} - \mathrm{x}_{\mathrm{i}2})^{2}}{2\mathrm{n}}}$$

SD(iR)

$$\mathrm{SD}(\mathrm{iR}) = \sqrt{\mathrm{SD}^{2}(\mathrm{b}) + \frac{1}{2} \times \mathrm{SD}^{2}(\mathrm{r})}$$

Where:

n is the number of (single or duplicate) determinations
x_i is the individual result within the set of single determinations with i going from 1 to n ;
x_{i1} and x_{i2} are the two results within the set of duplicate determination with i going from 1 to n ;
SD(b) is the standard deviation between the means of duplicates

2.3.3 Accuracy

Four matrices were selected that covered both infant formula and adult nutritionals (see Table 4 for details). They were analysed in duplicate on three different days by two different operators on two different instruments without spiking (level 0) and spiked with Vivinal GOS or Clasado GOS, each at two out of four levels (Table 4). The GOS ingredients used for spiking were separately analysed using method AOAC 2001.02 [1] to confirm their GOS content.

Table 4: Design of Spike-Recovery Experiment Using Three Fructan Ingredients in Six SPIFAN Matrices

Nestlé Code	Sample	Level 0 (0 g/100g)	Level 1 (0.2 g/100g)	Level 2 (1 g/100g)	Level3 (2.5 g/100g)	Level 4 (5.0 g/100g)
KBI-00046	Child Formula Powder	none	Clasado	Vivinal	Clasado	Vivinal
L3B-00037	Adult Nutritional RTF High Protein	none	Vivinal	Clasado	Vivinal	Clasado
KBI-00047	Infant Elemental Powder	none	Clasado	Vivinal	Clasado	Vivinal
KBI-00045	Adult Nutritional Powder Low Fat	none	Vivinal	Clasado	Vivinal	Clasado

2.3.4 Limit of Quantification

Because the method requires analysis of a complete profile of oligosaccharides, the detection and quantification limits depend on the GOS profile as well as the concentration of GOS in the product, making it extremely difficult to assess (except on an individual oligosaccharide basis, which is meaningless for GOS products). Therefore to demonstrate applicability at the LoQ defined in the SMPR, spike-recovery experiments have been performed at 0.2 g/100g.

5.1. System Calibration

A linear model was used to fit the data for calibration purposes (Figure 2) and the model seems to fit the data well. The relative residuals were calculated and plotted against the analyte concentration (Figure 3), in general the predicted concentration is within 5 % of the actual although there are a few outliers, particularly at lower concentrations.

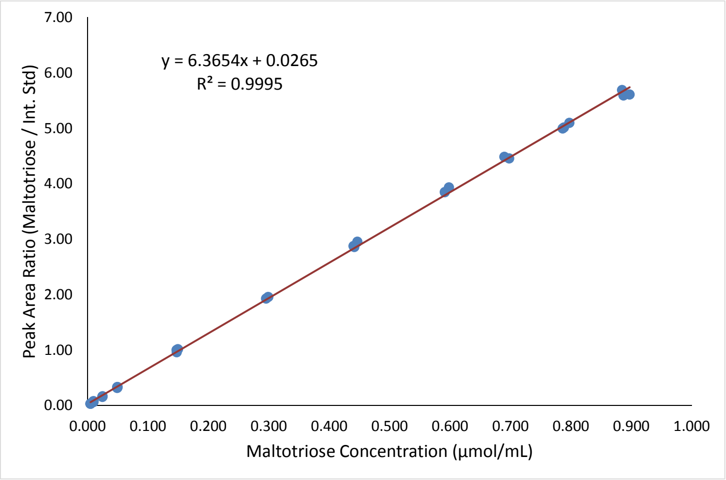


Figure 2: Calibration Curve

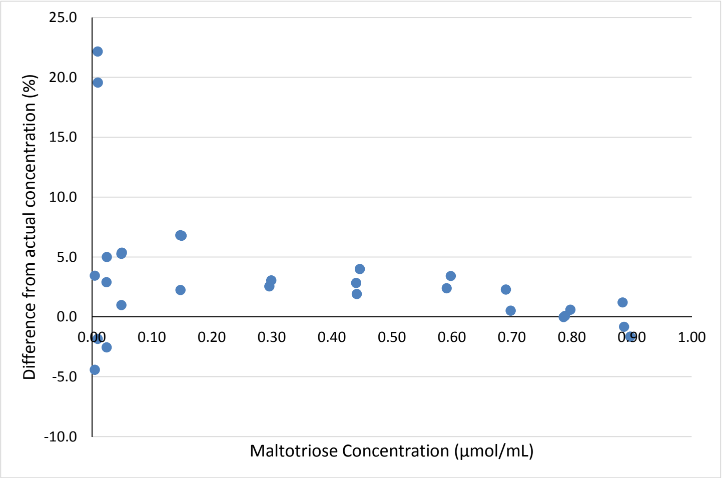


Figure 3: Plot of Relative Residuals (%) Against Maltotriose Concentration

5.2. Analytical Precision

(QSTAT 65117)
Method precision was assessed in a single laboratory by analyzing the samples on 6 days in duplicate. Analyses were performed by two operators on two different instruments (of same model) using two different columns (same column type, different batch number), results are shown in Table 5.

The SMPR [2] requires that the RSD(r) should be less than or equal to 6%. In all cases the achieved RSD(r) was less than 4%, thus the method meets the requirements of the SMPR for RSD(r). The RSD(iR) was less than 8% in all cases suggesting that the method should be able to achieve an RSD(R) of less than 12% during multi lab testing (as required in the SMPR).

Table 5: Analyses of GOS-Containing Samples Under Intermediate Reproducibility Conditions

Nestle Code	Sample Description	n	Mean Conc. (g/100g)	RSD(r) (%)	RSD(iR) (%)	Target RSD(r) (%)	Meets Target (Y/N)
KBI-00031	Infant Formula RTF, Milk Based	6×2	0.154	2.7	7.9	≤ 6	Y
KBI-00033	Infant Formula Powder Partially Hydrolysed, Milk Based	6×2	0.333	3.6	4.9	≤ 6	Y
KBI-00053	Infant Formula Powder FOS/GOS Based	6×2	0.309	2.0	2.4	≤ 6	Y
KBI-00048	Infant Formula Powder Milk Based	6×2	0.266	1.1	4.0	≤ 6	Y
KBI-00049	Infant Formula RTF, Milk Based	6×2	0.157	3.1	5.9	≤ 6	Y
L4B-00006	Infant Formula Powder with GOS	6×2	0.509	1.3	1.7	≤ 6	Y
L4B-00007	Infant Formula Powder with GOS/FOS	6×2	0.351	1.0	3.0	≤ 6	Y
Ref 56A	Infant Formula Powder with GOS/FOS	6×2	2.59*	2.0	2.9	≤ 6	Y

All results reported on a “ready-to-feed” basis except * reported as the concentration in the non-reconstituted powder

5.3. Method Accuracy

Four different blank matrices were spiked at four levels with two different GOS ingredients (as described in Table 4). Recoveries were calculated by comparing the spiked amount vs the measured amount (Table 6). Performance is poorest at the lowest spike level (0.2 g/100g) with spike recoveries ranging from 85-114%, at the other three levels the recoveries are in the range 86 – 94 %, but in most cases the recoveries are outside the 90 – 110% range specified in the SMPR, and in most cases the recoveries are slightly low.

