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Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN)

EXPERT REVIEW PANEL (NUTRIENTS)

REVIEWER FORMS
(March 16, 2016)



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AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

AMINO ACIDS

Amino-03

TUV-SUD

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method _____Amino-03_____#1

Title: Amino Acids in Infant Formula and Adult/Pediatric Formulas Ultra High Performance Liquid Chromatography

Author: TUV-SUD

Summary of Method: Three separate digestions (acid hydrolysis, oxidative acid hydrolysis (Cys, Met), and alkaline hydrolysis (Trp) for total amino acid analysis by dervitization with 6-aminoquinolyl-N-hydrolyysuccinimidyl carbamate (AQC) reagent (except Trp). Dervitized amino acids are separated by UHPLC and detected by DAD and FL (Trp) using Norvaline as internal standard.

Method Scope/Applicability: Total amino acid analysis in infant, adult, and/or pediatric formula (powders, RTF liquids and liquid concentrates) according to applicability statement however validation data only shown for NIST 1849a.

General comments about the method:
Overall data from NIST SRM 1849a looks very promising. Separate digestions to prevent degradation of Cys, Met, and Trp. Phenol addition in acid hydrolysis to prevent tyrosine degregation during hydrolysis. Norvaline IS and is historically robust for AA analysis methods. UHPLC separation resulting in shorter runtimes and high throughput sample preparation and analysis. Method also captures Taurine.

Method Clarity:
Phenol is added as a “speck” (approximately 10 mg)

Method Safety Concerns:
Acids and bases utilized in hydrolysis—exercise typical laboratory safety precautions.

Pros/Strengths:
- Utilizes internal standard (Norvaline)
- Addition of phenol to protect Tyr in acid hydrolysis
- Fast UHPLC separation
- Separate digestions to prevent degradation of Cys, Met, and Trp

Cons/Weaknesses

- Method does not show validation data for any of the SPIFAN matrices (only NIST 1849a)
- Methionine sulfone separation has very low peak resolution
- Internal standard is added after hydrolysis, transfer and filter steps so it is only adjusting for instrument recovery and no prep losses.

Supporting Data

- General Comment: Method has supporting validation data in NIST SRM 1849a only

    - Method Optimization: Concerns regarding resolution of methionine sulfone peak.

- Performance Characteristics:

Analytical Range: 3.6 mg/100g-22,500mg/100g. (SMPR: 0.4 mg/100g-2500 mg/100g)

LOQ: 3.6 mg/100g on an as is basis (SMPR: 0.4 mg/100g on reconstituted powder basis)

Accuracy/Recovery: For NIST 1849a recoveries reported ranged from 92.4-107.6 based on result obtained vs NIST SRM reference value. No spike recovery data reported in any SPIFAN matrices.

Precision (RSD_r): On NIST SRM 1849a ranged from 0.6-3.2% RSD (not listed for Trp). It is not clear how many replicates were performed to determine this value.

Reproducibility (RSD_R): Reported in NIST SRM 1849a to range from 1.4-4.9% RSD(R) however it is not clear what values were included and/or how this was determined.

- System suitability:

Not specified in current documents.

Recommendation:
Request for SLV data in SPIFAN matrices.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Amino -03 #2

Title: **Amino Acids in Infant Formula and Adult/Pediatric Formulas Ultra High Performance Liquid Chromatography**

Author: TUV-SUD

Summary of Method:
Method uses 3 different protein hydrolysis procedures:

- acid hydrolysis for Histidine, Taurine, Serine, Arginine, Glycine, Aspartic acid (sum of Aspartic acid and Asparagine), Glutamic acid (sum of Glutamic acidand Glutamine), Threonine, Alanine, Proline, Lysine, Tyrosine,Valine, Isoleucine, Leucine, Phenyl alanine.
- Oxidative hydrolysis with performic acid for Cysteine, Cystine and Methionine
- Alkaline hydrolysis for Tryptophan

Resulting amino acids (except Tryptophan) are derivitized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQQ-Tag derivatization reagent from Waters) reagent and separated using UHPLC system. (AA The detection of the derivatives is done using UV/Vis detector, Tryptophan is detected using Fluorescence detector.

Norvaline is used as Internal Standard

Method Scope/Applicability: Applicable for the determination of amino acids (including Taurine) in all forms of infant, adult, and/or pediatric formula (powders, ready to feed liquids and liquid concentrates).

General comments about the method:
3 separate sample preparation procedures and HPLC methods are required to analyze all the amino acids so essentially these are 3 separate methods. The hydrolysis procedures used are well-established and are part of AOAC and ISO methods.
Experimental part is clearly written and should be is easy to follow.
The only data available are for SRM 1849a. No SPIFAN matrices were analyzed.

Validation study design is not described so though repeatability and reproducibility data are listed for SRM 1849a it is not clear how reproducibility data were obtained or how many samples were analyzed.

Method Clarity:
Method is clearly written and should be easy to follow; unfortunately the validation study and results are not well described making it difficult to make comparison with SMPR

Pros/Strengths:
- 3 procedures described are capable of analyzing all amino acids specified by SPMR.
- Hydrolysis procedures are well known and proven to be effective
- The method uses commonly available instrumentation and is easy to implement
- Fast chromatographic analysis

Cons/Weaknesses
- Data for only a single matrix are presented – SRM 1849a
- No SPIFAN matrixes were used
- Full validation results are not presented
- Need at least 6-point calibration curves for all amino acids covering wider ranges (right now, 3-point for Cysteic acid and Methionine Sulfone, 6-ponit for Tryptophan and 5-point for all other amino acids)

Supporting Data
- General Comment:

    - Method Optimization:

- Performance Characteristics:

    Analytical Range: Not stated

LOQ: stated as 2 mg/100 g but how this number was calculated is not clear. SPMR sets LOQ at 0.4 mg/100g so the specifications are not met

Accuracy/Recovery:
Accuracy is validated by analyzing SRM 1849a. All recoveries for this sample met SMPR

Precision (RSD_r):
Repeatability for all amino acids except for Tyrosine (2.6 % >2.0% requirement) met SMPR. Tryptophan repeatability is not listed

Reproducibility (RSD_R): Reproducibility listed meets specifications for most amino acids though how reproducibility was calculated is not clear since all data seem to belong to a single laboratory.

- **System suitability:** not stated

Recommendation:

The method uses common methodology that should be well suited for analysis of total amino acids but more data are needed to fully evaluate the method against SMPR. It is recommended that all SPIFAN matrices were analyzed using this method. In addition the following questions need to be addressed:

- Describe clearly the validation study and the results
- Clarify the information on analytical range and LOQ
- Provide information on back-calculated errors for standards
- Add information on check samples and check standards to system suitability section
- Increase the number of calibrators to 6 for all amino acids
- It is not necessary to include all chromatograms and full data reports for each sample and standard. Several representative chromatograms and summary of the results would be sufficient

Decision on recommending this method as AOAC First Action is better made after data for more SPIFAN matrices are collected.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Amino-03 #3

Title:
Amino Acids in Infant Formula and Adult/Pediatric Formulas

Author:
TUV/SUD

Summary of Method:
Proteins hydrolysis followed by derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent. The derivatized forms of amino acids are separated by UHPLC and quantitated by Diode Array Detector (Tryptophan by Fluorescence detector) using Norvaline as internal standard.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:
Standard technique for analyzing amino acids using proprietary AccQ Tag derivatisation followed by LC-UV analysis.

Method Clarity:
Clear directions for operation of the method, instruments and calculations

Method Safety Concerns:
None other than standard laboratory safety

Pros/Strengths:
Very well established technique for amino acids analysis

Cons/Weaknesses
Use of proprietary AccTag reagents

Supporting Data
Values obtained for NIST 1849a are acceptable against certified values.
No SPIFAN kit used in the validation of the method.

Performance Characteristics:

Analytical Range:
Not specified

LOQ:

Not specified

Accuracy/Recovery:
Spike recovery not specified.
Recovery measured as % bias against NIST 1849a, within acceptable limits

Precision (RSD_r):
Mostly meets SMPR limits for repeatability

Reproducibility (RSD_R):
Reported, however no context of how reproducibility measured (number labs, etc.). Values acceptable against SMPR.

System suitability:
No details

Recommendation:
Not recommended First Action at this time as the SPIFAN kit was not used as required by ERP. Also issue of proprietary nature of analysis should be discussed.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Amino-03 #4

Title: Amino Acids in Infant Formulas and Adult/Pediatric Formula

Author: Devi Prasad

Reviewer Name:

Summary of Method:
Amino Acids ~~from~~ by hydrolyzed (Acid, Alkaline + oxidative Acid) Protein Then ~~derivatized~~ The AA are derivatized by AQC (except tryptophan) and detected by UV + FLD detection Using UHPLC

Method Scope/Applicability:
Method is applicable for SPI/RMP project
Measures analytes of interest

General comments about the method:
~~Note~~ method uses straight weight. No liquid weighing procedure involved

Method Clarity:
Fairly Easy to follow

Method Safety Concerns:
None

Pros/Strengths:
- Common Equipment
  ~~Short~~ typical prep for Aminoacids

Cons/Weaknesses

- Uses specific products from Waters

Supporting Data

- General Comment:

  NO DATA outside of 18-49% Need ~~test~~ SPIKED 2 DATA

  - Method Optimization:

- Performance Characteristics:

  Analytical Range:

  LOQ:

  ?

  Accuracy/Recovery:

  93 - 106.6

  meets most of SMPR

  Precision (RSD_r):

  .6 - 3.2% mostly meets SMPR

  Reproducibility (RSD_R):

  X

- System suitability:

Recommendation:

Need more data to make decision

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

BIOTIN

Bio-02

AsureQuality

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Bio-02 #1

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

Author:
George Joseph, Ranjani Devi and Raj Naganaboyina
AsureQuality Ltd, PO Box 41, Shortland Street, Auckland 1140, New Zealand

Elaine C. Marley and David Leeman
R-Biopharm Rhône Ltd, West of Scotland Science Park, Glasgow, Scotland G20 0XA

Summary of Method:
A portion of sample is dispersed in phosphate buffered saline(pH 7.4) and autoclaved at 121±2^{o}C for 25 minutes, cooled to room temperature and diluted to 100mL. The extract is clarified (centrifuged, filtered) and a portion of the resulting solution loaded to a Biotin immunoaffinity column mounted in an SPE manifold. The SPE column is washed w PBS then biotin is eluted with methanol, which is evaporated, and the residue re-constituted in water.

A portion of the resulting sample preparation is then injected onto a reversed-phase HPLC system using an isocratic separation (0.1% phosphoric acid / acetonitrile) with a column wash performed after elution of the peak of interest. Detection is by UV at 200nm. Quantification is by external standard against a multi-point standard curve. The method does not specify whether peak height or peak area is used.

Method Scope/Applicability:
The SMPR refers to the analysis of “total biotin”. The fortificant used in formulated nutritional products is free biotin. Biotin in foods may be covalently bound to L-lysine, either in protein-bound forms or as biocytin. The validation report did not specifically address free v total biotin, nor demonstrate recovery of a bound form of biotin.

General comments about the method:

Method Clarity:
Sample Preparation, Table 1, specifies the mass of product to process based on the expected biotin content in “ug/100g”; the values appear to be “as fed” or “as reconstituted” (rather than “as powder e.g.”); the basis of the expression should be stated.

The method does not include:

- Example calculations
    - Height or area?
    - How the standard curve data is treated to create a calibration;
    - Sample calculation.
- System suitability
    - What sequence of injections should be used for calibrants and unknowns;
    - Performance criteria for standard curve (r) and read-backs.

Method Safety Concerns:
None

Pros/Strengths:

- The sample preparation is straightforward and uses conventional, widely practiced techniques
- The quantification uses conventional HPLC w UV detection.
- The validation report documented similar analytical results for the majority of samples from a second brand of immunoaffinity column; most samples produced results within ± 5%. The milk based IF powder result was 10% lower - 3 mcg/100g powder, or c 0.4 mcg/100mL (4 mcg/L) as fed; although the absolute difference is small, further investigation is warranted because of the commonness of this matrix. (Table 5)

Cons/Weaknesses

- Text needs to be added to describe system suitability and calculations.
- The values in the SLV are not consistently reported in the SMPR units (mcg/100g reconstituted)

Supporting Data

- General Comment:

The validation report stated that while a reagent blank and a matrix blank prepared from infant elemental powder were devoid of interferences, the placebo (non-fortified) products child formula powder, adult nutritional RTF high fat, and infant formula RTF milk based showed a response near the retention time of biotin. The report does not show these chromatograms nor clarify if the “response” was attributed to (endogenous) biotin or was an interference.

- Method Optimization:

- Performance Characteristics:

Analytical Range:

- The method describes the processing of samples with biotin content over the entire concentration range stated in the SMPR (0.1 – 150 mcg/100g as fed)
- In practice, the concentration encountered in the SLV using the SPIFAN sample set was ~1.5 – 65 reconstituted.

LOQ:

- The SMPR LoQ (0.1 mcg/100g reconstituted) is achieved at the lowest standard curve concentration using the typical sample weight specified in the method for this concentration of analyte.

Accuracy/Recovery:
SMPR:
0.1 – 1 mcg/100g reconstituted: 80 - 120%
> 1 mcg/100g reconstituted: 90 – 110%

- Spike and recovery: Four placebo samples (Child Formula, Infant Elemental, Adult RTF High Fat, IF Milk RTF) were spiked at +50% and +150% of typical target, 3 days in duplicate, with recveries meeting the SMPR requirements ranging from 95.2% – 100%. One fortified sample (Adult RTF High Protein) was spiked similarly and showed recovery of 96.7% - 104%, also meeting the SMPR requirement. (Table 3)

- SRM 1849a: the sample was analyzed on 6 different days; the results (range 196.5 – 200.0 mcg/100g as is) were all well within the Certified Mass Fraction range (199 ± 13 mcg/100g as is). (Table 2)

Precision (RSD_r):
SMPR:
0.2 – 1 mcg/100g reconstituted: ≤ 8%
> 1 mcg/100g reconstituted: ≤ 6%

Reproducibility (RSD_R):
SMPR:
0.1 – 1 mcg/100g reconstituted: ≤ 16%
> 1 mcg/100g reconstituted: ≤ 12%

RSD_{IR} was characterized by duplicate analysis of the 12 fortified SPIFAN samples across 6 days (2 analysts, 2 instruments). All values were below 3.2%. Although they were not calculated individually, none of the within day precision values appeared to be greater than this value. The method could likely meet the SMPR requirements if extended to MLT. (Table 4)

- System suitability:
  - Not stated.

Recommendation:

Add system suitability and calculations. Although the entire SPIFAN set was not spiked, acceptable for First Action status.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method BIO-02 #2

Title: Determination of Biotin by HPLC coupled with EASI-EXTRACT Biotin Immunoaffinity column cleanup extraction

Author: George Joseph, Ranjani Devi, Raj Naganaboyina, AsureQuality

Summary of Method:

1. Biotin extraction with sodium phosphate buffer, pH 7, and autoclaving at 121°C for 25 minutes.
2. Sample cleanup and concentration with immunoaffinity cleanup columns
3. Gradient HPLC analysis with Kinetex phenyl hexyl solid-core column and UV detection at 200 nm.

Method Scope/Applicability:

Method applicable for use with infant, adult, and pediatric formulas.

General comments about the method:

Because of the use of immunoaffinity columns the method is specific for biotin.

Method Clarity:

Overall method document was clear and easy to follow, but may need a little more detail and some clarification. It would be helpful to explain how to back flush the column three times when eluting the sample and system suitability parameters would be helpful.