Table 6: GOS Recoveries

					Recovery							
Nestlé	**Level 1**			**Level 2**			**Level 3**			**Level 4**		
Code	Spike (g/100g)	Rec (%)	RSD (%)	Spike (g/100g)	Rec (%)	RSD (%)	Spike (g/100g)	Rec (%)	RSD (%)	Spike (g/100g)	Rec (%)	RSD (%)
KBI-00045	0.211	84.8	4.9	0.980	86.9	2.6	2.47	89.3	1.6	4.91	85.6	2.9
KBI-00046	0.211	91.5	13	0.990	90.7	5.1	2.45	89.2	3.3	4.97	86.6	1.6
KBI-00047	0.203	97.6	5.0	0.980	93.6	3.9	2.45	89.4	3.4	4.96	93.9	3.6
LB3-00037	0.202	114	2.6	1.11	88.7	4.2	2.36	93.1	3.3	4.46	89.2	3.9

5.4. Limit of Quantification

Based on the spike/recovery data at 0.2 g/100g the method is capable of determining GOS at 0.2 g/100g as specified in the SMPR. However, two of the samples in the SPIFAN matrix kit appear to contain lower GOS contents (around 0.15 g/100g), and at this level the method still appears to be performing well. We would thus estimate the LoQ of the method to be below 0.15 g/100g.

CONCLUSIONS

The method tested has can determine the GOS content of most infant formulae and adult nutritional products within the concentrations typically used in such products. The precision of the method is better than required in the SMPR, however spike recoveries are, in general, slightly below the target range of 90-110%. Nevertheless, the method is a significant improvement over current solutions for the determination of GOS in lactose-containing products.

REFERENCES

[1] Official Methods of Analysis of AOAC International (2005), 18th Ed., AOAC International, Gaitherburg, MD, Method 2001.02.

[2] J. AOAC Int. (2015) **98**: 1040. SMPR 2014.003

1. EXAMPLE CHROMATOGRAMS

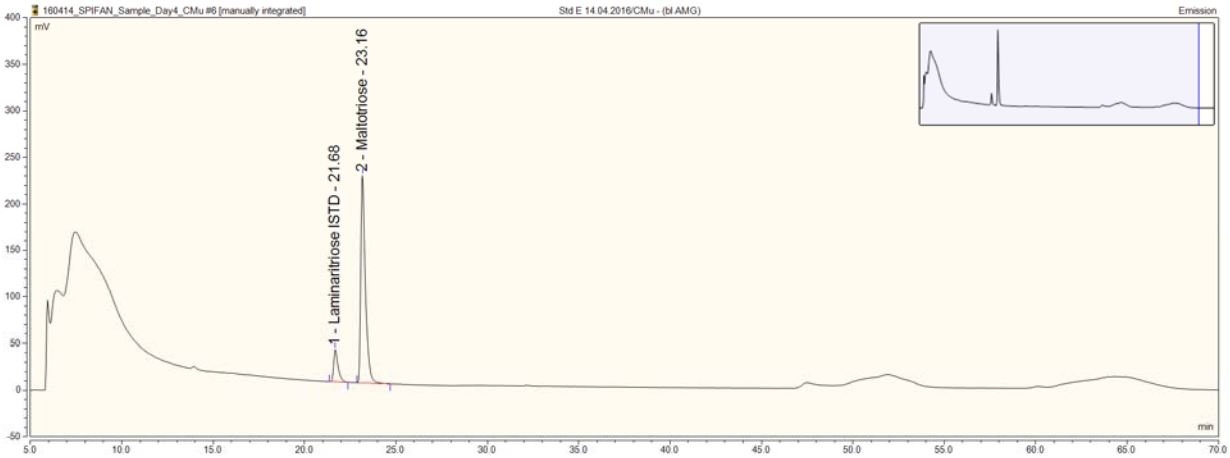


Figure 4: Chromatogram of Standards

Figure 5: Chromatograms of Infant Formula Powder Partially Hydrolysed Milk Based Containing 0.33 g/100g GOS. Upper Chromatogram Untreated, Lower Chromatogram After β-Galactosidase Treatment.

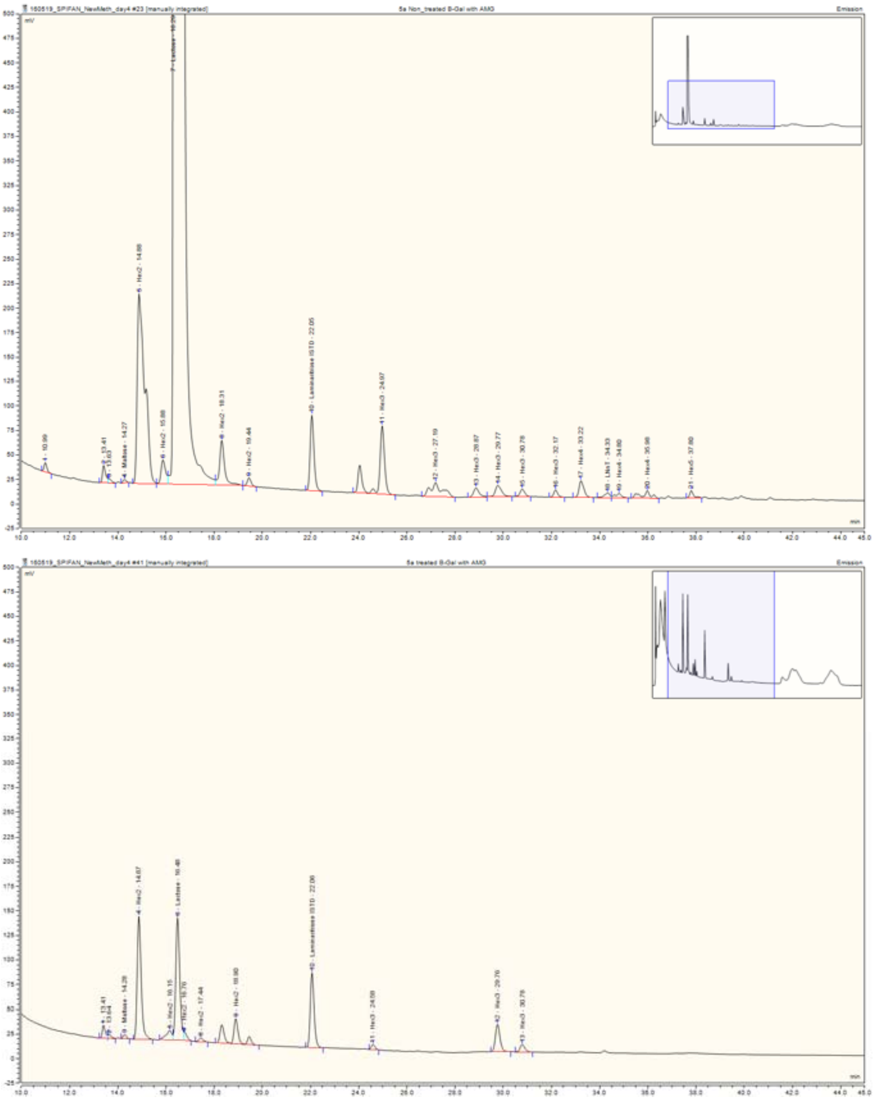


Figure 6: Chromatograms of Infant Formula RTF Milk Based Containing 0.15 g/100g GOS. Upper Chromatogram Untreated, Lower Chromatogram After β-Galactosidase Treatment.