Pros/Strengths:

-
  1. Method should specific for biotin because of the use of a monoclonal antibody.
  2. At least two immunoaffinity column suppliers were identified – R-Biopharm Biotin Easy Extract and BioTeZ Immunoaffinity columns Berlin-Buch Germany- technology not proprietary.

Cons/Weaknesses

- Although samples are autoclaved during the sample preparation procedure, they are autoclaved in a pH 7 phosphate saline buffer which would not be expected to release bound biotin. For total biotin analyses samples are typically autoclaved in sulfuric acid or hydrolyzed enzymatically.

Supporting Data

- General Comment:

1. Precision data were generated with all SPIFAN I matrices. Accuracy (spike recovery) data were generated with 1 fortified and 4 placebo SPIFAN I matrices.
2. 10 of the SPIFAN matrices were analyzed with immunoaffinity columns from two different manufacturers, EASI-EXTRACT (Biopharm) and BioTeZ IAC (Biotez)
3. Representative chromatograms provided.

- Method Optimization:

- Performance Characteristics:

Analytical Range: 0.1-300 µg/100g. SMPR = 0.1-150 µg/100g

LOQ: Estimated to be 0.1 µg/100g. SMPR $\leq$0.1 µg/100g.

Accuracy/Recovery:

Results for SRM 1849a are within the certified range.
Spike recoveries range from 95-105%. SMPR 90-110%

Precision (RSD_r): Repeatability was not calculated. Overall RSD of all results ranged from 0.55-3.19%. SMPR repeatability $\leq$6%.

Reproducibility (RSD_R): NAP

- System suitability: No system suitability requirements listed, but authors noted that during the SLV, r^2 was not less than 0.998.

Recommendation:

All of the data from analyses of SPIFAN matrices met the SMPR requirements. I would recommend this method for first action status.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Bio-02 #3

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

Author:
George Joseph, AsureQuality

Summary of Method:
The sample is dissolved, then autoclaved. After cooling, the diluted sample is centrifuged and filtered. The filtrate is collected for clean-up and extraction using an immunoaffinity column is mounted onto a SPE manifold. Biotin from the column is eluted with methanol and collected in a reacti-vial. The eluent is evaporated to dryness and the sample is re-constituted in 1mL of water. The biotin in the reconstituted sample is quantified by HPLC using a UV detector set at 200nm

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:

Method Clarity:
Clear directions for operation of the method, instruments. Clear equations describing how the standards and samples are quantitated is required.

Method Safety Concerns:
None, beyond standard laboratory safety

Pros/Strengths:
Simple straight forward analysis.

Cons/Weaknesses
Proprietary components (immunoaffinity cartridge)

Supporting Data
SPIFAN Kit 1 used.
Data not calculated on RTF basis

Performance Characteristics:

Analytical Range:
0.1 to 300 µg/100g meets SMPR limits (0.1-150 µg/100g)

LOQ:
≤0.1 µg/100g meets SMPR limits (<0.1 µg/100g)

Accuracy/Recovery:
95 to 105 % meets SMPR limits (90-110%).
Acceptable bias against NIST1849a SMPR

Precision (RSD_r):
≤4% meets SMPR limits (<6%)

Reproducibility (RSD_R):

System suitability:
No details

Recommendation:
TBD

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Bio-02 #4

Title: Determination of Biotin by LC Coupled with Immunoaffinity Column Cleanup Extracts

Author: G. Joseph

Reviewer Name:

Summary of Method:
Sample dispersed in Phosphate Buffer, Autoclaved, diluted to final vol.
Extract centrifuged + filtered before placed on immunoaffinity colu
Extract from colu dried + reconstituted befor LC determ @ 200nm

Method Scope/Applicability:

Applicable to Spiffon

General comments about the method:

Measurement @ 200nm is concern
Not sure if samples were recovered

Method Clarity:

Very clear + easy to follow

Method Safety Concerns:

None

Pros/Strengths:

- Easy method to follow
  Meets/exceeds SMPL requirements

Cons/Weaknesses

- 200nm
  unsure about recov
  immunoaffinity col

Supporting Data

- General Comment:

  - Method Optimization: USED 2 IMAff columns

- Performance Characteristics:

  Analytical Range: 0.1 – 300 mcg/100g Exceeds

  LOQ: ≤ 0.1 mcg/100g meets

  Accuracy/Recovery: 95-105% meets SLM 198 VS 199

  Precision (RSD_r): ≤ 4% worst case

  Reproducibility (RSD_R):

- System suitability:

Recommendation: 1ST ACTION

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

BIOTIN

Bio-03

Abbott Nutrition

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method BIO-03 #1

Title: Determination of Biotin in Infant, Pediatric and Adult Nutritionals by HPLC and Fluorescence Detection

Author: Abbott Nutrition, Columbus, OH. USA

Summary of Method:
This reverse phase HPLC method with post column protein conjugation and fluorescence detection allows for the quantitative determination of biotin in infant, pediatric, and adult nutritionals. Sample of appropriate size is mixed with 6% meta-phosphoric acid to precipitate out the protein to produce a filtrate. The filtrate is injected onto a C18 HPLC column where biotin and riboflavin are separated with a mobile phase (20% methanol in 0.02 M phosphate buffer at pH7.0). The biotin after eluting from the column binds with the streptavidin fluorescein to become a fluorescent conjugate. The conjugate is then detected by fluorescence at an excitation wavelength of 495 nm and an emission wavelength of 518 nm.

Method Scope/Applicability:
Applicability of this method includes determination of Biotin in Infant, Pediatric and Adult Nutritionals.

General comments about the method:
The method is suitable to determine biotin in a short time with the used of LC-FLD methodology. The basis of this method is the strong affinity between biotin and streptavidin.

Method Clarity:
The description of the method is clear and easy to follow.

Pros/Strengths:
A column switch is used in the method to shorten the run time from 30 min to 15 min, by eluting out riboflavin at a higher flow rate.

Cons/Weaknesses

- The method requests appropriate sample size for analysis. If the concentration of the prepared sample is higher than 60 ng/ml, a further dilution with mobile phase is required.
- It is necessary to equilibrate the system before standard and sample injections with several standards injections. The fluorescence intensity of the post column reaction end product did not show a linear relationship with biotin concentration

Supporting Data

- **General Comment:**

- **Method Optimization**:

Several parameters were varied during validation to establish method ruggedness.
Samples were prepared by three analysts and analyzed with C18 columns with 4 different lots.
New mobile phase, post column reagents, intermediate standards and working standards were made daily and used during validation.

- **Performance Characteristics:**

Analytical Range: No data
SMPR requirements : 0.1–150 mcg/100g

LOQ: 0.8 and 1.5 mcg/100g
SMPR Requirements : ≤0.1 mcg/100 g reconstituted final product
The limit of quantitation 0.1 mcg/100 g was not met.

Biotin detection and quantitation limits were determined experimentally by spiking a very low level biotin into placebos. Blank mean and standard deviation were obtained from 8 injections.

The LOQ was estimated to be 0.8 mcg/100g reconstituted final product for powder assuming 4 gram sample was diluted to 50 ml and **1.5 mcg/100g for RTF** assuming 20 gram sample was diluted to 50 ml.

Accuracy/Recovery: 95 – 109 %
SMPR Requirements: 0.1 – 1 mcg/100g : 80 – 120 %
> 1.0 mcg/100g : 90 – 110 %

Sample	**Replicates**	**Native level (mcg/100g reconstituted)**	**Level 1 (50%) Avg (%)**	**Level 2 (150% for placebo, 100% for fortified) Avg (%)**
Child formula powder, placebo	6	Not detected	**103**	**105**
Adult nutritional RTF high protein, placebo	6	Not detected	**102**	**103**
Adult nutritional RTF high fat, placebo	6	Not detected	**102**	**104**
Infant formula powder partially hydrolyzed soy based	6	4.43	**105**	**101**
Infant formula powder milk based	6	5.11	**104**	**103**
Adult nutritional powder low fat	6	31.9	**111**	**102**
Child formula powder	6	21.6	**109**	**104**
Infant elemental powder	6	10.7	**106**	**95.1**
Infant formula powder FOS/GOS based	6	1.66	**105**	**99.9**
Infant formula powder milk based	6	5.11	**104**	**103**
Adult nutritional RTF high protein	6	56.8	**109**	**102**
Adult nutritional RTF high fat	6	76.2	**109**	**101**

Precision (RSD_r): 1.5 – 3.0 %
SMPR requirements: 0.1 – 1 mcg/100g ≤ 8 %
>1.0 mcg/100g ≤ 5 %

All fortified and unfortified SPIFAN matrices were freshly prepared and analyzed in duplicate on six days.
All SPIFAN matrices meet repeatability requirements.

Sample	Replicates (duplicates on 6 days)	Concentration level (mcg/100g reconstituted)	RSD_r
SRM 1849_a	12	22.4	**1.5**
Infant formula powder partially hydrolyzed milk based	12	4.07	**0.5**
Infant formula powder partially hydrolyzed soy based	12	4.43	**0.5**
Toddler formula powder milk based	12	10.7	**1.9**
Infant formula powder milk based	12	2.90	**2.5**
Adult nutritional powder low fat	12	31.9	**0.7**
Child formula powder	12	21.6	**1.2**
Infant elemental powder	12	10.7	**1.9**
Infant formula powder FOS/GOS based	12	1.66	**2.6**
Infant formula powder soy based	12	5.15	**3.0**
Infant formula RTF milk based	12	3.86	**1.7**
Adult nutritional RTF high protein	12	56.8	**1.0**
Adult nutritional RTF high fat	12	76.2	**0.6**

Reproducibility (RSD_R): ---
SMPR requirements: 0.1 – 1 mcg/100g ≤ 16 %
>1.0 mcg/100g ≤ 12 %

System suitability:
Certified value of NIST 1849a is 1.99 ± 0.13 mg/kg biotin.
.
During each analytical run were injected 7 standards with biotin concentrations ranging from 5 to 100 ng/ml before and after each sample set.
The method demonstrated good polynomial regression (cubic) fit, over a standard range of 5-100 ng/ml biotin with r^2 greater than 0.999. The calibration error for the lowest 2 levels (near LOQ level) is around 25% and 10% respectively. The calibration error for the rest levels is less than 8%.

Recommendation:

This method is suitable to proceed to First Action Status.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Bio-03 #2

Title:
Determination of Biotin in Infant, Pediatric and Adult Nutritionals by HPLC and Fluorescence Detection

Author:
Abbott

Summary of Method:
Sampleis mixed with meta-phosphoric acid to precipitate out the protein with filtrate is injected onto a C18 HPLC column where biotin and riboflavin are separated. After elution biotin after eluting from the column binds with the streptavidin fluorescein to become a fluorescent conjugate. The conjugate is then detected by fluorescence at an excitation wavelength of 495 nm and an emission wavelength of 518 nm. A column switch is used in the method to shorten the run time from 30 min to 15 min, by eluting out riboflavin at a higher flow rate.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:
Well establish technique based on the high affinity of streptavidin for biotin

Method Clarity:
Clear and well written method description.

Method Safety Concerns:

Pros/Strengths:

Cons/Weaknesses
Non-linear response of formation of biotin-streptavidin complex.
Column switching not used in all labs.

Supporting Data
Values obtained for NIST 1849a are acceptable against certified values.
SPIFAN kit was used in the validation of the method.

Performance Characteristics:

Analytical Range:
1.66-142 mcg/100g reconstituted final product

Just outside the range specified in SMPR

LOQ:
~0.8 mcg/100g in reconstituted powder product
Just outside the range specified in SMPR

Accuracy/Recovery:
95-111% acceptable recovery
% bias against NIST 1849a, within acceptable limits

Precision (RSD_r):
0.5-3.0% within limits specified in SMPR

Reproducibility (RSD_R):

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

Recommendation:
Recommended First Action

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method BIO-03 #3

Title: Determination of Biotin in Infant, Pediatric + Adult Nutritionals by HPLC-FLD

Author: ABBOTT

Reviewer Name

Summary of Method:

Simple sample extraction w/ centrifuge step. Filtered + injected on LC-FLD 495 518 using column switching

Method Scope/Applicability:

Meets submission requirements

General comments about the method:

Simple LC-FLD method using column switching

Method Clarity:

Method clear + easy to follow

Method Safety Concerns:

NONE

Pros/Strengths:

- Quick sample prep
  Short injection time

Cons/Weaknesses

- Column Switching

Supporting Data

- General Comment:
  - Method Optimization:
- Performance Characteristics:

  Analytical Range: Meets SMPR

  LOQ: Higher Leg 0.8

  Accuracy/Recovery: 95-118% Spike 199 vs 199 for SRM

  Precision (RSD_r): 0.5 → 3.0% meets SMPR

  Reproducibility (RSD_R):

- System suitability:

Recommendation:

1st Review

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

B VITAMINS (B_3)

BVit-02

Abbott Nutrition

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: BVit-02 (B3 only) #1

Title: Simultaneous Determination of Total Vitamin B6, B2, B3 and B1 in Infant Formula Products by LC-MS/MS Using Enzymatic Digestion

Author: Abbott Nutrition

Summary of Method:
Powdered samples are reconstituted in room temperature water. An accurately measured portion of an internal standard mixture is added containing an isotope-labeled form of each vitamer of interest. An enzyme mixture is added containing papain, α-amylase, and acid phosphatase. The sample solution is mixed for 18 – 24h at 37C, and then made to final volume. A portion of the extract is filtered then injected into an LC-MS/MS system (ESI+) for quantification. Internal Standard calibration is performed for each component using multi-point standard curves.

Method Scope/Applicability:
The measurement and expression of the 4 B-Vitamins agrees w the respective SMPR meaning that:

- B1: total thiamine, including any phosphorylated forms;
- B2: total riboflavin, including any phosphorylated forms;
- B3: niacinamide and nicotinic acid
- B6: pyridoxine, pyridoxal, pyridoxamine including any phosphorylated forms

General comments about the method:
This method was originally validated by its authors for the determination of Vitamins B1, B2, B6. The data reviewed by ERP in 9/2015 contained precision and accuracy data for the total forms of these vitamins, on non-SPIFAN samples, generated in 2009, and precision data for the total forms of these vitamins, from the SPIFAN I sample set, in 2012.

B3 was later added to the scope of the method. The B3 data presented to ERP in 9/2015 consisted of a single day's precision data for 4 of the SPIFAN II matrices.

The method was adopted as First Action for Vitamins B1, B2, B6 on 9/29/2015.

The method was not adopted as a First Action for Vitamin B_3 due to concerns over potentially low recovery, caused by the fairly lengthy enzymatic sample digestion at 37C. It was noted that the niacinamide values reported for the SRM 1849a, while in the Certified Mass Fraction range (108 ± 10 mg/kg) were near the lower bound. No spike and recovery data was presented to ERP.

It was reported by other observers that for samples which had been analyzed by both BVit-02 (AN) and BVit-01 (MJ), results from the former showed a consistent low bias relative to the latter.

Sample	BVit-02 (AN)	BVit-01 (MJ)

SRM-1849a	99804 ppb	106.6 mg/kg

Method Clarity:

- Section D.2.e. (p33 of PDF) does not describe the preparation of the nicotinic acid internal standard
- Section D.2.f. (p33 of PDF) does not describe the addition of either niacinamide or nicotinic acid to the internal standard solution ISSM
- Section D.2.g. (p34 of PDF) does not describe the preparation of either niacinamide or nicotinic acid native standards
- Section D.2.h. (p35 of PDF) does not describe the addition of either niacinamide or nicotinic acid native standards to the intermediate working standard solution IWSS

Method Safety Concerns:

Pros/Strengths:

- Measures all forms of all vitamins required by the SMPR.