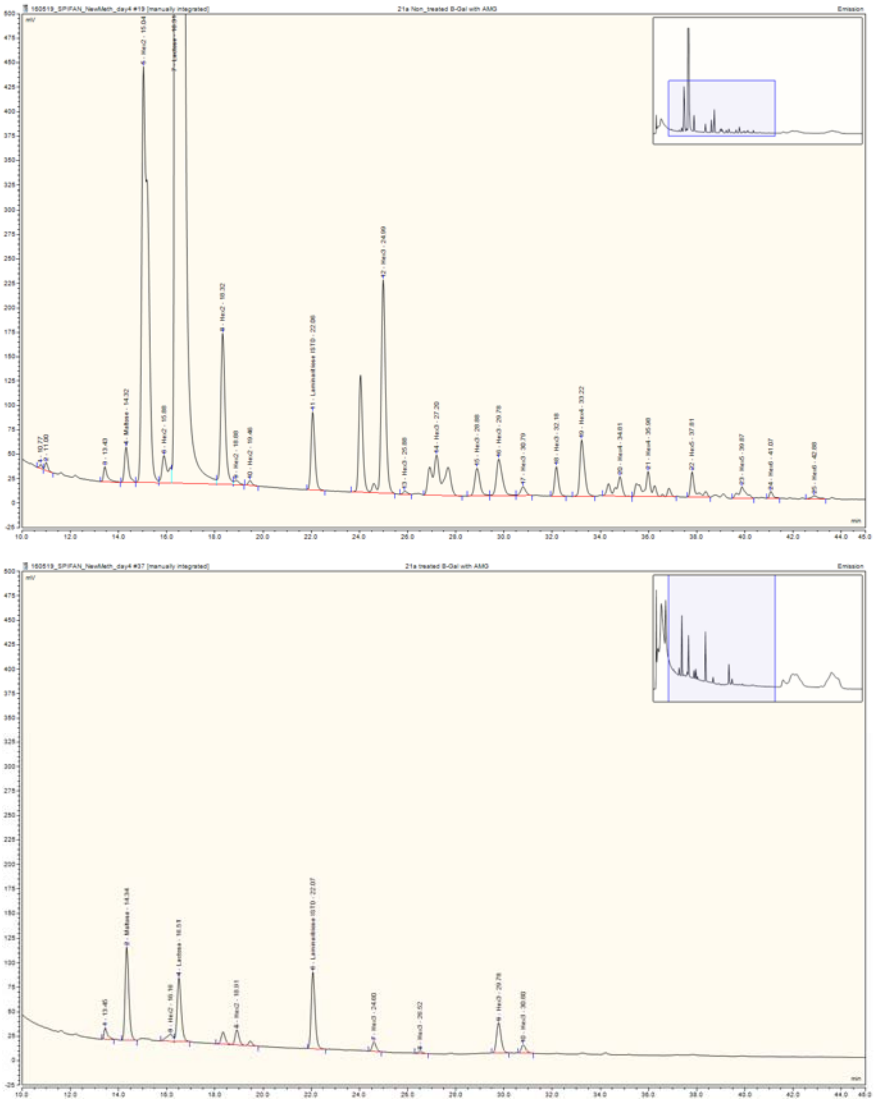


Figure 7: Chromatograms of Infant Formula Powder With GOS, Containing 0.51 g/100g GOS.Upper Chromatogram Untreated, Lower Chromatogram After β-Galactosidase Treatment.

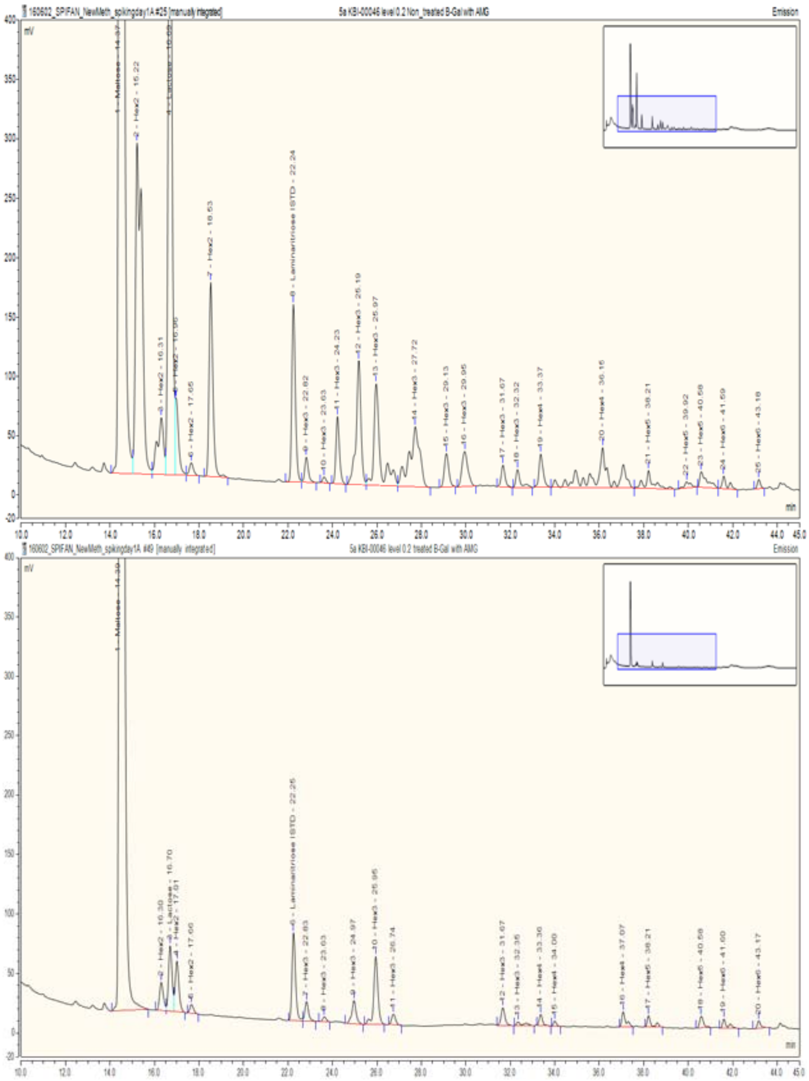


Figure 8: Chromatogram of Child Formula Powder (KBI-00046) Spiked with 0.2g/100g Clasado GOS. Upper Chromatogram Untreated, Lower Chromatogram After β-Galactosidase Treatment.

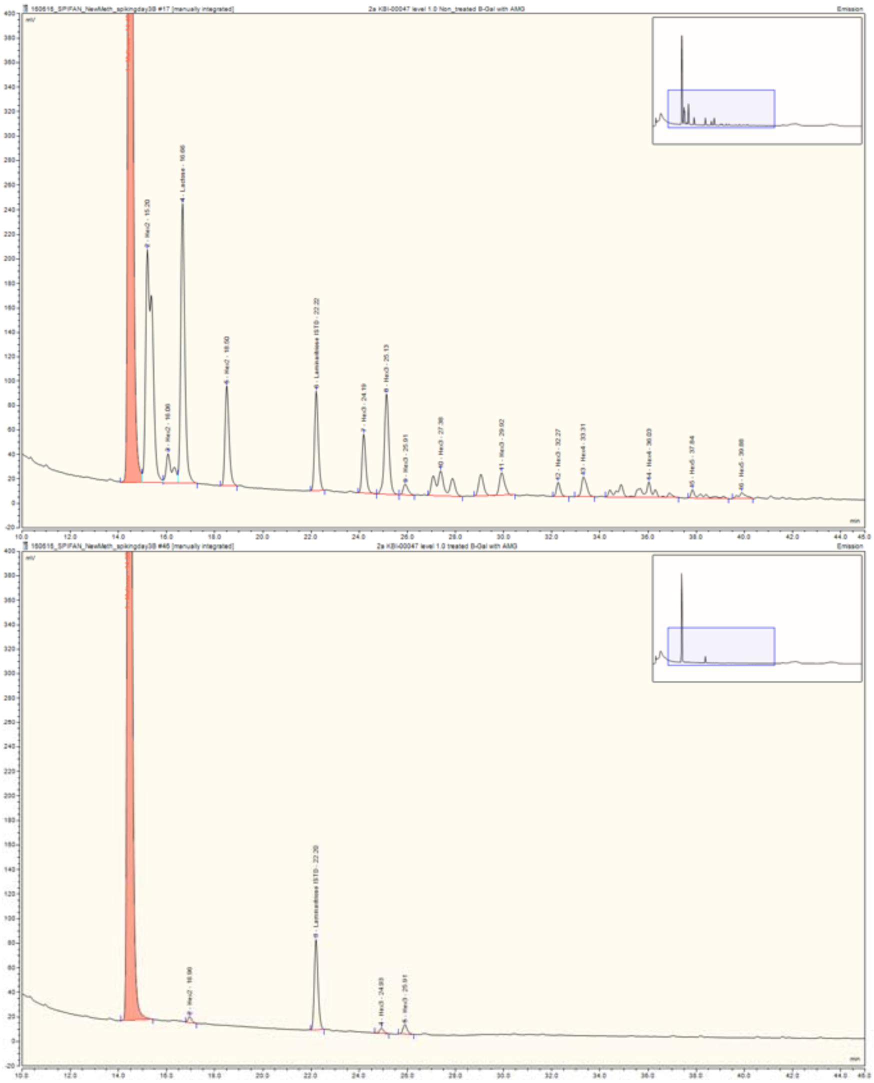


Figure 9: Chromatogram of Infant Elemental Powder (KBI-00047) Spiked with 1.0g/100g Vivinal GOS. Upper Chromatogram Untreated, Lower Chromatogram After β-Galactosidase Treatment.

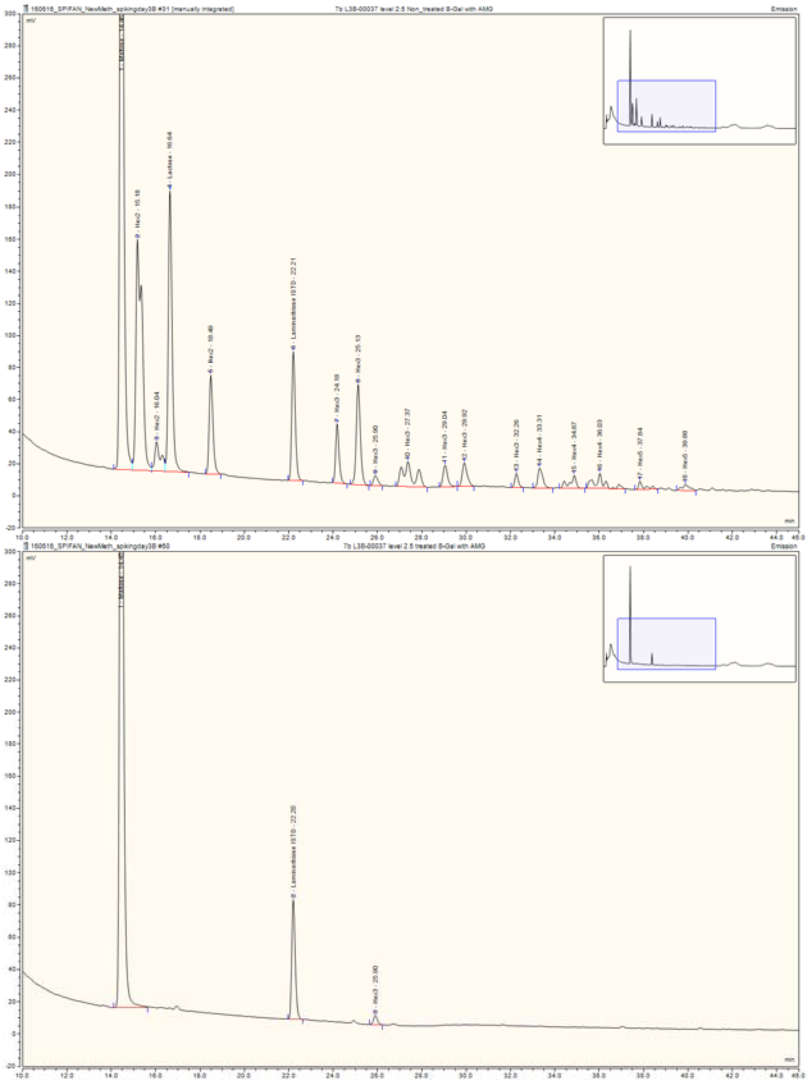


Figure 10: Chromatogram of Adult Nutritional RTF High Protein (L3B-00037) Spiked With 2.5g/100g Vivinal GOS. Upper Chromatogram Untreated, Lower Chromatogram After β-Galactosidase Treatment.