Cons/Weaknesses

-

Supporting Data

- General Comment:

New comparison data was provided for this ERP for samples in the SPIFAN II set, between the fortification level (where known), and the values obtained by microbiological assay, BVit-01 (MJ), and BVit-02 (AN). On average, the BVit-01 (MJ) values were 107% of the BVit-02 (AN) values, and were higher in 12 of 14 cases. For 11 samples in which the fortification was known, the values produced by BVit-01 (MJ) were 111% and by BVit-02 (AN) 104% of these fortification values.

  - Method Optimization:

- Performance Characteristics:
  B_3 SMPR: Total niacin as niacinamide and nicotinic acid

  - Analytical Range:
    B_3 SMPR: 200 – 10000 mcg/100g as fed

    The samples tested ranged in concentration from ~1300 - ~7200 ppb as is for nicotinic acid and 29000 – 110000ppb for niacinamide (as is, not as reconstituted)

    LOQ:
    B_3 SMPR: 200 mcg/100g as fed

    LoQ was calculated using the lowest standard concentration and typical sample mass and dilution, which met the SMPR requirement.

    Accuracy/Recovery:

B_3 SMPR: 90 – 110%

Validation report from 9/2015: Section 2 Table 4:
SRM 1849a: 99804 ppb niacinamide
CofA: 108 ± 10 mg/kg
4127 ppb nicotinic acid
Precision (RSD_r):
B_3 SMPR: RSDr: < 5%

Validation report from 9/2015: Section 2 Table 4, for SPIFAN 2 set: 4 of 4 samples met requirement for RSDr for niacinamide (RSDr: range 1.5 – 2.2%) and nicotinic acid (RSDr: range 0.8 – 1.8%) individually.

Reproducibility (RSD_R):

- System suitability:
  System suitability criteria are specified for calibration curve correlation coefficient, the number of standards which may be excluded from a given analysis, and the residuals observed for the lowest standards.

Recommendation:
The supplemental comparison data appears to show that methods BVit-01 (MJ) and BVit-02 (AN) produce values which are in reasonable agreement, with the values from the latter perhaps slightly closer to fortification levels. However, the lack of spike and recovery data leaves open the question of whether the method completely recovers niacinamide and nicotinic acid. Without this data the method cannot be recommended for First Action.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: BVit-02 #2

Title:
Simultaneous Determination of Total Vitamin B6, B2, B3 and B1 in Infant Formula Products by LC-MS/MS Using Enzymatic Digestion

Author:
Abbott

Summary of Method:
First Action method for B1, B2, B6 and discussion of submission relates to possible bias for B3.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:

Method Clarity:

Method Safety Concerns:

Pros/Strengths:

Cons/Weaknesses

Supporting Data
Data provided illustrates that there is no bias between Abbott method and that from Mead Johnson. At previous meeting it was found that initial there was a high bias for Abbott method that this may be caused by B3 coming from the enzyme.
No report attached which does not address the concern that B3 is not present in enzyme
The data just shows that this method gives same result as MJ method.
However, since this was only a hypothesis, the fact that there is no bias suggests that no problem for B3 exists.

Performance Characteristics:

Analytical Range:

LOQ:

Accuracy/Recovery:

Precision (RSD_r):

Reproducibility (RSD_R):

System suitability:

Recommendation:
Recommended First Action for B3

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method BVit-02 (Added information for Vit B3) #3

Title: Simultaneous Determination of Total Vitamin $B_{6,}$ $B_{2,}$ B_3 and B_1 in Infant Formula Products by LC-MS/MS Using Enzymatic Digestion

Author: Abbott Nutrition

Summary of Method:

This is a method utilizing an enzymatic digestion and LC/MS analysis for the determination of the total content of all the vitamins of this group

Powder samples were first reconstituted in water at 10% (w/w). Two grams of the reconstituted sample was weighed into 50 mL disposable centrifuge tubes, internal standard was added, and 5mL of a three enzyme cocktail was added. The enzyme cocktail was prepared in 50 mM ammonium formate solution adjusted to a pH between 4.0 and 4.5. The samples were incubated in a 37° water bath and shaken for a minimum of 12 hours. Upon removal from the bath, the samples were filled to 30 mL with 50 mM ammonium formate solution. The samples were then mixed thoroughly and filtered through 0.20 µm PTFE syringe filters into autosampler vials.

Method Scope/Applicability:

This method is applicable to determination of vitamin B1, B2, B3 and B6 in infant formulas.

General comments about the method:
The method is reported to perform well and meets all the SMPR requirements. The method demonstrates high specificity.

Method Clarity:
The method is clear, descriptive and easy to follow in its current format.

Supporting Data

- General Comment:

Method Optimization:
No data about the use of blanks and no data about calibration curve of standards included in the report.

- Performance Characteristics:

Analytical Range: data not included

SMPR 200 – 10000 μ/100g

LOQ: data not included

SMPR ≤ 200 μ/100g

Accuracy/Recovery: 96 – 110 %
SMPR 90-110% for Vit B3
The recovery was calculated on all SPIFAN samples and on NIST reference material SRM 1849a. The results obtained (96 – 110 %) show that the method meets de SMPR recovery for niacin.

Precision (RSD_r): ≤ 5 %
SMPR : ≤ 5 % for Vit B3
The precision was calculated on 3 SPIFAN samples, in triplicate and the method meets de SMPR.

Product	Niacinamide	Nicotinic Acid
1849a-1	101452	4122
1849a-2	97718	4202
1849a-3	100242	4058
Ave.	99804	4127
RSD	**1.9**	**1.8**
Child Nut. -1	83461	3909
Child Nut. -2	85461	3848
Child Nut. -3	85795	3862
Ave.	84906	3873
RSD	**1.5**	**0.8**

Adult RTF HF -1	29748	1337
Adult RTF HF -2	28519	1371
Adult RTF HF -3	29429	1338
Ave.	29232	1349
RSD	**2.2**	**1.4**
Elemental-1	111317	7135
Elemental-2	113188	7213
Elemental-3	109756	7056
Ave.	111420	7135
RSD	**1.5**	**1.1**

Reproducibility (RSD_R): ---

- System suitability:

No data available.

Recommendation:

My recommendation is to move the method to First Action.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method BVIT -02 #4

Title: Simultaneous determination of Vit B_6 B_2, B_3 + B_1 in infant-formulas by LC-MS using enzymatic digest

Author: ABBOTT

Reviewer Name:

Summary of Method:
Sample weighed, enzymes (3) added, sample PH'd incubated for NLT 12 hrs, filtered + injected on LC-MS

Method Scope/Applicability:

Meets SMPR

General comments about the method:

Method Clarity:

Easy to follow

Method Safety Concerns:

Pros/Strengths:

- Can do all Vitamins under 24 hrs

Cons/Weaknesses

- B_3 low RS formation

Supporting Data

- General Comment:
  - Method Optimization:
- Performance Characteristics:

  Analytical Range:

  LOQ:

  Accuracy/Recovery:

  Precision (RSD_r):

  Reproducibility (RSD_R):

- System suitability:

Recommendation: 1st Acceptance

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

B VITAMINS (B_1, B_2, B_3, B_6)

BVit-03

TUV-SUD

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: BVit-03 #1

Title:
Determination of B-Vitamins in Infant Formula and Adult/Pediatric Formulas

Author:
TUV/SUD

Summary of Method:
Samples are extracted with water in presence of ascorbic acid and adjusted to pH 4.8 with ammonia solution to precipitate proteins. Extract is filtered and subjected to LC-MSMS. Results are quantified against certified reference materials by external standard method.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:
Method describes the analysis of a number of B-group vitamins using LC-MS/MS. Only free supplemental vitamin forms are measured and the method does not include measurement of phosphorylated forms specified in the SMPR applicability.

Method Clarity:
Method clarity and detail sufficient, however equations detailing the calculation of standard concentrations needed.

Method Safety Concerns:
None

Pros/Strengths:
Relatively simple straightforward analysis.

Cons/Weaknesses
Quantitation is by external standardisation only which can be problematic in LC-MS assays.

Supporting Data
Data from the SPIFAN kit has not been reported. Of the analytes under consideration as part of the B-group SMPRs, repeatability is within the SMPR limits for riboflavin and pyridoxine only. Recovery is within SMPR limits for riboflavin and niacinamide.
The only sample reported is the NIST 1849a and unfortunately it is unclear from the report how the results obtained compare to certified value.
Performance Characteristics:

Analytical Range:

LOQ:

Accuracy/Recovery:
Recovery is within SMPR limits for riboflavin(107%) and niacinamide (94.5%). Recovery exceeds limits in SMPR (90-110%) for thiamine (88.9%), pyridoxine(112.7%)

Precision (RSD_r):
Repeatability is within the SMPR limits for riboflavin (2.2%), niacinamide (4%) and pyridoxine (1.9%) only. Repeatability exceeds limits in SMPR (<5%) for thiamine (7.5%).

Reproducibility (RSD_R):

System suitability:
None

Recommendation:
Recommended First Action for B3

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method BVIT 03 #2

Title: Determination of B-Vitamins in Infant Formula + Adult/Pediatric Formulas

Author: THV

Reviewer Name:

Summary of Method:

Samples extracted w/ H_2O in Presence of ascorbic acid
Extract is filtered + analyzed by LC-MS/MS

Method Scope/Applicability:

General comments about the method:

Method Clarity:

Method Safety Concerns:

Pros/Strengths:

- 8min injection
  Simple extraction

Cons/Weaknesses

- Full spiffy kit
  MISS

Supporting Data

- General Comment:

  NEED FULL KIT for DATA

  - Method Optimization:

- Performance Characteristics:

  Analytical Range:

  LOQ:

  Accuracy/Recovery:

  Precision (RSD_r):

  Reproducibility (RSD_R):

- System suitability:

Recommendation:

DO NOT MOVE UNTIL FULL KIT DATA PRESENTED

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

CAROTENOIDS

Carot-01

Ausnutria Hyproca

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Carot-01 #1

Title: Determination of Carotenoids in Infant Formula and Adult/Pediatric Nutritional Formula using High Performance Liquid Chromatography with Photo Diode Array Detection.

Author: Martijn Vermeulen# , Edith de Haan* , Arjan de Vries* , and Fariha Bouraada* . # Ausnutria Hyproca Analytics, Lelystad, The Netherlands, to whom correspondence should be addressed. * TNO Triskelion, Department of Analytical Chemistry, Utrechtseweg 48, Zeist, The Netherlands.

Summary of Method: Carotenoid esters are saponified using potassium hydroxide in ethanol/water in the presence of sodium ascorbate, sodium sulfide and glycerol (to prevent oxidation). Dimethyltocol (DMT) as internal standard. Carotenoids are extracted using diisopropyl ether. Carotenoids are determined by HPLC-UV at 450 nm and DMT is measured at 292 nm. Separation is performed using 2 suplex pkb-100 columns of 25 cm with a gradient.

Method Scope/Applicability: Infant Formula and Adult/Pediatric Nutritional Formula

General comments about the method: Well done work which incorporates the major learnings in the carotenoid arena of the past 40 years (except for the accurate quantitation of the beta carotene stock standard solution-see below)

Method Clarity: Well Done

Method Safety Concerns:
Warning on handling diisopropyl ether should be added, since peroxide formation is a major concern as an explosion hazard. (Not only that, but DIPE that has peroxides is not only dangerous, but will really mess with the carotenoids). Assure it is stabilized and not stored so long the stabilizer protection has run out.

Pros/Strengths:
- Simple workup and isolation
- Routine HPLC for a well equipped laboratory.

Cons/Weaknesses
- **Step E (b) . Concentration of beta carotene stock standard will be inaccurate. Simply measuring the absorbance at 453 nm only tells you there is something absorbing at 453nm. HPLC must also be run on the solution at 453 nm to measure all the peaks at 453nm, then correct the total absorbance for the beta carotene absorbance by multiplying the total absorbance by the peak area of the beta carotene over the total peak area at 453nm. Or by calculating the concentration based strictly on absorbance and correcting by the ratio of the peak areas.**
- **I assume working standard solution mentioned in G(b) is the HPLC standard solution prepared above.**

Supporting Data
- General Comment: Meets all the requirements of the SMPR (see the SMPR).
    - Method Optimization:
- Performance Characteristics: Meets all the requirements of the SMPR (see the paper).

Analytical Range:

LOQ:

Accuracy/Recovery:

Precision (RSD_r):

Reproducibility (RSD_R): NA

- System suitability: Acceptable

Recommendation: Move to First Action Status.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Carot-01 #2

Title:
Determination of Carotenoids in Infant Formula and Adult/Pediatric Nutritional Formula using High Performance Liquid Chromatography with Photo Diode Array Detection

Author:
Martijn Vermeulen, Ausnutria

Summary of Method:
Carotenoid esters are saponified using potassium hydroxide in ethanol/water in the presence of sodium ascorbate, sodium sulfide and glycerol to prevent oxidation. Dimethyltocol (DMT) is added as an internal standard and carotenoids are extracted using diisopropyl ether. The extract is subsequently washed and concentrated. Carotenoids are determined by RPLC-UV at 450 nm and DMT is measured at 292 nm.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:
Method appears to measure carotenoid forms specified in the SMPR, however, this is not explicitly stated. Using this chromatography, it is not possible to get sufficient resolution to unambiguously integrate the cis-isomers of lutein and a-carotene which are explicitly to be determined in the applicability statement.

Method Clarity:
Clear directions for operation of the method, instruments. No details about how to make up a-carotene, lutein, lycopene standards, or mixed calibration standards. Clear equations describing how the standards and samples are quantitated is required.

Method Safety Concerns:
None other than standard laboratory safety

Pros/Strengths:
Very simple straightforward analysis

Cons/Weaknesses

Supporting Data
SPIFAN kit was used in the validation of the method.
Results reported on dry weight basis, making interpretation against SMPR difficult.

Performance Characteristics:

Analytical Range:
1–2800 µg/100g meets SMPR if units are RTF

LOQ:
<1 µg/100g meets SMPR if units are RTF

Accuracy/Recovery:
Spike recovery 97-109% within acceptable limits of SMPR (90-110%)

Precision (RSD_r):
0.9-4.5%, meets SMPR (<5%)

Reproducibility (RSD_R):

System suitability:
No details

Recommendation:
TBD

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method CAROT -01 #3

Title: CAROTENOIDS IN IF + Adult/Ped Nutritional Formula using HPLC - DAD

Author: TNO

Reviewer Name:

Summary of Method:

Sample saponified using KOH in EtOH/H_2O in Presence of Sodium ascorbate
Sodium sulfide + glycerol w/ use of IS. Carotenoids extracted
using diisopropyl ether

Method Scope/Applicability:

General comments about the method:

Promising method — Wants full list

MULTIplicity data

Method Clarity:

method clear + understandable

Method Safety Concerns:

None

Pros/Strengths:

- good separation
  Common instruments

Cons/Weaknesses

- 50 minute injection

Supporting Data

- General Comment:

No Lycopene But Results Spikes WGC close to My Results @ CVD

  - Method Optimization:

- Performance Characteristics:

Analytical Range: Was Kit run more than 1 day?

No spike in Spiked II placebo

LOQ:

Accuracy/Recovery:

Precision (RSD_r):

Reproducibility (RSD_R):

- System suitability:

Recommendation:

Full Kit Needed to Move to First Action

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

CAROTENOIDS

Carot-02

Perrigo

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Carot-02 #1

Title: Determination of Lutein and β-Carotene in Infant Formula and Adult Nutritionals Reversed-Phase High Performance Liquid Chromatography

Author: Greg Hostetler, Perrigo Nutritionals, Georgia, VT USA

Summary of Method: Samples are spiked with an internal standard and saponified with potassium hydroxide, extracted with MTBE, THF, and 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 and α-carotene.