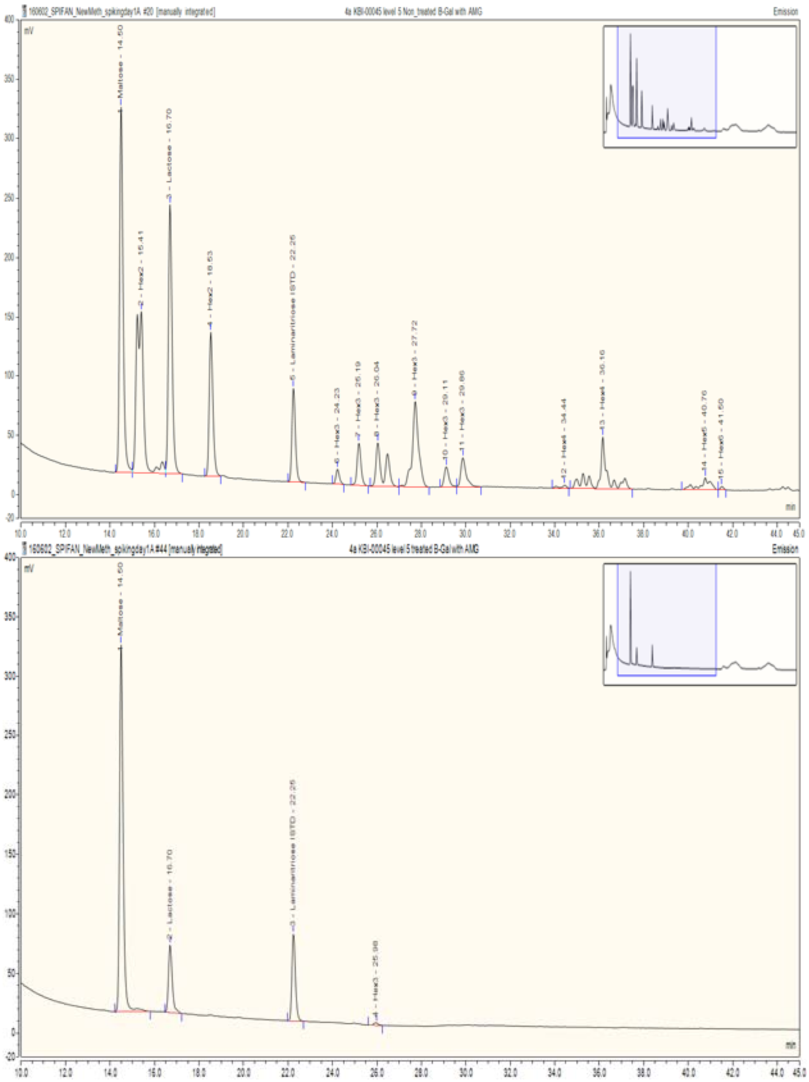


Figure 11: Chromatogram of of Child Formula Powder (KBI-00045) Spiked with 5.0g/100g Clasado GOS. Upper Chromatogram Untreated, Lower Chromatogram After β-Galactosidase Treatment.

2. RAW DATA (PRECISION)

NRC Ref	Batch	Sample Description	Date	Analyst	Result A (g/100g)	Result B (g/100g)
Ref 56A	Ref 56A	Infant Formula Powder with GOS/FOS	26.04.2016	C	2.66	2.68
			29.04.2016	D	2.60	2.46
			12.05.2016	C	2.54	2.48
			19.05.2016	C	2.54	2.62
			24.05.2016	D	2.65	2.67
			03.06.2016	D	2.64	2.59
KBI-00033		Infant Formula Powder Partially Hydrolysed Milk Based	26.04.2016	C	0.339	0.375
			29.04.2016	D	0.334	0.328
			12.05.2016	C	0.325	0.326
			19.05.2016	C	0.313	0.323
			24.05.2016	D	0.319	0.331
			03.06.2016	D	0.336	0.346
KBI-00053		Infant Formula Powder FOS/GOS Based	26.04.2016	C	0.315	0.312
			29.04.2016	D	0.308	0.306
			12.05.2016	C	0.313	0.295
			19.05.2016	C	0.300	0.300
			24.05.2016	D	0.313	0.317
			03.06.2016	D	0.308	0.318
KBI-00048		Infant Formula Powder Milk Based	29.04.2016	D	0.277	0.281
			12.05.2016	C	0.255	0.261
			19.05.2016	C	0.260	0.259
			24.05.2016	D	0.266	0.259
			03.06.2016	D	0.278	0.278
			09.06.2016	C	0.255	0.257
L4B-00006		Infant Formula Powder With GOS	29.04.2016	D	0.512	0.512
			12.05.2016	C	0.503	0.508
			19.05.2016	C	0.502	0.507
			24.05.2016	D	0.501	0.517
			03.06.2016	D	0.526	0.519
			16.06.2016	C	0.497	0.509
L4B-00007		Infant Formula Powder With GOS/FOS	26.04.2016	C	0.367	0.364
			29.04.2016	D	0.351	0.354
			12.05.2016	C	0.337	0.344
			19.05.2016	C	0.334	0.342
			24.05.2016	D	0.356	0.357
			03.06.2016	D	0.352	0.355
KBI-00031		Infant Formula RTF Milk Based	26.04.2016	C	0.171	0.164
			29.04.2016	D	0.165	0.163
			12.05.2016	C	0.141	0.145
			19.05.2016	C	0.139	0.136
			24.05.2016	D	0.150	0.159
			03.06.2016	D	0.163	0.156
KBI-00049		Infant Formula RTF Milk Based	26.04.2016	C	0.166	0.167
			29.04.2016	D	0.163	0.169
			12.05.2016	C	0.149	0.154
			19.05.2016	C	0.144	0.147
			24.05.2016	D	0.146	0.160
			03.06.2016	D	0.160	0.163

3. RAW DATA (TRUENESS)

Spike-Recovery: Sample KBI-00045

								Recovery	
Date	Analyst	Instrument	Sample	GOS	GOS Spike Level (g/100g)	Measured GOS (g/100g)	Recovery (%)	Average (%)	RSD (%)
02.06.2016	CMu	RS #2	KBI-00045	Vivinal	0.211	0.177	83.9	84.8	4.9
02.06.2016	CMu	RS #2	KBI-00045	Vivinal	0.211	0.178	84.4		
10.06.2016	dc	RS #3	KBI-00045	Vivinal	0.211	0.188	89.1		
10.06.2016	dc	RS #3	KBI-00045	Vivinal	0.211	0.19	90.0		
09.06.2016	CMu	RS #2	KBI-00045	Vivinal	0.211	0.168	79.6		
09.06.2016	CMu	RS #2	KBI-00045	Vivinal	0.211	0.172	81.5		
02.06.2016	CMu	RS #2	KBI-00045	Clasado	0.980	0.857	87.4	86.9	2.6
02.06.2016	CMu	RS #2	KBI-00045	Clasado	0.980	0.856	87.3		
10.06.2016	dc	RS #3	KBI-00045	Clasado	0.980	0.873	89.1		
10.06.2016	dc	RS #3	KBI-00045	Clasado	0.980	0.872	89.0		
09.06.2016	CMu	RS #2	KBI-00045	Clasado	0.980	0.822	83.9		
09.06.2016	CMu	RS #2	KBI-00045	Clasado	0.980	0.827	84.4		
02.06.2016	CMu	RS #2	KBI-00045	Vivinal	2.47	2.24	90.7	89.3	1.6
02.06.2016	CMu	RS #2	KBI-00045	Vivinal	2.47	2.23	90.3		
10.06.2016	dc	RS #3	KBI-00045	Vivinal	2.47	2.18	88.3		
10.06.2016	dc	RS #3	KBI-00045	Vivinal	2.47	2.18	88.3		
17.06.2016	dc	RS #3	KBI-00045	Vivinal	2.47	2.16	87.4		
17.06.2016	dc	RS #3	KBI-00045	Vivinal	2.47	2.24	90.7		
02.06.2016	CMu	RS #2	KBI-00045	Clasado	4.91	4.26	86.8	85.6	2.9
02.06.2016	CMu	RS #2	KBI-00045	Clasado	4.91	4.27	87.0		
10.06.2016	dc	RS #3	KBI-00045	Clasado	4.91	4.29	87.4		
10.06.2016	dc	RS #3	KBI-00045	Clasado	4.91	4.31	87.8		
09.06.2016	CMu	RS #2	KBI-00045	Clasado	4.91	4.04	82.3		
09.06.2016	CMu	RS #2	KBI-00045	Clasado	4.91	4.06	82.7		