Method Scope/Applicability: Beta Carotene and Lutein in Infant Formula and Adult/Pediatric Nutritional Formula

General comments about the method: Well done work which incorporates the major learnings in the carotenoid arena of the past 40 years.

Method Clarity: Very clearly written, and exceptionally clear calculations sections. Well Done

Method Safety Concerns:
Nothing out of ordinary laboratory safety practices.

Pros/Strengths:

- Simple workup and isolation
- Clear and concise calculation sections.
- Routine HPLC for a well-equipped laboratory.
- Exceptionally well done illustrative chromatograms.

Cons/Weaknesses

- **None of concern.**

Supporting Data

- General Comment: Meets all the requirements of the SMPR (see the SMPR).
    - Method Optimization:

- Performance Characteristics: Meets all the requirements of the SMPR (see the paper) except LOQ just slightly above SMPR.

-

Analytical Range:

LOQ:

Accuracy/Recovery:

Precision (RSD_r):

Reproducibility (RSD_R): NA

- System suitability: Acceptable

Recommendation: Move to First Action Status.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method : Carot 02 #2

Title: Determination of Lutein and β-Carotene in Infant Formula and Adult Nutritionals Reversed-Phase High Performance Liquid Chromatography

Author: Greg Hostetler, Perrigo Nutritionals, Georgia, VT USA

Summary of Method:

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 Methyl tert butyl ether (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 and α-carotene.

Method Scope/Applicability:

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.

General comments about the method:

Method uses sound methodology
It is clearly written and should be easy to follow
Has description of spectrophotometric as well as chromatographic determination of standards purity
Uses internal standard (Apocarotenal)
Saponification step is short and done at room temperature

No data are presented for alpha-carotene and Lycopene – doesn't meet the scope specified in SMPR
Not enough validation data:

- Accuracy by spike recovery of SRM1849a at 2 levels of all-trans lutein and all-trans b-carotene – meet SMPR for recoveries and repeatability (no reference or certified carotenoid values is available from NIST)

- Repeatability determined for only 2 SPIFAN matrices for cis and trans lutein and cis and trans b-carotene –data meet SMPR

Method Clarity:

Method clearly written and should be easy to follow.

Method Safety Concerns:

No safety concerns are noted by the authors

Pros/Strengths:

- Well-established methodology
- Clearly written and easy to follow
- Good chromatography for listed carotenoids
- Repeatability and recovery data, though very limited, show that method could meet SMPR
- Acceptable linearity

Cons/Weaknesses

- Not all carotenoids specified by SMPR are determined by this method
- Only SRM 1849a and 2 SPIFAN (?) matrices were analyzed
- Not enough validation data

Supporting Data

- General Comment:

    - Method Optimization:

- Performance Characteristics:

Analytical Range:

Not clearly specified

LOQ:
Based on spike and recovery data a LOQ of 1.3 µg/100 g was observed for lutein and 1.5 µg/100 g for β-carotene. SMPR is < 1 ug/100 g but since there is a concentration step in sample preparation LOQ could probably go down if more sample is taken for evaporation

Accuracy/Recovery:

Limited data but meet SMPR:
Accuracy by spike recovery of SRM1849a at 2 levels of all-trans lutein and all-trans b-carotene – meet SMPR for recoveries and repeatability (no reference or certified carotenoid values available from NIST)

Precision (RSD_r):

Limited data but meet SMPR

Reproducibility (RSD_R): no data available

- **System suitability:** there is some information on how to determine resolution between cis and trans isomers but need to specify acceptance criteria

Recommendation:
Expand the scope to include a-carotene and lycopene
Complete validation to determine repeatability and accuracy for different samples
All SPIFAN matrices need to be analyzed
Clarify system suitability requirements
Add data about analytical range
See if LOQ could be improved to meet SMPR

Decision on recommending this method as AOAC First Action is better made after data for more SPIFAN matrices are collected.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method CAROT-02 #3

Title: Determination of Lutein + Beta Carotene in IF + Adult Nutrition

Author: Perrigo

Reviewer Name:

Summary of Method:

Sample is saponified then extracted w/ MTBE + THF Along w/ Internal. Extracted down and Reconstituted in IPA Analyzed on HPLC

Method Scope/Applicability:

General comments about the method:

Does Zea Beta not separate

Method Clarity:

Easy to follow

Method Safety Concerns:

None

Pros/Strengths:

- Robust method
  Common Extraction

Cons/Weaknesses

- Lycopene not included

Supporting Data

- General Comment:
  - Method Optimization:
- Performance Characteristics:

Analytical Range:

LOQ:

Accuracy/Recovery:

Precision (RSD_r):

Reproducibility (RSD_R):

Full Kit not run

- System suitability:

Recommendation:

NEED FULL KIT DATA.
what was Then is acceptable

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

CHLORIDE

Chlor-02/Chlor-04 – Combination Method

Nestlé/CAIQ

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Chlor-02 and Chlor-04 #1

Title: Chloride in Milk, Milk Powder, Whey Powder, Infant Formula and Adult Nutritionals Potentiometric titration method.

Author: Nestle and CAIQ

Reviewer Name:

Summary of Method: Potentiometric titration using silver nitrate and a silver electrode

Method Scope/Applicability: Milk, milk powder, infant formula (RTD and powder) and adult nutritionals

General comments about the method: Method is a combination of Chlor-02 and Chlor-04. Two very good and similar methods were combined into one very good method. Both original methods had successful SLV data submitted

Method Clarity: Method instructions are very clear with step by step instructions.

Method Safety Concerns: Only 2 sentences on safety. There should be a few more safety warnings about some of the reagents like glacial acetic acid and nitric acid.

Pros/Strengths:

- Simple method

- Fast
- Does not require expensive instrument

Cons/Weaknesses

-

Supporting Data

- General Comment: SLV done with SPIFAN matrix sample set

  - Method Optimization:

- Performance Characteristics:

  Analytical Range: Meets SMPR 2014.015

  LOQ: Meets SMPR 2014.015

  Accuracy/Recovery: Meets SMPR 2014.015

  Precision (RSD_r): Meets SMPR 2014.015

  Reproducibility (RSD_R): Meets SMPR 2014.015

- System suitability:

Recommendation: Move method forward

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Chlor-02/Chlor-04 combined method #2

Title: Chloride in Milk, Milk Powder, Whey Powder, Infant Formula and Adult Nutritionals
Potentiometric titration method
Combined AOAC 1st action methods 2015.07 and 2015.08

Author: Nestlé / CAIQ

Summary of Method:
The application describes a procedure for the determination of chloride by potentiometric titration using silver nitrate. The present method represents a harmonized method of Nestlé (Chlor-02) and CAIQ (Chlor-04).

Nestlé (Chlor-02):
Sample preparation: 25 g of powder sample are dissolved in 200 g warm demin. water of 40 °C. 50 ml 2% nitric acid are added and put on a magnetic stirrer until dissolved. Under continuous stirring the solution is titrated with 0.1 mol/l $AgNO_3$.

CAIQ (Chlor-04):
Sample preparation: 5 g powdered sample or 20 g liquid in a 50 ml centrifuge tube. 25 ml demin. water of 40 °C is added. For removal of protein respectively 2.5 ml Carrez reagents are added, filled up to 50 ml with demin. water and centrifuged at 8.000 rmp for 5 min. at 4 °C. 10 ml of the supernatant or an appropriate aliquot into a 120 ml autosampler cup. 5 ml nitric acid solution and 50 ml water are added before titration. For the titration with 0.1 M $AgNO_3$ a combined silver electrode is applied. The titration is performed until the end potential.

Combined method:
25 g powder is dissolved in 200 g warm (40 °C) water, while ready-to-feed products are used as it is. For high protein samples an aliquot is transferred into a 50 ml centrifuge tube, respectively 2.5 ml Carrez I and II are added and filled up with demin. water up to 50 ml. Centrifuged at 12.000 g for 5 min. at 4 °C.
10 ml of the supernatant or an appropriate aliquot of RTF or reconstituted powder (if no protein precipitation was necessary resp. performed) is transferred into a 150 ml sample beaker, 5 ml nitric acid solution and 50 ml water is added before the automatic titration with 0.1 mol/l $AgNO_3$ is started until the end point.

Conclusion:
For higher protein levels in the test sample the precipitation of protein by adding Carrez I and II, followed by a centrifugation step is included (taken from the sample preparation of Chlor 04). The added nitric acid solution is stronger (16 %) than those of Chlor 2, where a 2% nitric acid is applied to get a low pH value. The automatic titration with 0.1 mol/l $AgNO_3$ and a combined silver electrode is in both methods equal.

Method Scope/Applicability:
The method is applicable for the determination of chloride in milk, milk powder, whey powder, infant formula and adult nutritionals.

General comments about the method:
The sample preparation is easy to perform and does not require special glassware or equipment.

Method Clarity:
The combined method is very clearly described and easy to follow.

Pros/Strengths:
- Simple sample preparation
- Method also applicable for other food matrices than IMF

Cons/Weaknesses
- The method is not specific for chloride as the analyt is not determined directly. Interferences from bromide and iodine may occur, but is unlikely due to low levels of those anions in IMF and Adult Nutritionals.

Supporting Data
- General Comment:
  - Method Optimization: no remark
- **Performance Characteristics:**

Analytical Range:	0.35 – 1060 mg/100g reconstituted product SMPR: 5 – 500 mg/100g reconstituted product
Linearity:	- SMPR: not required
LOQ:	Chlor 2: 1,4 mg/100g Chlor 4: 2,2 mg/100 ml SMPR: ≤ 5 mg/100g reconstituted product
Accuracy/Recovery:	Chlor 2: 100 % Chlor 4: NIST 1849a: 98 % SMPR: 95 – 105 %
Precision (RSD_r):	Chlor 2: 0,03 – 1,6 % Chlor 4: 0,1 – 1,8 % % SMPR: ≤ 2 %

Reproducibility (RSD_R) = Chlor 2: 0,54 % (0,1 – 2,8%)
Chlor 4: 0,7 – 2,1 %
SMPR: ≤ 4 %

- System suitability: checked by analyzing daily standards at lowest and midpoint of the analytical range

Recommendation:
It is recommended to perform a MLT by applying the proposed combined method for the determination of chloride.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Clor-02/Chlor-4 #3

Combined AOAC 1st action methods 2015.07 and 2015.08 - Chloride in Milk, Milk Powder, Whey Powder, Infant Formula and Adult Nutritionals Potentiometric titration method - For AOAC / ISO / IDF MLT study. No new data presented.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Chlor 02 | 04 #4

Title: Combined 2015.07 + 2015.08

Author: NESTLE | China CIAQ

Summary of Method:

Reconstitute sample, precipitate proteins, acidify and titrate chloride ions against standardized silver nitrate

Method Scope/Applicability:

Applicable

General comments about the method:

Easy method & perform
Commonly used method

Method Clarity:

method easy to follow

Method Safety Concerns:

No major concerns

Pros/Strengths:

- Easy method
  Common Chemicals
  MOST LABS DO THIS NOW

Cons/Weaknesses

- Can method be tempremental

Supporting Data

- General Comment:

  NO DATA IN FILE But
  Data Previously Shown

  - Method Optimization:

- Performance Characteristics:

  Analytical Range:

  LOQ:

  Accuracy/Recovery:

  Precision (RSD_r):

  Reproducibility (RSD_R):

- System suitability:

Recommendation:

~~DATA?~~ → E

Make 2015.07/2015.08 one Method
This is for Action Items Move to MLT

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

CHLORIDE

Chlor-03/Fluor-02

ThermoFisher

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Chlor-03 / Fluor-02 #1

Title: Single Lab Validation of Fl + Cl analysis in IF + Adult nutritionals

Author: Thermo

Reviewer Name:

Summary of Method:

Sample prepared using various column filtration
and analyzed by IC

Method Scope/Applicability:

Meets Applicability
Required

General comments about the method:

Method is simple dilute based on paper assessment
Warned about proprietary filters

Method Clarity:

Easy to follow

Method Safety Concerns:

None

Pros/Strengths:

- Both Fl + Cl @ once
  About 30 minutes/each

Cons/Weaknesses

- Fl seems to be under Recovered All Spikes < 100% Recov

Supporting Data

- General Comment:

Report per template
Easy to follow

  - Method Optimization:

- Performance Characteristics:

Analytical Range: 4-800 mg/kg Fl
4-2400 mg/kg Cl

LOQ:

		Fl	Cl
Accuracy/Recovery:	50%	93.9	100
	100%	91.9	101

Precision (RSD_r):

1.4 — 7.4 Fl
1.2 - 3.2 Cl

Reproducibility (RSD_R):

- System suitability:

Recommendation:

If filters cannot be purchased from
another manufacturer then
consideration should be made to MLR

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Chlor-03 / Fluor-02 #2

Title: Determination of chloride and fluoride in infant formula and adult nutritionals:

Author: Thermo Scientific

Reviewer Name:

Summary of Method:
The application describes a procedure for the determination of chloride and fluoride by ion chromatography with suppressed conductivity measurement. Sample preparation: 2,5 g sample is weighted in a 20 ml polypropylene tube and filled up to 20 ml with demin. Water. X g of demin. water is added and the weight is recorded. After shaken well for shaken for 2 – 3 minutes 12 ml are transferred into an Amicon Ultra-15 centrifugal filter device and centrifuged for 60 min at 5000 rpm. 5 ml of the filtrate are passed through an onguard cartridge, 3 ml are discarded and the remaining 2 ml are filtered through 0,2 µm before analysis The determination of chloride and fluoride are performed by ion chromatography with suppressed conductivity measurement.

Method Scope/Applicability:
The method is applicable for the determination of chloride (and fluoride) in IMF and Adult Nutritionals (shown in single lab validation report)

General comments about the method:
The test sample preparation is easy to perform and does not require special glassware or equipment. The used ion chromatograph requires some experience of the analyst. The used chromatography columns IonPac 15 is a protected trademark (functional group: alkanol quaternary ammonium salt).

Method Clarity:
The method is clearly described and easy to follow.
One remark:
Step 2: Add ?g of DI water and record the total weight. According the SMPR the reconstitution of the powder should be 25g in 200g deionized water). In the method it is not clear described how much water should added to which total weight.

Pros/Strengths:
- Simple sample preparation
- Simultaneous determination of chloride and fluoride is prescribed
- Method also applicable for other food matrices

Cons/Weaknesses

- Dionex ion chromatograph with suppressed conductivity measurement needs experienced analysts
- Standard dilution given in the SMPR is not followed (homogeneity of the test sample given by using 2,5 g test material?)