Spike-Recovery: Sample KBI-00046

								Recovery	
Date	Analyst	Instrument	Sample	GOS	GOS Spike Level (g/100g)	Measured GOS (g/100g)	Recovery (%)	Average (%)	RSD (%)
02.06.2016	CMu	RS #2	KBI-00046	Vivinal	0.211	0.175	82.9	91.5	12.7
02.06.2016	CMu	RS #2	KBI-00046	Vivinal	0.211	0.166	78.7		
10.06.2016	dc	RS #3	KBI-00046	Vivinal	0.211	0.191	90.5		
10.06.2016	dc	RS #3	KBI-00046	Vivinal	0.211	0.181	85.8		
17.06.2016	dc	RS #3	KBI-00046	Vivinal	0.211	0.225	106.6		
17.06.2016	dc	RS #3	KBI-00046	Vivinal	0.211	0.221	104.7		
16.06.2016	CMu	RS #2	KBI-00046	Clasado	0.990	0.858	86.7	90.7	5.1
16.06.2016	CMu	RS #2	KBI-00046	Clasado	0.990	0.844	85.3		
10.06.2016	dc	RS #3	KBI-00046	Clasado	0.990	0.891	90.0		
10.06.2016	dc	RS #3	KBI-00046	Clasado	0.990	0.891	90.0		
09.06.2016	CMu	RS #2	KBI-00046	Clasado	0.990	0.961	97.1		
09.06.2016	CMu	RS #2	KBI-00046	Clasado	0.990	0.94	94.9		
02.06.2016	CMu	RS #2	KBI-00046	Vivinal	2.45	2.09	85.3	89.2	3.3
02.06.2016	CMu	RS #2	KBI-00046	Vivinal	2.45	2.11	86.1		
10.06.2016	dc	RS #3	KBI-00046	Vivinal	2.45	2.18	89.0		
10.06.2016	dc	RS #3	KBI-00046	Vivinal	2.45	2.22	90.6		
09.06.2016	CMu	RS #2	KBI-00046	Vivinal	2.45	2.24	91.4		
09.06.2016	CMu	RS #2	KBI-00046	Vivinal	2.45	2.27	92.7		
02.06.2016	CMu	RS #2	KBI-00046	Clasado	4.97	4.35	87.5	86.6	1.6
02.06.2016	CMu	RS #2	KBI-00046	Clasado	4.97	4.38	88.1		
10.06.2016	dc	RS #3	KBI-00046	Clasado	4.97	4.36	87.7		
10.06.2016	dc	RS #3	KBI-00046	Clasado	4.97	4.26	85.7		
09.06.2016	CMu	RS #2	KBI-00046	Clasado	4.97	4.24	85.3		
09.06.2016	CMu	RS #2	KBI-00046	Clasado	4.97	4.23	85.1		

Spike-Recovery: Sample KBI-00047

Date	Analyst	Instrument	Sample	GOS	GOS Spike Level (g/100g)	Measured GOS (g/100g)	Recovery (%)	Recovery Average (%)	Recovery RSD (%)
02.06.2016	dc	RS #3	KBI-00047	Vivinal	0.203	0.206	101.5	97.6	5.0
02.06.2016	dc	RS #3	KBI-00047	Vivinal	0.203	0.212	104.4		
07.06.2016	dc	RS #3	KBI-00047	Vivinal	0.203	0.195	96.1		
07.06.2016	dc	RS #3	KBI-00047	Vivinal	0.203	0.201	99.0		
16.06.2016	CMu	RS #2	KBI-00047	Vivinal	0.203	0.188	92.6		
16.06.2016	CMu	RS #2	KBI-00047	Vivinal	0.203	0.187	92.1		
02.06.2016	dc	RS #3	KBI-00047	Clasado	0.980	0.955	97.4	93.6	3.9
02.06.2016	dc	RS #3	KBI-00047	Clasado	0.980	0.937	95.6		
07.06.2016	dc	RS #3	KBI-00047	Clasado	0.980	0.918	93.7		
07.06.2016	dc	RS #3	KBI-00047	Clasado	0.980	0.946	96.5		
16.06.2016	CMu	RS #2	KBI-00047	Clasado	0.980	0.875	89.3		
16.06.2016	CMu	RS #2	KBI-00047	Clasado	0.980	0.873	89.1		
02.06.2016	dc	RS #3	KBI-00047	Vivinal	2.45	2.23	91.0	89.4	3.4
02.06.2016	dc	RS #3	KBI-00047	Vivinal	2.45	2.22	90.6		
07.06.2016	dc	RS #3	KBI-00047	Vivinal	2.45	2.26	92.2		
07.06.2016	dc	RS #3	KBI-00047	Vivinal	2.45	2.24	91.4		
16.06.2016	CMu	RS #2	KBI-00047	Vivinal	2.45	2.09	85.3		
16.06.2016	CMu	RS #2	KBI-00047	Vivinal	2.45	2.1	85.7		
02.06.2016	dc	RS #3	KBI-00047	Clasado	4.96	4.83	97.4	93.9	3.6
02.06.2016	dc	RS #3	KBI-00047	Clasado	4.96	4.86	98.0		
07.06.2016	dc	RS #3	KBI-00047	Clasado	4.96	4.66	94.0		
07.06.2016	dc	RS #3	KBI-00047	Clasado	4.96	4.64	93.5		
16.06.2016	CMu	RS #2	KBI-00047	Clasado	4.96	4.49	90.5		
16.06.2016	CMu	RS #2	KBI-00047	Clasado	4.96	4.45	89.7		

Spike-Recovery: Sample L3B-00037

								Recovery	
Date	Analyst	Instrument	Sample	GOS	GOS Spike Level (g/100g)	Measured GOS (g/100g)	Recovery (%)	Average (%)	RSD (%)
02.06.2016	dc	RS #3	L3B-00037	Vivinal	0.202	0.229	113.4	113.8	2.6
02.06.2016	dc	RS #3	L3B-00037	Vivinal	0.202	0.239	118.3		
07.06.2016	dc	RS #3	L3B-00037	Vivinal	0.202	0.234	115.8		
07.06.2016	dc	RS #3	L3B-00037	Vivinal	0.202	0.229	113.4		
16.06.2016	CMu	RS #2	L3B-00037	Vivinal	0.202	0.225	111.4		
16.06.2016	CMu	RS #2	L3B-00037	Vivinal	0.202	0.223	110.4		
02.06.2016	dc	RS #3	L3B-00037	Clasado	1.110	1.05	94.6	88.7	4.2
02.06.2016	dc	RS #3	L3B-00037	Clasado	1.110	1.02	91.9		
07.06.2016	dc	RS #3	L3B-00037	Clasado	1.110	0.975	87.8		
07.06.2016	dc	RS #3	L3B-00037	Clasado	1.110	0.95	85.6		
16.06.2016	CMu	RS #2	L3B-00037	Clasado	1.110	0.961	86.6		
16.06.2016	CMu	RS #2	L3B-00037	Clasado	1.110	0.953	85.9		
02.06.2016	dc	RS #3	L3B-00037	Vivinal	2.36	2.28	96.6	93.1	3.3
02.06.2016	dc	RS #3	L3B-00037	Vivinal	2.36	2.24	94.9		
07.06.2016	dc	RS #3	L3B-00037	Vivinal	2.36	2.24	94.9		
07.06.2016	dc	RS #3	L3B-00037	Vivinal	2.36	2.21	93.6		
16.06.2016	CMu	RS #2	L3B-00037	Vivinal	2.36	2.11	89.4		
16.06.2016	CMu	RS #2	L3B-00037	Vivinal	2.36	2.11	89.4		
02.06.2016	dc	RS #3	L3B-00037	Clasado	4.46	4.02	90.1	89.2	3.9
02.06.2016	dc	RS #3	L3B-00037	Clasado	4.46	4.12	92.4		
07.06.2016	dc	RS #3	L3B-00037	Clasado	4.46	4.04	90.6		
07.06.2016	dc	RS #3	L3B-00037	Clasado	4.46	4.11	92.2		
16.06.2016	CMu	RS #2	L3B-00037	Clasado	4.46	3.82	85.7		
16.06.2016	CMu	RS #2	L3B-00037	Clasado	4.46	3.75	84.1		