Supporting Data

- General Comment:
  - Method Optimization: no remark

- **Performance Characteristics:**
  NIST 1894a Infant formula was used as reference sample

Chloride:

Analytical Range:	4 - 2400 mg/100g, shown by 3 independent tests with independent prepared standard solutions SMPR: 5 – 500 mg/100g reconstituted final product (corresponds to 20 – 2000 mg/100g powder)
Linearity:	R^2 = 0.9995 SMPR: not required
LOQ:	0.02 mg/100 g SMPR: ≤ 5 mg/100g reconstituted product
Accuracy/Recovery:	94,7 % – 106 % SMPR: 95 % – 105 % Except of two data points the SMPR is met NIST: 12 determinations: recovery: 103 – 110 % (7207 – 7677 mg/kg) NIST 1849a reference fraction mass for chlorine: 7010 mg/kg +/- 170 mg/kg
Precision (RSD_r):	NIST SRM 1849a: 1.90 % 1,22 % – 3,16 % for SPIFAN samples SMPR: ≤ 2 %
Reproducibility (RSD_R):	has to be determined in a multi lab study

- System suitability: checked by analyzing daily standards at lowest and midpoint of the analytical range. System recalibrated, when percent error of the check standard is > 5 %

- SPIFAN samples: 13 fortified SPIFAN samples were analysed and validation data are provided

Recommendation:

The method should be formulated manufacturer neutral.
Method cannot be recommended at first action status as the precision and the accuracy/recovery data do not meet the SMPR requirements as well as the test sample amount (should be 25 g in 200 ml). But the method has potential to get first action status after some adjustments resp. improvements.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Chlor-03/Fluor-02 #3

Title:
Ion Chromatography method for the determination of Fluoride and Chloride in Infant Formula and Adult Nutritionals

Author:
Thermofisher

Summary of Method:
Ultra-centrifugation is used to filter the sample and remove the fats and proteins from the sample. Following the filtration, solution was passed through cation exchange cartridge to remove heavy metals (such as Iron) from the samples. Samples were then separated using an anion-exchange column and detected by suppressed conductivity.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:

Method Clarity:
Clear directions for operation of the method, instruments. Clear equations describing how the standards and samples are quantitated is required.

Method Safety Concerns:
None other than standard laboratory safety

Pros/Strengths:
Very simple straightforward analysis.

Cons/Weaknesses
Unclear as to whether only Thermofisher columns and cartridges are required or if alternatives are available.

Supporting Data
SPIFAN kit has been used for both fluoride and chloride validation.

Performance Characteristics:

Analytical Range:
4-800 µg/100g fluoride, meets SMPR
4-2400 µg/100g chloride, meets SMPR

LOQ:
4.2 µg/100g fluoride, meets SMPR (80-120%)
0.02 mg/100g chloride, meets SMPR (90-110%)

Accuracy/Recovery:
81-104% fluoride, meets SMPR
94-104% chloride, meets SMPR

Precision (RSD_r):
Repeatability fluoride average meets SMPR (5%) although with a few samples exceed 5%
Repeatability chloride average meets SMPR (2%) although with a few samples exceed 2%
Although intermediate precision not SMPR requirements, values for fluoride and chloride are similar or less than values required for repeatability.

Reproducibility (RSD_R):

System suitability:
No details

Recommendation:
Recommended First Action for both Chloride and Fluoride

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

CHOLINE

Chol-08

Covance

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Chol-08 / Carn-07 #1

Title: Determination of Free and Total Choline and Carnitine in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)

Author: Covance; David Ellingson, Study Director

Summary of Method:
Method is applicable to the determination of free and total L-carnitine and choline. L-carnitine portion of the method (2015.10) was approved to move to MLT in 9/2015. The SMPR for choline requires "determination of total choline" so the method as it applies to free choline determination is not considered.

A portion of reconstituted powder or liquid sample is accurately weighed into a microwave digestion vessel. Labeled internal standards for both compounds of interest are added. The sample is acidified, the vessel sealed, and it is processed through the microwave digestion unit at 120C for c 40 minutes. The contents are then transferred to a polypropylene tube, and diluted with water, then a portion of the resulting solution is diluted with ACN, centrifuged and filtered. A reagent blank and the assay calibrants are processed in the same manner. Quantification is by LC/MS/MS (ESI+) w MRM using a multi-point ISTD calibration for each compound.

Method Scope/Applicability:

General comments about the method:

Method Clarity:
Clearly written.

Method Safety Concerns:
None.

Pros/Strengths:
- Recovery of two endogenous forms of choline was demonstrated (acetylcholine and phosphatidylcholine)

Cons/Weaknesses
- In the repeatability study, the validation report indicated that outliers had been removed and replaced, but did not show the data.

Supporting Data
- General Comment:

The method validation report is very strong.

- Method Optimization:

- Performance Characteristics:

  Analytical Range:
  SMPR 2012.013: 2 – 250 mg/100g as RTF / reconstituted powder

  Validation report: The lower analytical range was verified by spiking a placebo sample, achieving a concentration of 1.5 mg/100g, with a mean recovery across 3 days / 6 replicates of 99.0%. (Table 11). Verification of upper range described below (Table 16).

  LOQ:
  SMPR 2012.013: 2.0 mg/100g as RTF / reconstituted powder

  Validation report: LOQ = 0.05 mg/100g, from back calculation of lowest standard and default weights and dilution (p19), thus well below SMPR requirement.

  Accuracy/Recovery:
  SMPR 2012.013: 90 – 110%

  Validation report: A total of 4 SPIFAN II matrices were spiked. Each product had endogenous levels of choline, so 50 and 100% overspikes were utilized to assess recovery. Each of the products was spiked in duplicate across three days by multiple analysts. The marginal recovery (recovery of spiked amount) plus total recovery (recovery of endogenous + spiked amount) are provided in these tables. Outliers were not removed from the recovery analysis and calculations used all recovery results.

  - AN High Fat RTF: spiked with choline to achieve the upper SMPR range for total choline at 250 mg/100g. Mean recovery from 3 days, 5 total replicates: 99.9% of added choline (Table 16).
  - SPIFAN BLANK: spiked w choline at +50% and +100%. Mean of 3 days, 6 replicates each: 96.5%, 93.3% of added choline, respectively (Table 18).
  - Soy Based IF: spiked w choline at +50% and +100%. Mean of 3 days, 6 replicates each: 95.2%, 98.8% of added choline, respectively (Table 20).
  - AN High Protein RTF: spiked w choline at +50% and +100%. Mean of 3 days, 6 replicates each: 94.9%, 97.2 of added choline, respectively (Table 22).

  An additional assessment was performed to determine the efficiency of hydrolyzing bound forms choline. Acetylcholine and phosphatidylcholine were processed through the total analysis at levels at the upper range of the SMPR. Both compounds showed acceptable recoveries: acetylcholine, mean recovery 104.6%; phosphatidylcholine mean recovery 96.7%. (Table 23)

  NIST SRM 1849a was analyzed over four days in triplicate and produced results averaging 105 mg/100g (range 102 – 108 mg/100g). The results obtained meet the Certified Mass Fraction Range of 109 ± 11 mg/100g. (Table 25)

Precision (RSD_r):
SMPR 2012.013: ≤ 10% @ 2 mg/100g as RTF / reconstituted powder
≤ 5% @ 20 - 200 mg/100g as RTF / reconstituted powder

Validation report: All 17 SPIFAN matrices were run over at least 4 days in triplicate. New working standards were prepared each day. Each sample was analyzed by at least 2 analysts, on 2 instruments, and 2 columns. "Results that calculated to be outliers based on a 95% confidence interval using Grubb's outlier test were replaced with re-analysis to obtain 12 data points overall for each product."

15 of the 17 matrices produced results for total choline within the analytical range of the method. The range of RDSr values was 1.2% - 3.4% (Table 14).

Reproducibility (RSD_R):
SMPR 2012.013: ≤ 15% @ 2 mg/100g as RTF / reconstituted powder
≤ 10% @ 20 - 200 mg/100g as RTF / reconstituted powder

- System suitability:
  The method does not describe the exact sequence of calibrants and unknowns to be injected, the requirement for the linearity of the calibration curve, the requirement for the reproducibility of any standard readbacks, or the agreement required between the results from quantitative and confirmatory transitions.

Recommendation:
Approve as First Action.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Chol-08/Carn-07 #2

Title: Determination of Free and Total Choline and Carnitine in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)

Author: David Ellingston, Covance

Summary of Method:
An analytical method for the analysis of free as well as total carnitine and choline in infant formula and adult/pediatric nutritional formula was developed with a simultaneously analysis of both the analytes using strong cation exchange (SCX) chromatography**.** The free portion is analyzed using water extraction, while the total extraction employs acid assisted microwave hydrolysis. The total amount can include contribution from bound sources such as phosphatidylcholine or acetylcarnitine. Both extraction methods employ LCMSMS analysis with electrospray ionization (ESI). Calibration standards are included through each extraction procedure.

Method Scope/Applicability:
The analysis of free as well as total carnitine and choline in infant formula and adult/pediatric nutritional formula

General comments about the method:
It's an easy method to specifically analyze total as well as free choline and carnitine in the samples. The extract ion of analytes present in free form is a simple water extraction. The extraction of the analytes in the bound form is a relatively faster (about 1 hr) using microwave hydrolysis. The analysis is MS/MS based and thus specific. The method employs labeled choline and carnitine as internal stds.

Method Clarity:
Good. The authors may provide a Table providing concentrations of analytes in each level of working standard including internal std.

Method Safety Concerns:

The authors may need to add safety section to the method which is lacking now particularly with the use of concentrated nitric acid in microwave.

Pros/Strengths:

- The method is able to analyze choline as well as carnitine simultaneously.
- The method can analyze choline and carnitione present in free form as well as bound form separately.
- The method uses internal std for both analytes during extraction as well as analysis.
- Specific for analytes
- The method is relatively faster and simple

Cons/Weaknesses

- The method is not able to differentiate between D & L carnitine.

Supporting Data

- General Comment:

Satisfactory & complete

- Method Optimization:

No method optimization data provided

- Performance Characteristics:

Analytical Range:
The analytical range of the method is from the stated 0.05 to 250 mg/100g choline and 0.05 to 20 mg/100g carnitine.

LOQ:
An LOQ of 0.05 mg/100g was obtained for both free and total choline and carnitine. The LOQ was calculated from the lowest working standard concentration through the default weights and dilutions used.

Accuracy/Recovery:
Analysis of bound sources of carnitine and choline analyzed in duplicate over three days gave average recoveries of 104.6% for acetylcholine, 96.7% for phosphatidylcholine, and 104.1% for acetylcarnitine.

Precision (RSD_r):
An overall repeatability for free and total choline of 1.9 and 2.3 %RSDr, while the overall Intermediate precision obtained for free and total choline was 2.4 and 2.7 %RSDINT, respectively. Free and total carnitine had an overall repeatability of 2.9 and 2.7 %RSDr, while the overall intermediate precision obtained for free and total carnitine was 3.3 and 3.1 %RSDINT, respectively

Reproducibility (RSD_R):

- System suitability: No specific data provided about system suitability other than calibration data and ion ratios of transitions.

Recommendation:

1. The method meets all the SMPR requirements for choline analysis listed in the SMPR 2012.13. The ERP may consider the method for the analysis of free and total choline for First action method.
2. The method meets all requirements of SMPR (2012.010) for analysis of free and bound carnitine except its inability to distinguish between L- and D-carnitine. The ERP may have to decide whether the requirement stated in the SMPR of reporting results as L-carnitine can be over looked or is it too important for further consideration of the method. 0.6-1.3% contamination by D-carnitine in L-carnitine pharmaceutical preparation have been reported.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Chol-08/Carn-07 #3

This method is currently AOAC First Action for Carnitine (2015.10)

Title:
Determination of Free and Total Choline and Carnitine in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)

Author:
Covance

Summary of Method:
The total method involves extraction of choline and carnitine using acid assisted microwave hydrolysis with nitric acid, followed by filtration, addition of acetonitrile, and injection. The separation utilizes strong cation exchange (SCX) with electrospray ionization (ESI) in positive mode.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:
The acid-digestion method will under-recover phosphocholine, so it may not meet the SMPR. However, a similar issue ie under-recovering carnitine esters due to absence of alkaline digestion did not prevent the method becoming First Action for carnitine.

Method Clarity:
Clear directions for operation of the method, instruments. Clear equations describing how the standards and samples are quantitated is required.

Method Safety Concerns:
None, beyond standard laboratory safety

Pros/Strengths:
Modern technique with simple straight forward analysis.

Cons/Weaknesses

Supporting Data
SPIFAN Kit used.

Performance Characteristics:
Results reported refers to Total Choline

Analytical Range:
10 ng/mL to 5000 ng/mL

LOQ:
2 mg/100g meets SMPR limits (<2 mg/100g)

Accuracy/Recovery:
97.1-102.1 % meets SMPR limits (90-110%).
Method shows no bias against NIST 1849a

Precision (RSD_r):
2.3% meets SMPR limits (<5%)

Reproducibility (RSD_R):

System suitability:
No details

Recommendation:
Recommended First Action

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

FLUORIDE

Fluor-02/Chlor-03

ThermoFisher

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Chlor-03 / Fluor-02 #1

Title: Single Lab Validation of Fl + Cl analysis in IF + Adult nutritionals

Author: Thermo

Reviewer Name:

Summary of Method:

Sample prepared using various column filtration
and analyzed by IC

Method Scope/Applicability:

Meets Applicability
Required

General comments about the method:

Method is simple dilute based on paper assessment
Warned about proprietary filters

Method Clarity:

Easy to follow

Method Safety Concerns:

None

Pros/Strengths:

- Both Fl + Cl @ once
  About 30 minutes/each

Cons/Weaknesses

- Fl seems to be under Recovered All Spikes < 100% Recov

Supporting Data

- General Comment:

Report per template
Easy to follow

  - Method Optimization:

- Performance Characteristics:

Analytical Range: 4-800 mg/kg Fl
4-2400 mg/kg Cl

LOQ:

		Fl	Cl
Accuracy/Recovery:	50%	93.9	100
	100%	91.9	101

Precision (RSD_r):

1.4 — 7.4 Fl
1.2 - 3.2 Cl

Reproducibility (RSD_R):

- System suitability:

Recommendation:

If filters cannot be purchased from
another manufacturer then
consideration should be made to MLR

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Chlor-03 / Fluor-02 #2

Title: Determination of chloride and fluoride in infant formula and adult nutritionals:

Author: Thermo Scientific

Reviewer Name:

Summary of Method:
The application describes a procedure for the determination of chloride and fluoride by ion chromatography with suppressed conductivity measurement. Sample preparation: 2,5 g sample is weighted in a 20 ml polypropylene tube and filled up to 20 ml with demin. Water. X g of demin. water is added and the weight is recorded. After shaken well for shaken for 2 – 3 minutes 12 ml are transferred into an Amicon Ultra-15 centrifugal filter device and centrifuged for 60 min at 5000 rpm. 5 ml of the filtrate are passed through an onguard cartridge, 3 ml are discarded and the remaining 2 ml are filtered through 0,2 µm before analysis The determination of chloride and fluoride are performed by ion chromatography with suppressed conductivity measurement.

Method Scope/Applicability:
The method is applicable for the determination of chloride (and fluoride) in IMF and Adult Nutritionals (shown in single lab validation report)

General comments about the method:
The test sample preparation is easy to perform and does not require special glassware or equipment. The used ion chromatograph requires some experience of the analyst. The used chromatography columns IonPac 15 is a protected trademark (functional group: alkanol quaternary ammonium salt).

Method Clarity:
The method is clearly described and easy to follow.
One remark:
Step 2: Add ?g of DI water and record the total weight. According the SMPR the reconstitution of the powder should be 25g in 200g deionized water). In the method it is not clear described how much water should added to which total weight.

Pros/Strengths:
- Simple sample preparation
- Simultaneous determination of chloride and fluoride is prescribed
- Method also applicable for other food matrices

Cons/Weaknesses

- Dionex ion chromatograph with suppressed conductivity measurement needs experienced analysts
- Standard dilution given in the SMPR is not followed (homogeneity of the test sample given by using 2,5 g test material?)