4. PROTOCOL FOR MW DETERMINATION OF GOS BY UHPLC-FLD-MS

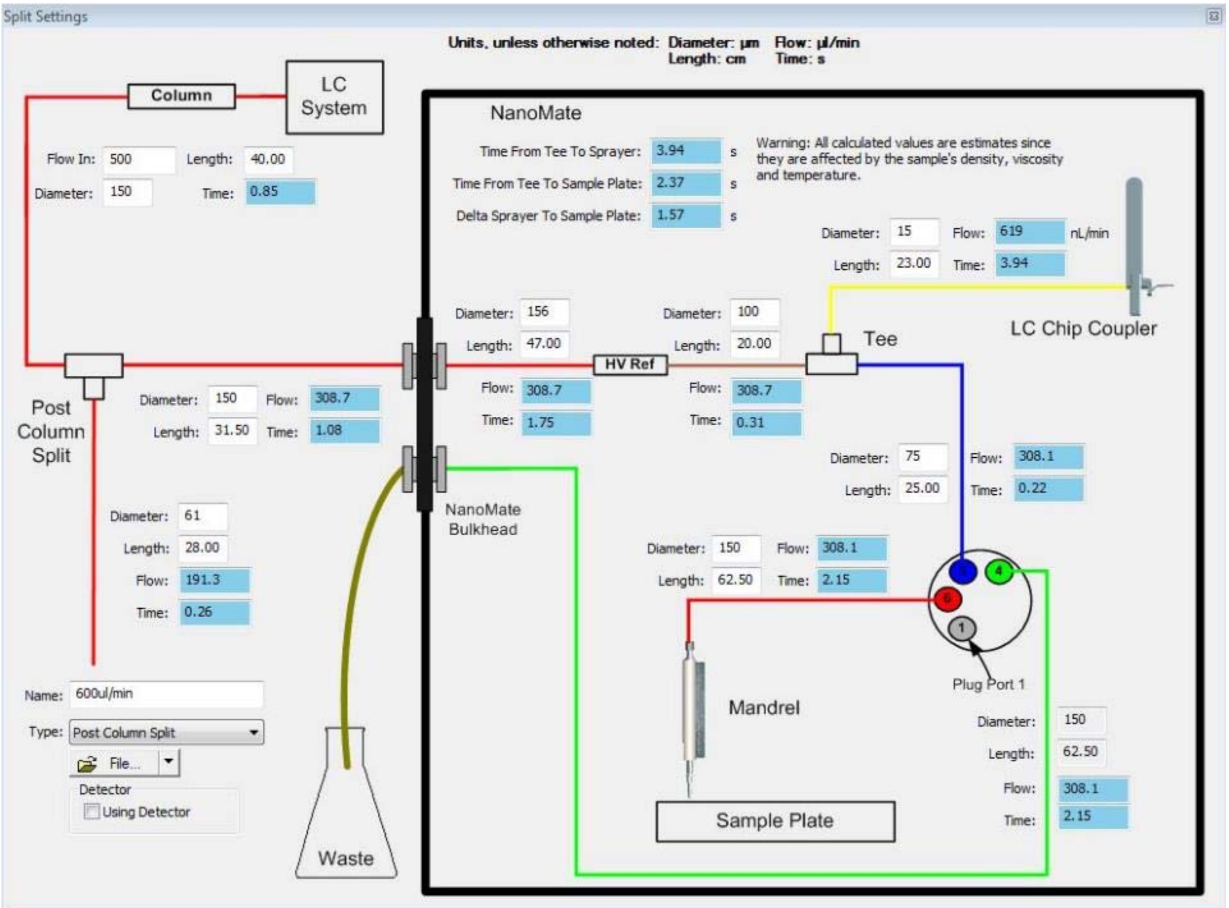
Sample preparation:
The same sample preparation is the same for both quantitative analysis and for the MW assignment of signals, thus follow the normal sample preparation procedure (the same vial prepared for the quantitative determination of GOS can thus also be used for the analysis of MW assignment). Alternatively one may prepare a sample using only the GOS ingredient. In case the mass spectrometer has insufficient sensitivity it is possible to prepare a sample having ten times greater GOS concentration for the purposes of peak identification only (in this case it is recommended to use the GOS ingredient).

Mass Spectrometer Set-up:
In addition to the UHPLC-FLD instrument, an API 4000 QTRAP (Applied Biosystems) mass spectrometer detector was attached via a Triversa Nanomate (Advion) to work in nanoelectrospray mode. The UHPLC flow was split at the exit of the LC column, approximately half of the flow (0.3 mL/min) goes to the FLD, and the remaining 0.3 mL/min is directed to the nanomate source). The nanomate further splits the flow down to about 600 nL/min for introduction to the mass spectrometer. The following describes the setup of the MS system in our lab. The mass spectrometer settings in other labs should be optimised locally.
NOTE: Do not connect the FLD and the mass spectrometer in series. In general the FLD cells cannot withstand the generated backpressure

LC parameters:
Use the same LC conditions as described in the quantitative method

Triversa Nanomate source parameters:
Method type: LC Chip Coupling
Trigger acquisition when input singnal received: Yes. Signal: DigIn 1
Voltage: 1.40 kV
Polarity: Negative
Output contact closure signal: MS Relay Duration: 2.5s
Always use new nozzle: No

Split setting:



Units, unless otherwise noted: Diameter: µm Length: cm Flow: µl/min Time: s

Flow In: 500
Length: 40.00
Diameter: 150
Time: 0.85

Time From Tee To Sprayer: 3.94 s
Time From Tee To Sample Plate: 2.37 s
Delta Sprayer To Sample Plate: 1.57 s

Warning: All calculated values are estimates since they are affected by the sample's density, viscosity and temperature.