Supporting Data

- General Comment:
  - Method Optimization: no remark

- **Performance Characteristics:**
  NIST 1894a Infant formula was used as reference sample

Chloride:

Analytical Range:	4 - 2400 mg/100g, shown by 3 independent tests with independent prepared standard solutions SMPR: 5 – 500 mg/100g reconstituted final product (corresponds to 20 – 2000 mg/100g powder)
Linearity:	R^2 = 0.9995 SMPR: not required
LOQ:	0.02 mg/100 g SMPR: ≤ 5 mg/100g reconstituted product
Accuracy/Recovery:	94,7 % – 106 % SMPR: 95 % – 105 % Except of two data points the SMPR is met NIST: 12 determinations: recovery: 103 – 110 % (7207 – 7677 mg/kg) NIST 1849a reference fraction mass for chlorine: 7010 mg/kg +/- 170 mg/kg
Precision (RSD_r):	NIST SRM 1849a: 1.90 % 1,22 % – 3,16 % for SPIFAN samples SMPR: ≤ 2 %
Reproducibility (RSD_R):	has to be determined in a multi lab study

- System suitability: checked by analyzing daily standards at lowest and midpoint of the analytical range. System recalibrated, when percent error of the check standard is > 5 %

- SPIFAN samples: 13 fortified SPIFAN samples were analysed and validation data are provided

Recommendation:

The method should be formulated manufacturer neutral.
Method cannot be recommended at first action status as the precision and the accuracy/recovery data do not meet the SMPR requirements as well as the test sample amount (should be 25 g in 200 ml). But the method has potential to get first action status after some adjustments resp. improvements.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Chlor-03/Fluor-02 #3

Title:
Ion Chromatography method for the determination of Fluoride and Chloride in Infant Formula and Adult Nutritionals

Author:
Thermofisher

Summary of Method:
Ultra-centrifugation is used to filter the sample and remove the fats and proteins from the sample. Following the filtration, solution was passed through cation exchange cartridge to remove heavy metals (such as Iron) from the samples. Samples were then separated using an anion-exchange column and detected by suppressed conductivity.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:

Method Clarity:
Clear directions for operation of the method, instruments. Clear equations describing how the standards and samples are quantitated is required.

Method Safety Concerns:
None other than standard laboratory safety

Pros/Strengths:
Very simple straightforward analysis.

Cons/Weaknesses
Unclear as to whether only Thermofisher columns and cartridges are required or if alternatives are available.

Supporting Data
SPIFAN kit has been used for both fluoride and chloride validation.

Performance Characteristics:

Analytical Range:
4-800 µg/100g fluoride, meets SMPR
4-2400 µg/100g chloride, meets SMPR

LOQ:
4.2 µg/100g fluoride, meets SMPR (80-120%)
0.02 mg/100g chloride, meets SMPR (90-110%)

Accuracy/Recovery:
81-104% fluoride, meets SMPR
94-104% chloride, meets SMPR

Precision (RSD_r):
Repeatability fluoride average meets SMPR (5%) although with a few samples exceed 5%
Repeatability chloride average meets SMPR (2%) although with a few samples exceed 2%
Although intermediate precision not SMPR requirements, values for fluoride and chloride are similar or less than values required for repeatability.

Reproducibility (RSD_R):

System suitability:
No details

Recommendation:
Recommended First Action for both Chloride and Fluoride

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

FOLATE

Fol-20

University of New South Wales (UNSW)

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Fol-20 #1

Title:
Analysis of Folic acid and 5-Methyltetrahydrofolate in Infant and Adult Nutritional formula using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

Author:
University New South Wales

Summary of Method:
Folate extraction involved treatment in a boiling water bath, solid phase extraction and ultra-filtration. Quantitation of folic acid and 5-methyltetrahysdrofolate is done by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:
This method is unchanged since previous review by ERP. Previous concern expressed at higher results obtained from analysis without tri-enzyme compared to results using tri-enzyme digestion.

Method Clarity:
Clear directions for operation of the method, instruments. Clear equations describing how the standards and samples are quantitated is required.

Method Safety Concerns:

Pros/Strengths:

Cons/Weaknesses

Supporting Data
No new data since last review.

Performance Characteristics:

Analytical Range:

LOQ:

Accuracy/Recovery:

Precision (RSD_r):

Reproducibility (RSD_R):

System suitability:
No details

Recommendation:
TBD

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method FOL-20 #2

Title: Analysis of Folic acid and 5-Methyltetrahydrofolate in Infant and Adult Nutritional formula using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

Author: Maria V. Chandra-Hioe, Martin P. Bucknall, and Jayashree Arcot

Summary of Method:
Folate extracted by heating in a boiling water bath, followed by cleanup using SPE and Amicon membrane filters. Determination by UPLC-MS/MS using 13C5 labeled internal standards.

Method Scope/Applicability:
Applicable to all infant formula matrices. Demonstrated for SPIFAN II matrices. Note from method authors indicates that the method is not rugged for high fat/high protein SPIFAN matrices.

General comments about the method:
Seems straightforward and logical. Removes need for triple enzyme treatment and qualifications that go along with that approach (cleaning and/or validating cleanliness of enzymes, evaluating activity of enzymes, etc.). Doesn't address 5-MTHF polyglutamate, which is a grey area in the SMPR. Folic acid detected in 2 of the placebo materials, which seems strange and may indicate an interference.

Method Clarity:
The procedure is clearly written and described.

Method Safety Concerns:
None

Pros/Strengths:
- Does not require enzyme treatment

Cons/Weaknesses
- Values for SRM 1849a are very high for both folic acid and 5-MTHF
- Values do not agree with (are much higher than) tri-enzyme method
- Performance characteristics do not meet SMPR

Supporting Data
- General Comment: the source of the validation data is not clearly described. Only values are reported in each SPIFAN matrix compared to the tri-enzyme treatment; nothing about recovery from each matrix. Values are highly variable between the heat treatment and tri-enzyme treatment.

    - Method Optimization: not described

- Performance Characteristics:

  Analytical Range: *Requirement for analytical range (0.50 – 300 µg/100 g) not met;* reported ranges were 0.31 – 140 µg/100 g for folic acid, 1.6 – 140 µg/100 g for 5-MTHF

  LOD: *Requirement for LOD (≤0.10 µg/100 g) not met*; reported values were 0.14 µg/100 g for folic acid, 0.75 µg/100 g for 5-MTHF

  LOQ: reported values were 0.31 µg/100 g for folic acid, 1.6 µg/100 g for 5-MTHF. This meets the requirement in the SMPR for folic acid of ≤0.50 µg/100 g. There is no stated requirement for 5-MTHF.

  Accuracy/Recovery: *Requirement for recovery (90 – 110 %) for folic acid not met;* values reported were 85 % for folic acid, 95 % for 5-MTHF

  Precision (RSD_r): *Requirement for repeatability precision (≤11 % or ≤7 %) not met;* repeatability information is reported at 8 %, but unclear at what level and for what samples.

  Reproducibility (RSD_R): within lab reported as 6 %, which is within the limits in the SMPR.

- System suitability: not described

Recommendation: I do not recommend this method be moved forward to first action.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method Fol-20 #3

Title: Folic Acid + 5-Methyltetrahydrofolate in IF + AN Formula using UPLC Tandem MS

Author: UNSW - Australia

Reviewer Name:

Summary of Method:

Samples heat treated in Boiling H_2O Then solid phase extraction + treatment using Amicon membrane filters, UPLC-MS Then used to determine

Method Scope/Applicability:

~~Applicable~~

Meets our needs Pertaining to scope

General comments about the method:

Very promising

Method Clarity:

Clear + Easy to understand

Method Safety Concerns:

NO

Pros/Strengths:

- Quick NOT 3day micro method
  Use of common enzyme

Cons/Weaknesses

- SEEMS to have high bias vs Micro

  Cont Tell how many days were done on spike sample (Spl from II? or I)

Supporting Data

- General Comment:

  - Method Optimization:

    Only monitoring one transition

- Performance Characteristics:

  Analytical Range:

  LOQ:

  Accuracy/Recovery:

  Low (85% Some high

  Precision (RSD_r):

  Reproducibility (RSD_R):

- System suitability:

Recommendation:

Promising Needs some more work to improve

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

FOLATE

AOAC # 2011.06 (Fol-22)

Mérieux NutriSciences (formerly Silliker)

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method FOL-22 #1

Title: Report of validation of LC-MS/MS for folate analysis

Author: Sillen

Reviewer Name:

Summary of Method:

Method Scope/Applicability:

Multi SMPL

General comments about the method:
A lot of work was done to take
Dual method for food + optimize it
For IF + AN

Method Clarity:
Would be nice to have
Final method spelled out

Method Safety Concerns:

None

Pros/Strengths:

- Already have Method
  optimized for IF + AN

Cons/Weaknesses

-

Supporting Data

- General Comment:

  Minor folates NOT Big contributor

  Remark?

  - Method Optimization:

    What is Method going forward

- Performance Characteristics:

  Analytical Range:

  LOQ: < 0.4 mg/liter

  Accuracy/Recovery:

  94-108%

  Precision (RSD_r):

  ~~[illegible]~~

  Reproducibility (RSD_R):

- System suitability:

Recommendation:

Go to Downselect VS Nestle Method

Need final method after optimization

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: Fol-22 #2

- **Currently an AOAC First Action method (2011.06)**

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

FRUCTANS (FOS)

FOS-01

Beneo-Orafti

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method FOS-01 #1

Title:
SPIFAN FOS-01 Single Lab Validation (SLV) for fructan in Infant Formula and Adult Nutritionals

Author:
Steegmans (Beneo-Orafti)

Summary of Method:
Samples are first defatted with hexane. Subsequently, the fructans are extracted from the sample with boiling water. An aliquot of the extract is hydrolyzed with inulinase. The concentrations of glucose, fructose and sucrose are chromatographically determined by gaschromatography: an internal standard is added to the sample followed by drying under vacuum. After treatment of the dried sample with hydroxylamine, the sugars are transformed to derivatives with N-(Trimethylsilyl)imidazole (TMSI). After addition of water the volatile derivatives are extracted with iso-octane and this phase is injected on a GC column. Fructan content is calculated from fructose, corrected for free fructose and fructose released from sucrose.

Method Scope/Applicability:
The method is applicable for Infant formula, adult / pediatric nutritional formula, and commodity ingredients.

General comments about the method:
- The method used is AOAC 997.08 with an adaptation according to Stöber (the incubation with amyloglucosidase is skipped).

Method Clarity:
- In general clearly written
- For the calculation of the fructan content, DP is an important factor. It is unclear how to calculate the fructan content, when the DP is unknown.

Pros/Strengths:
- Widely available equipment is used
- No correction needed for missing part of Fm chains

Cons/Weaknesses
- Elaborate sample preparation
- DP needs to be known in order to calculate fructan content
- Less precise when sucrose content exceeds about three times the total fructan content (according to Stöber)

Supporting Data

General Comment:

- Generally, set-up seems suitable for SLV
- As only four of the SPIFAN matrices contained FOS, the number of tested matrices for precision is rather limited

- Performance Characteristics:
  Analytical Range: 0.03 – 5 %
  LOQ: 0.03 %
  Accuracy/Recovery: average 100%; recoveries are well within 90 – 110% range
  Precision (RSD_r): average 6.3%; 2 out of 4 are outside acceptability limit
  Reproducibility (RSD_R): average 7.1%; within acceptability limits

- System suitability: ok

Recommendation:
Although the precision results are just outside the acceptability limits, we recommend this method for First Action approval if clarity is given around the calculation of DP.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method FOS-01 #2

Title: AOAC Official Method 997.08 Fructans in Foods
Single Lab validation for fructan in Infant Formula and Adult Nutritionals.

Author: Beneo-Orafti – Central Department Research

Summary of Method:

The method is based on AOAC Method 997.08 with some modifications, the chromatographic analyses were performed by Gas Chromatography.
Based on Stober method, the incubation with amyloglucosidase (AG) is skipped as the performance of the method is not influenced. Calculations are performed accordingly Strober at all. The samples contain fat are first defatted by use of hexane.
The fructans are extracted from the sample with boiling water. An aliquot of the extract is hydrolyzed with inulinase. The concentrations of glucose, fructose and sucrose are chromatographically determined by gaschromatography: an internal standard is added to the sample followed by drying under vacuum. After treatment of the dried sample with hydroxylamine, the sugars are transformed to derivatives with N-(Trimethylsilyl) imidazole (TMSI). After addition of water the volatile derivatives are extracted with iso-octane and this phase is injected on a capillary column.

Method Scope/Applicability:
Applicable to the determination of added fructans in infant formula and adult nutritionals.

General comments about the method:
Depending on the matrix of the sample, is possible to perform different chromatographic technique as GC or HPLC.
The samples analized were SPIFAN infant formula an adult nutritionals.
The pure products Orafti HP (inulin), Orafti P95 (FOS) and Nutraflora P95 (FOS) are part of the test materials kit and were screened in duplicate by 2 different chromatographic techniques, being GC and HPLC.

Method Clarity:
The method description is clear and easy to follow.

Pros/Strengths:
- The determination of the sugars can be done accurately without interferences.

Cons/Weaknesses

Supporting Data

- General Comment:

As a first step the samples were screened in duplicate for the presence of the fructans.

Method Optimization:

- Performance Characteristics:

Analytical Range: 0.03 – 5.0 g/100g
SMPR : 0.03 – 5.0 g/100g
The method meets the requirements

LOQ: 0.03g/100g
SMPR requirements : ≤ 0.03 g/100g

The LOQ was checked by adding fructans to a placebo infant formula product at the level of 0.03g/100g reconstituted final product. At this low level recovery is ranging from 90 to 98%.

Accuracy/Recovery: 90- 107 %
SMPR requirements: 90 -110 %

This recovery was measured by spiking 9 different matrices at different levels covering the proposed range from 0.05 to 5 g/100g reconstituted product.
The 3 raw materials available in the Test Materials Kit were used for this spiking.

Precision (RSD_r): 2.9 – 5.8 %
SMPR requirements: ≤ 6 %
The samples were analyzed in double on 6 different days by different operators.

Sample	Fructan (g/100g)	RSD_r %
Adult nutritional powder milk protein based J4G-00019	0.59	3,2
Adult nutritional RTF high fat J4G-00980	0.06	**8.0**
Child formula powder J4G-00013	0.35	4.5
Child formula powder J4G-00974	0.28	**9.5**

Reproducibility (RSD_R): ---

- **System suitability:**
  No data about calibration curve for standards.

Recommendation:

This method is suitable to proceed to First Action Status .

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method FOS-01 #3

Title: ~~Benco~~ FOS in IF and Adult Nutritionals

Author: Benco-Orafti

Reviewer Name:

Received, extracted in Boiling H_2O. Inulinase added. Glucose, fructose + sucrose determined by GC.

Summary of Method:

Method Scope/Applicability:

Meets FOS Std Needed

General comments about the method:

Method Clarity:

Method Safety Concerns:

Pros/Strengths:

- Uses existing Benc Method But Tests by GC

Cons/Weaknesses

-

Supporting Data

- General Comment:

  - Method Optimization:

- Performance Characteristics:

  Analytical Range:

  0.03 – 5

  LOQ:

  .03

  Accuracy/Recovery:

  98 – 107

  Precision (RSD_r):

  3.2 – 9.5% 6% is SMPR
  Some products fail to meet SMPL

  Reproducibility (RSD_R):

- System suitability:

Recommendation:

FIRST ACTION

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: FOS-01 #4

Title:
Fructan in Infant Formula and Adult Nutritionals.

Author:
Monique Steegmans, Beneo

Summary of Method:
Based upon AOAC 997.08 except method incubation with amyloglucosidase (AG) is skipped and the chromatographic analyses were performed by gas chromatography.
The concentrations of glucose, fructose and sucrose are chromatographically determined by gas chromatography: an internal standard is added to the sample followed by drying under vacuum.
The samples are first defatted by use of hexane. The fructans are extracted from the sample with boiling water. An aliquot of the extract is hydrolyzed with inulinase. After treatment of the dried sample with hydroxylamine, the sugars are transformed to derivatives with N-(Trimethylsilyl)imidazole (TMSI). After addition of water the volatile derivatives are extracted with iso-octane and this phase is injected on a capillary column.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas with fructan % of 0.03g to 5g/100g

General comments about the method:
A comparatively simple approach for analysis of fructans

Method Clarity:
Not the easiest method to follow and method would need to be written in clearer, concise language.

Method Safety Concerns:
Standard laboratory safety, however uses pyridine which can be concern for some labs.

Pros/Strengths:
Relative simple assay and GC-FID are found in many industry labs and have been for decades and provide a good work horse for many analyses.
Similar to AOAC 997.08

Cons/Weaknesses

Supporting Data

SPIFAN kit (1) used in the validation of the method.

Performance Characteristics:

Analytical Range:
0.03g to 5g/100g

LOQ:
0.03g/100g

Accuracy/Recovery:
Spike recovery of 100.3% (96.9% -107.1%)within SMPR limits

Precision (RSD_r):
6% RSDr(3.2-9.5%), partially (2.4 reported results) meets SMPR

Reproducibility (RSD_R):

System suitability:
No details

Recommendation:
TBD

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

FRUCTANS (FOS)

FOS-04

Abbott Nutrition

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method FOS-04 #1

Title:
Determination of Fructans in Infant and Adult/Pediatric Nutritional Formula as well as ingredient commodities

Author:
Haselberger (Abbott)

Summary of Method:
A two part analysis is performed. Part 1 is the qualitative determination of the type of fructan present in the sample. Part 2 is the quantitative determination of fructan in the sample. The sample to be analyzed is weighed and diluted with lab water. In part 1 the type of fructan is determined using certain rules based on retention time. In part 2 samples are treated with sucrase to hydrolyze any sucrose present. Glucose and fructose released by the enzymatic hydrolysis, as well as inherent glucose and fructose, are then reduced to sugar alcohols by the addition of sodium borohydride. Internal standard (glucoheptose) and fructanase are added to the sample solution. After the fructanase hydrolysis is completed, the samples are analyzed for fructose on HPAEC-PAD instrumentation. The total fructan content is calculated from the peak areas, adjusted by a commodity factor (CF, determined by part 1).

Method Scope/Applicability:
The method is applicable for Infant formula, adult / pediatric nutritional formula, and commodity ingredients.

General comments about the method:

- Based on the principle of AOAC 999.03: glucose and fructose (also from sugar) are reduced to sugar alcohols; then fructans are hydrolyzed to fructose, which is subsequently analyzed
- As in Cuany *et al.*, HPAEC-PAD is used for fructose analysis instead of the PAHBAH spectro-photometric method
- Using a borate trap reduces sample preparation
- The approach is to solve the problem of reduced precision of certain fructan types (high Fm type fructans with low chain length) by first determining the type of fructan that is added

Method Clarity:

- In general clearly written, including supporting tables and figures
- Extensive examples explaining how to determine the type of fructan
- Gradient in part 1 is now clarified
- Calculation of Commodity Factors is now included
- Calculation of fructan content was adapted to prevent double inclusion of Commodity Factor

Pros/Strengths:

- Method is straightforward, without complex sample preparation
- Widely available equipment is used
- Considerable reduction in analysis time

Cons/Weaknesses

- Previous remarks were taken up satisfactorily

Supporting Data

General Comment:

- Wide range of matrices tested for 3 types of fructans, including SPIFAN samples
- Multiple labs, analysts, and equipment used

- Performance Characteristics:
  Analytical Range: 0.03 – 5 % as-fed, 45-100% ingredient commodity
  LOQ: 0.03 % (empirically determined using low spike level in sugar solution)
  Accuracy/Recovery: average recoveries within 90 – 110% range
  Precision (RSD_r): well within acceptability limits
  Reproducibility (RSD_R): well within acceptability limits

- System suitability: ok

Recommendation:

Clear method with extensive supporting validation data. We recommend this method for First Action approval.

Infant Formula and Adult Nutrition - Review Form

Evaluation of Method: FOS-04 #2

Title: FOS-04

Author: Philip Haselberger

Summary of Method:

Method is in 2 parts:-

Part 1:-

- Sample is diluted in water at room temperature and stirred for 30 min
- Sample is then further diluted depending on expected fructan concentration (table provided)
- An aliquot is injected on HPAEC-PAD and the oligosaccharide profile recorded
- Depending on the profile it is possible to identify fructan ingredient present

Part 2:-

- 0.2 g of diluted sample (from above) is treated with sucrase and incubated at 40°C for 2h
- Sodium borohydride is then added and reaction proceeds for 1h at 40°C
- Add acetic acid to stop borohydride reaction
- Add 100ul of internal standard (glucoheptose)
- Add 100ul of furctanase
- Incubate at 40°C for 30min
- Filter and analyse released fructose by HPAEC-PAD

Fructan content is calculated as:-

Fructan = fructose x dilution factor x commodity factor

Where: dilution factor depends on the weight of sample used and amount of dilution
Commodity factor depends on the type of fructan in the sample. The commodity factor varies from 0.953 to 1.23 depedning on the fructan ingredient present.

Method Scope/Applicability:

Method has been applied to the SPIFAN kit (so formula and adult nutritionals) as well as some of Abbots own samples.

General comments about the method:

- Method is an improved version of the method of Cuany, et.al
- Improvements include the addition of a method for determining type of fructan
- Reduction of sample handling steps
- Introduction of internal standard
- Reduction of instrument time
- Overall I think the method should be OK
- Only concern would be the difficulty to assign the appropriate commodity factor in samples with a complex background containing other OS such as maltodextrins, GOS, resistant dextrin etc…
- Sample preparation has not strictly followed SPIFAN guidelines.

Method Clarity:

- The method is clear, but should be re-written in the appropriate AOAC format before going to first action.
-

Pros/Strengths:

- Avoids issues with sucrose interference
- Step to identify fructan and thus the commodity factor

Cons/Weaknesses

- Two step process requiring identification of fructan ingredient as a first step.
- Requires a different chromatographic set-up for the 2 different procedures (could part 1 also be ran with the borate trap?)

Supporting Data

General Comments:

- Precision data has been collected on 6 SPIFAN samples (new kit?) and 4 of Abbots own products. In addition 1 product has been analysed in 3 different labs. All precision data looks good. **(RSD (r) below 4% in all cases** which meets SMPR of ≤ 6%)
- The analysis of spiked samples has been devised in such a way as to cover many matrices. It is likely the have used several fructan ingredients – but it is not clear (except in the case of the Abbott matrix). Spiking levels are also not clearly defined (i.e. what is the actual spike level of the 50% and 150% overspikes – especially in blank matrices). However reported **recoveries are : 92.9 – 108% (within the 90-110% specified in SMPR)**
- It seems that the whole range 0.03 – 5.0 g/100g defined in the SMPR has not been covered. Maximum level measured is 3 g/100g.
- LoQ of 0.03 g/100g has been established by spiking fructan in to a concentrated sucrose solution. Might have been better to also demonstrate in a sample. However one SPIFAN kit sample has a concentration of 0.035 g/100g and the performance of the method at this level seems to be acceptable. .

- A quadratic curve has been used for calibration. Only 4 points are employed for making the quadratic curve (may be the limit for a quadratic) – plot of relative error indicates in most cases the error is less than 5% (one exception at lowest level).

- Performance Characteristics:

Analytical Range: tested **0.05 – 3.0 (SMPR goes up to 5.0)**

LOQ: assessed to be 0.03 g/100g in water solution containing sucrose. Might also be interesting to assess in blank matrix.

Accuracy/Recovery: **92.9 – 108%** (meets SMPR 90 – 110).

Precision (RSD_r): **1.09 – 3.67** (meets SMPR <= 6)
(RSD iR): 2.46 – 6.79 (no specification in SMPR)

Reproducibility (RSD_R): n/a

- System suitability:
  - Blank samples have been analysed and found to be blank.
  - Otherwise no mention of the use of check standards or blank check samples

Recommendation:

- Write method in appropriate format
- Better describe the actual spiking levels in spike/recovery experiments
- Good candidate for 1st action

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method FOS-04 #3

Title: Determination of Fructans in Infant Adult/Pediatric Nutritional Formula

Author: ABBOTT

Reviewer Name:

Summary of Method:

Determination of FOS By IC

Method Scope/Applicability:

General comments about the method:

IC Method That Appears it Needs to BE "Perfect" or Method Will Not Perform Well i.e Finicky

Method Clarity:

Method Safety Concerns:

None

Pros/Strengths:

•

Cons/Weaknesses

- Sucrase from Mega Zyme Unit to be substituted

Supporting Data

- General Comment:

  - Method Optimization:

- Performance Characteristics:

  Analytical Range:

  LOQ:
  .032

  Accuracy/Recovery:
  90-115

  Precision (RSD_r):
  2-7%

  Reproducibility (RSD_R):

- System suitability:

Recommendation:

1st Action

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: FOS-04 #4

Title:
Determination of Fructans in Infant and Adult/Pediatric Nutritional Formula as well as ingredient commodities

Author:
Abbott

Summary of Method:
A two part analysis with Part 1 is the qualitative classification of the fructan present in the sample, if not already known. This classification is based on rules related to presence/absence of GF3 (Nystose) and GF4 (fructofuranosylnystose), specifically, and higher oligomers, generally. It groups the fructans into one of three DP ranges and allows selection of an appropriate commodity factor for converting fructose content to total fructan content. Part 2 is the quantitative determination of total fructan in the sample. The sample to be analyzed is weighed and diluted with lab water, as appropriate. Part 1 of the analysis is then performed.
Part 2 can be run in parallel, whereby an aliquot of the diluted product is treated with sucrase to hydrolyze any sucrose present. Glucose and fructose released by the enzymatic hydrolysis, as well as inherent glucose and fructose, are then reduced to sugar alcohols by the addition of sodium borohydride. Excess borohydride is neutralized by the addition of acetic acid. An aliquot of internal standard (glucoheptose) and an aliquot of fructanase are added to the sample solution. After the fructanase hydrolysis is completed the samples are analyzed for fructose on HPAEC-PAD instrumentation. The total fructan content is calculated from the fructose, adjusted by a commodity factor (Part 1).

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas and ingredient commodities

General comments about the method:
Time consuming in the qualitative work needed upfront before you can start analysis of an unknown sample, so the conversion factor can be determined. It uses HPAEC-PAD which is less common in labs than GC-FID and can be interfered with by maltodextrans which are common in dairy preps requiring further enzyme steps which are described.

Method Clarity:
Clearly written method although very complex approach.

Method Safety Concerns:
Standard laboratory safety.

Pros/Strengths:
Directly measures fructose liberated and every else is removed.

Cons/Weaknesses
Requires correct identification of raw ingredient to generate a “commodity factor”.
Requires a single source of sucrase (Megazyme)
Potential suppliers of glucoheptose mention but supply may not be available.

Supporting Data
SPIFAN kit (1) used in the validation of the method.

Performance Characteristics:

Analytical Range:
0.03g to 5g/100g

LOQ:
0.03g/100g

Accuracy/Recovery:
Spike recovery of 99.2% (92.9% -108%)within SMPR limits

Precision (RSD_r):
2.2% RSDr(1.1-3.7%); meets SMPR

Reproducibility (RSD_R):

System suitability:
No details

Recommendation:
TBD

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

VITAMIN D

VitD-17

CDC-SH

Infant Formula and Adult Nutrition - Review Form

Evaluation of Method VitD-17 #1

Title: Determination of Vitamin D in Milk Products by UPLC-MS-MS with 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) Derivatization

Author: CDC-SH

Summary of Method:

Method Principle

Samples are prepared using a procedure based on AOAC Method 2011.11. Powder samples are reconstituted per SMPR. Liquid samples are delivered to a 100-mL flask to which pyrogallol is added as an antioxidant and tri-deuterated vitamin D3 is added as an internal standard. Potassium hydroxide (50%, aq) is added to the sample which is then flushed with nitrogen, tightly sealed. Saponification is carried out at room temperature overnight.

The reaction solution is quantitatively transferred to a 250-mL separatory funnel to which Hexane (w/BHT) is added. The sample is shaken and the aqueous layer removed. The hexane extraction solution is then washed 4x20mL with water and cooled at -20C for 60 mins.

A six-mL aliquot of the Hexane solution is transferred to a small vial and dried under nitrogen. PTAD solution (60 mcL) is added to the dried sample, mixed by vortex and sonication. Derivatization is complete after 30 mins at RT. Water (400 mcL) is added to the sample and the sample filtered prior to analysis by UPLC-MS/MS.

Instrument:

Instrument analysis for this work was completed using a 1290 Infinity UPLC (Agilent) with a 50 x 2.1 mm, 1.7micron BEH C18 column (Waters) interfaced to a 6490 Triple Quadrupole Mass Spectrometer (Agilent). Ionization is by ESI (+). Two mass transitions, one for quantitation and a second for qualitative verification, are used for each analyte, vitamin D_2, D_3 and tri-deuterated D3 IS.

Method Scope/Applicability:

The method as submitted includes validation data for SRM 1849a, overspike data for vitamins D2 and D3 in four SPIFAN matrices (Amino Acid Based Powder, Milk-Based IF Powder, Soy-Based IF Powder, and High Fat Adult Nutritional RTF).

Applicability is consistent with SMPR definition for Infant Formula and Adult Nutritional products; Vitamins D_2 and D_3.

General comments about the method:

Applicability generally meets SMPR.
Method is an LC-MS/MS method offering selectivity as a strength.
Diels-Alder reaction is specific to vitamin D2 and D3 conjugated diene which are absent in common plant sterols and contributes to method selectivity
Internal standardization is used to facilitate analyte quantitation and method robustness.
Instrument operation detail (chromatography conditions and mass spectrometer parameter) are provided in good detail.
Multiple mass transitions are used, one for quantitation and qualitative confirmation.
The PTAD-D adducts produce simplified mass spectra contributing to good S/N.
Method repeatability, accuracy, recovery for both vitamin D2 and D3 meet SMPR. Over-spike recovery data for vitamin D2, while within SMPR range, are consistently below 100%. More data is needed.
Method suitability requirements, calculation detail, standard curve detail are generally sparse or absent.
Method written for internal use – more detail and are sparse as are reagent details
Linearity is discussed for both vitamin D2 and D3 with sparse details in how the curve is generated. I would also like to see additional curve quality criteria including residuals to assess concentration-dependent error.
Precision was evaluated but only in SRM 1849a and in overspike experiments. As such the data are somewhat limited

Method Clarity:
Method is generally well written but appears to be written as an internal document. Suggest more detail to be added to address consistent application between labs.

Pros/Strengths:
Method is an LC-MS/MS method offering selectivity as a strength.
Diels-Alder reaction is specific to vitamin D2 and D3 (conjugated diene) which is absent in common plant sterols.
Internal standardization is used to facilitate analyte quantitation and method robustness.
Multiple mass transitions are used, one for quantitation and qualitative confirmation.
The PTAD-D adducts produce simplified mass spectra contributing to good S/N.
Method repeatability, accuracy, recovery and LOQ for both vitamin D2 and D3 meet SMPR.

Cons/Weaknesses
Does not include an internal standard for vitamin D2 which could reduce robustness for this analyte relative to SMPR.
Method detail is a thin. The method would benefit from inclusion of more detail around the sample preparation procedure (eg, saponification overnight), method suitability requirements, typical method performance (chromatography), calculation of standard curve metrics, to ensure robustness.
Location of deuterium labelling may not be as robust as others available.
pre-vitamin D, D2 or D3, is not addressed as required by SMPR
Applicability to SPIFAN has been demonstrated but additional data is needed to fully evaluate the method.
Need more data – only four matrices presented in addition to SRM 1849a.