Diameter: 15
Flow: 619 nL/min
Length: 23.00
Time: 3.94

Diameter: 156
Length: 47.00
Flow: 308.7
Time: 1.75

Diameter: 100
Length: 20.00
Flow: 308.7
Time: 0.31

Diameter: 150
Flow: 308.7
Length: 31.50
Time: 1.08

Diameter: 75
Flow: 308.1
Length: 25.00
Time: 0.22

Diameter: 61
Length: 28.00
Flow: 191.3
Time: 0.26

Diameter: 150
Flow: 308.1
Length: 62.50
Time: 2.15

Diameter: 150
Length: 62.50
Flow: 308.1
Time: 2.15

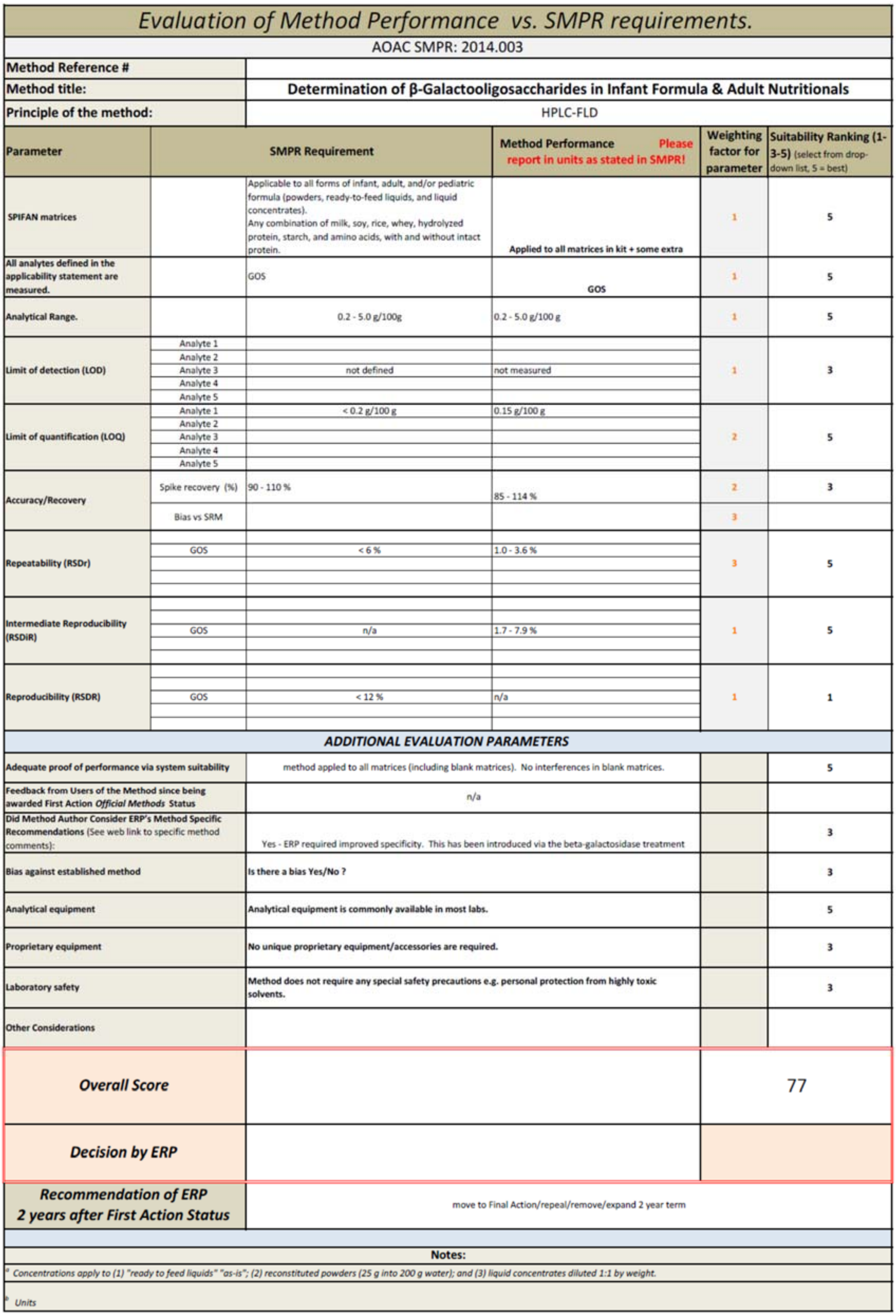
Name: 600ul/min
Type: Post Column Split
File...
Detector
☐ Using Detector

MS parameters:
Experiment type: Multiple ion monitoring (Q1 or EMS)
Mode: ESI negative.
CUR: 5.0
IS: -0.0 (spray voltage is applied by the Nanomate)
GS1: 0
GS2: 0
IHT: 80.0°C
DP: -110
RP: -10.0

Monitored masses:

Q1 Mass (Da)	Dwell time (ms)	Corresponding GOS
461.3	50.0	Hex_2 (including lactose)
623.4	50.0	Hex_3
785.4	50.0	Hex_4
947.4	50.0	Hex_5
1109.5	50.0	Hex_6
1271.6	50.0	Hex_7
1433.6	50.0	Hex_8

5. METHOD EVALUATION FORM



Evaluation of Method Performance vs. SMPR requirements.

AOAC SMPR: 2014.003

Method Reference #					
Method title:	**Determination of β-Galactooligosaccharides in Infant Formula & Adult Nutritionals**				
Principle of the method:	HPLC-FLD				
Parameter		**SMPR Requirement**	**Method Performance** **Please report in units as stated in SMPR!**	**Weighting factor for parameter**	**Suitability Ranking (1-3-5)** (select from drop-down list, 5 = best)
SPIFAN matrices		Applicable to all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates). Any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.	**Applied to all matrices in kit + some extra**	1	**5**
All analytes defined in the applicability statement are measured.		GOS	**GOS**	1	**5**
Analytical Range.		0.2 - 5.0 g/100g	0.2 - 5.0 g/100 g	1	**5**
Limit of detection (LOD)	Analyte 1			1	**3**
	Analyte 2				
	Analyte 3	not defined	not measured		
	Analyte 4				
	Analyte 5				
Limit of quantification (LOQ)	Analyte 1	< 0.2 g/100 g	0.15 g/100 g	2	**5**
	Analyte 2				
	Analyte 3				
	Analyte 4				
	Analyte 5				
Accuracy/Recovery	Spike recovery (%)	90 - 110 %	85 - 114 %	2	**3**
	Bias vs SRM			3	
Repeatability (RSDr)				3	**5**
	GOS	< 6 %	1.0 - 3.6 %		
Intermediate Reproducibility (RSDiR)				1	**5**
	GOS	n/a	1.7 - 7.9 %		
Reproducibility (RSDR)				1	**1**
	GOS	< 12 %	n/a		
ADDITIONAL EVALUATION PARAMETERS					
Adequate proof of performance via system suitability	method appled to all matrices (including blank matrices). No interferences in blank matrices.				**5**
Feedback from Users of the Method since being awarded First Action *Official Methods* Status	n/a				
Did Method Author Consider ERP's Method Specific Recommendations (See web link to specific method comments):	Yes - ERP required improved specificity. This has been introduced via the beta-galactosidase treatment				**3**
Bias against established method	**Is there a bias Yes/No ?**				**3**
Analytical equipment	**Analytical equipment is commonly available in most labs.**				**5**
Proprietary equipment	**No unique proprietary equipment/accessories are required.**				**3**
Laboratory safety	**Method does not require any special safety precautions e.g. personal protection from highly toxic solvents.**				**3**
Other Considerations					
Overall Score				77	
Decision by ERP					
Recommendation of ERP 2 years after First Action Status	move to Final Action/repeal/remove/expand 2 year term				
Notes:					

[a] *Concentrations apply to (1) "ready to feed liquids" "as-is"; (2) reconstituted powders (25 g into 200 g water); and (3) liquid concentrates diluted 1:1 by weight.*

[b] *Units*