This effort was significantly hindered by shipping difficulties.

Supporting Data

- General Comment:
  Data provided are consistent with SMPR requirements. More data is needed to complete a full SPIFAN SLV. This effort hindered by sample shipping issues.
  Method Optimization:

- **Performance Characteristics:**

  Analytical Range:
  7 working standard concentrations are indicated for both vitamin D2 and D3. Concentrations ranging from 5-100 ng/mL

  LOQ:
  LOQ was evaluated using blank samples, n=10, with measured standard deviation.
  VitD3 LOQ, x+10SD, is reported as 0.961 ng/100g in reconstituted/RTF product.
  Vit D2 LOQ, x+10SD, is reported as 0.813 ng/100g in reconstituted/RTF product
  As reported these pass SMPR requirement

  Accuracy/Recovery:
  Recovery of vitamin D2 was assessed by overspike experimentation using SRM 1849a at three spike levels. Analysis was completed in duplicate on each of 6 days. It is unclear if the within day replicates were separate preparations or duplicate injections from same sample
  Recovery reported was 101%-103% with very good precision.
  Three additional product samples, 3 powders and 1 liquid, were spiked at two levels with both vitamin D3 and D2. Each sample was analyzed in triplicate.
  Recoveries ranged from 89-97% (Vit D2) and 97%-102% (Vit D3)
  Precision values, n=3, ranged from <1% - 8.2% for vit D2 and vit D3
  vitamin D3 results are 97% relative to the NIST SRM 1849a certified value
  As reported these pass SMPR requirements

  Precision (RSD_r):
  Precision was evaluated using SRM 1849a (vitamin D3) and overspike results in product matrices.
  Data are limited.
  Vitamin D2:
    3.5% at 0.12mcg/100g RTF (SMPR ≤15%)
    5.2% at 1.5 mcg/100g RTF (SMPR ≤11%)
    4.1% at 2.5 mcg/100g RTF(SMPR ≤11%)
  Vitamin D3:
    3% at 1.5 mcg/100g RTF (SMPR ≤11%)

Reproducibility (RSD_R): NAP

- **System suitability:**
  As noted previously, method suitability requirements are sparse.
  Linearity is discussed for both vitamin D2 and D3 with few details in how the curve is generated.
  Performance requirements around injection precision, standards stability, curve performance (linearity, residuals, IS recovery), chromatography (eg. RT, symmetry, peak width, etc) should be added.

Recommendation:
This method is similar to VitD-18 and for same reasons has very strong potential. More detail is need on the assessment of pre-D, either via direct or indirectly measurement.
An IS for vitamin D2 is recommended.

Further work required.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: VitD-17 #2

Title: Determination of Vitamin D in Milk Products by UPLC-MS-MS with 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) Derivatization

Author: CDC_SH

Summary of Method:
Test samples are saponified, extracted, and the solvent evaporated. Vitamin D is derivatized with PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione, and then determined by UHPLC-MS/MS.

Method Scope/Applicability:
Determination of total vitamin D2 and vitamin D3 in all forms (powders, ready-to-feed liquids, and liquid concentrates) of infant, adult, and pediatric nutritional formulas.
Questionable on previtamin D determination.

General comments about the method:
This method is a well written method, it is easy to understand. The derivatization procedure is simple. Stable isotope labeled vitamin D3 was used as an internal standard. The detection is sensitive and selective.

Some of the difference between the two method:
The chromatography is within 10 min. The saponification is conducted at low temperature overnight. The derivatization reaction time is 30 min. After saponification, the hexane extract is dried before the PTAD reaction. NIST SRM 1849a and three powder samples and one liquid samples were used in recovery study.

Method Clarity:
The method was easy to follow. However, there is no info on how the results were calculated in the method.

Method Safety Concerns:
No particular safety concerns.

Pros/Strengths:
- Use of stable isotope labeled Vitamin D2 as internal standard.
- PTAD derivatization improves the method sensitivity and selectivity.

Cons/Weaknesses
- Need to have a purity check on standards
- Question about the previtamin determination.

Supporting Data

- General Comment:

Contains 1) linearity data, 0.5-100 ng/mL range, greater than 0.9997 coefficient correlation.
2) accuracy and precision on the NIST SRM 1849a excellent. The repeatability are less than 5.2% for concentration range from 0.12-2.5 ug/100g.
3) recovery results on NIST SRM on vitD2, 101-103%. On four samples within 89-108% at two spike levels. The four samples are infant formula powder, Milk powder with DHA prebiotics, DHA soy, and High fat AN ready to feed
4) There is no chromatograms available for review.

  - Method Optimization:

- Performance Characteristics:
  Meet the SMPR requirements in the analytical range, LOQ, Accuracy/Recovery, precision,

  Analytical Range:

  LOQ:

  Accuracy/Recovery:

  Precision (RSD_r):

  Reproducibility (RSD_R):

- System suitability:

There is no system suitability check written in the method.

Recommendation:
There is a concern about the previtamin D content determination in the samples. How accurate the final vitamin D result is if the previtamin D is not determined.
Recommendation:
1) Evaluate the effect of previtamin D on the final results of vitamin D
2) Regarding the method, suggest to add a purity check step for the standards, a calculation section, and system suitability check requirement.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: VitD-17 #3

Title:
Determination of Vitamin D in Milk Products by UPLC-MS-MS with 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) Derivatization

Author:
CDC-Shanghai

Summary of Method:
Powder samples were reconstituted and diluted in pyrogallic acid/ethanol, and KOH solutions. After saponification, liquid-liquid extraction was performed, followed by derivatization using PTAD. The derivatized sample solution was analyzed by UPLC-MS-MS analysis.

Method Scope/Applicability:
Infant Formula and Adult/Pediatric Formulas

General comments about the method:
The method describes the analysis of vitamin D2 and vitamin D3 using a PTAD derivatisation technique followed by UPLC-MS/MS. The use of PTAD derivatisation adds an extra degree of specificity to the analysis and provides clean chromatograms free from isobaric interferences.
Meets SMPR applicability statement by measuring both vitamin D and pre-vitamin D as single result.

Method Clarity:
Clear directions for operation of the method, instruments. Clear equations describing how the standards and samples are quantitated is required.

Method Safety Concerns:
None, beyond standard laboratory safety

Pros/Strengths:
VitD-PTAD analysis provides high molecular weight analyte which is free from matrix interferences that can cause problems in typical vitamin D methods. Additionally, the PTAD-adduct ionizes readily allowing for increase sensitivity.

Cons/Weaknesses
The choice of internal standard is not ideal given the site of deuteration coincides with the site of PTAD derivatisation. This has been shown in a recent publication to cause problems with accurate analysis.
No SIL vitamin D2 std is used and so questionable about whether accurate quantitation for this analyte.

Supporting Data
The SPIFAN kit was not used for validation.

Performance Characteristics:
The study director has made an error in the evaluation of bias against the NIST by reporting the wrong certified value for NIST 1849a. The method actually shows no bias against the NIST value when using the correct value.

Analytical Range:
0.05-10 meets SMPR limits (0.12-5.1 µg/100g)

LOQ:
4.58 ng/100g meets SMPR limits (<0.12 µg/100g)

Accuracy/Recovery:
103% average (101-103%) meets SMPR limits (90-110%).
Acceptable bias against NIST1849a SMPR

Precision (RSD_r):
2.3% meets SMPR limits (<11%)

Reproducibility (RSD_R):

System suitability:
No details

Recommendation:
Not recommended First Action at this time.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method VitD-17 #4

Title: Determination of Vitamin D in Milk products by UPLC-MS-MS with PTAD Derivatization

Author: CDC-SIT

Reviewer Name:

Summary of Method:
Method based on 2011.11 Method but PTAD Derivatization used Q Exactive

Method Scope/Applicability:
Applicable for SRM/PRM

General comments about the method:
SAYS uplc → should be UHPLC uplc Copyrighted by waters + Agilent used

Method Clarity:
Method clear

Method Safety Concerns:
NONE

Pros/Strengths:
•

Cons/Weaknesses

- NO PreD

NO SPIKE IN FULL KIT

Supporting Data

- General Comment:
  - Method Optimization:
- Performance Characteristics:

  Analytical Range:

  LOQ:

  Accuracy/Recovery:

  Precision (RSD_r):

  Reproducibility (RSD_R):
- System suitability:

Recommendation:

Need MORE DATA

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

VITAMIN D

VitD-18

Fonterra

Infant Formula and Adult Nutrition - Review Form

Evaluation of Method VitD-18 #1

Title: Analysis of Vitamin D2 and Vitamin D3 by LC-MS/MS in Milk Powders, Infant Formulas, and Adult Nutritionals

Author: Brendon Gill/Fonterra

Summary of Method:

Method Principle

Liquid samples are prepared directly with powder samples reconstituted per SMPR guidelines prior to sampling. Pyrogallol is added to protect against oxidation and hexa-deuterated analogues for vitamin D2 and D3 are added as internal standards prior to sample preparation. Samples are prepared by alkaline saponification at 70C for 1 hr. Lipid-soluble components including vitamins D2 and D3 are extracted into isooctane. A portion of the isooctane extraction layer is transferred to a second vial and washed. Following centrifugation, an aliquot of the isooctane layer is transferred to a separate centrifuge tube to which 75-mcL of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) solution is added. The Diels-Alder reaction is completed in 5 minutes at ambient temperature producing high molecular mass, easily ionisable Vitamin D-PTAD adducts. The vitamin D-adducts are re-extracted into a small volume of acetonitrile and analysed by reversed-phase liquid chromatography. Detection is by MS/MS using MRM.

Instrument:

Chromatographic separation for the SLV study was completed using a Shimadzu UHPLC system equipped with a 2.1 mm x 50 mm x 2.6micron Phenomenex Kinetex C18 column.

Mass Spectral analysis was completed using ABSciex 6500 QTrap triple quadrupole mass spectrometer with Electrospray (+ion) ionization. Two mass transitions, one for quantitation and a second for qualitative verification, are used for each analyte, vitamin D_2, D_3 and each IS compound.

Method Scope/Applicability:

The method submitted includes single-laboratory validation data for all SPIFAN materials.

Applicability is consistent with SMPR definition for Infant Formula and Adult Nutritional products; Vitamins D_2 and D_3.

General comments about the method:

The method is very well written and thorough.

Hexa-deuterated internal standards are used for both vitamin D2 and D3 which contribute significantly to the robustness of the method.

The resulting chromatography is very clean and addresses a primary chromatography concerns observed in some LC-MS/MS methods previously evaluated. S/N is also significantly improved vs direct measurement of vitamins D2 and D3.

Method Clarity:
Method is very well written.

Pros/Strengths:
Method is an LC-MS/MS method offering selectivity as a strength.
Diels-Alder reaction is specific to vitamin D2 and D3 (conjugated diene) which are absent in common plant sterols.
Internal standardization is used to facilitate analyte quantitation and method robustness.
Location of deuterium labelling is robust
Multiple mass transitions are used, one for quantitation and qualitative confirmation.
The PTAD-D adducts produce simplified mass spectra contributing to good S/N.
Method repeatability, accuracy, recovery and LOQ for both vitamin D2 and D3 meet SMPR.
Method performance was evaluated against AOAC 2002.05 for bias. While not an SMPR requirement, the method compares quite favorably to this commonly used method.

Cons/Weaknesses
There are very few weaknesses in the method as presented.
Vitamin D and pre-D are measured as an aggregate, that is pre-D is not directly measured. Vitamins D2 and D3, along with their pre-vitamin D isomers, contain the necessary conjugated diene structure to facilitate the Diels-Alder reaction to form PTAD-D adduct.

Supporting Data

- General Comment:
  A full set of SLV data are provided and completed per Appendix L; AOAC Recommended Guidelines for Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Single-Laboratory Validation.

- **Performance Characteristics:**

  Analytical Range:
  Vitamin D2: 0.04 – 7.3 mcg/100g RTF
  Vitamin D3: 0.06 – 11.3 mcg/100g RTF
  SMPR: 0.12 – 5.1 mcg/100g RTF

  LOQ:
  Vitamin D2: 0.016 mcg/100g RTF (≤0.120 mcg/100g RTF)
  Vitamin D3: 0.028 mcg/100g RTF (≤0.120 mcg/100g RTF)

Accuracy/Recovery:
Vitamin D2: 97 – 101% (mean = 98%); (SMPR 90-110%)
Vitamin D3: 94 – 101% (mean = 98%); (SMPR 90-110%)

vs. NIST SRM
Vitamin D2: NAP
Vitamin D3: within certified range

Precision as Repeatability (RSD_r):
Vitamin D2: NAP, none of SPIFAN matrices contain vitamin D2
Vitamin D3: 1.5 – 5.3% (mean = 3.0%); (SMPR ≤11%)

Reproducibility (RSD_R):
NAP

- **System suitability:**
  I did not see any defined system or method performance suitability criteria defined within the method text. Performance requirements around injection precision, standards stability, curve performance (linearity, residuals, IS recovery), chromatography (eg. RT, symmetry, peak width, etc) should be added.

Recommendation:
First Action

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: VITD-18 #2

Title: Analysis of Vitamin D2 and Vitamin D3 by LC-MS/MS in Milk Powders, Infant Formulas, and Adult Nutritionals

Author: Gill, B.D

Summary of Method:

Samples are saponified at high temperature then lipid soluble components are extracted into isooctane. A portion of the isooctane layer is transferred, washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione is added to derivatise vitamin D to form a high molecular mass, easily ionisable adduct. The vitamin D-adduct is then re-extracted into a small volume of acetonitrile and analysed by reverse-phase liquid chromatography. Detection is by triple quadrupole mass spectrometer. Stable isotope labelled vitamin D2 and vitamin D3 internal standards are used for quantitation.

Method Scope/Applicability:

The method is applicable for vitamin D_2 or vitamin D_3 supplemented milk powders, infant formulas, and adult nutritional products.
This method does not distinguish between contribution from pre-vitamin D and vitamin D.
and measures only an aggregate result.

General comments about the method:

1. The method involves hot saponification followed by solvent extraction of vit D.
2. Vitamin D is not measured directly but measured after drivatization.
3. It does not distinguish between contribution from pre-vitamin D and vitamin & measures only an aggregate vitamin D2 and/or D3 value.

Method Clarity: Satisfactory

Method Safety Concerns: Safe handling of the chemicals involved listed

Pros/Strengths:

- Quite sensitive method
- The method lists steps to establish purity of standards.

Cons/Weaknesses

- Involves hot saponification
- Not able to distinctly separate previtamin D from vitamin D

Supporting Data

- General Comment: Required data are available

  - Method Optimization: Data not provided

- Performance Characteristics:

Analytical Range:
The average range for vitamin D_2 concentration of 0.04–7.3 µg hg^{-1} RTF which extends beyond limits of the range specified in the vitamin D SMPR.
The average range for vitamin D_3 0.06–11.3 µg hg^{-1} RTF which exceeds the range specified in the vitamin D SMPR.

LOQ:
The LOQ for vitamin D_2 = 0.016 µg hg^{-1}.
The LOQ for vitamin D3 = 0.028 µg hg^{-1}.
LOQ for both vitamin D_2 and vitamin D_3 are lower than those defined in the vitamin D SMPR.

Accuracy/Recovery:
Recovery was evaluated using unfortified samples within the SPIFAN kit. Each matrix was spiked at two levels: 50% (≈ 0.6 µg hg^{-1} RTF) and 100% (≈ 1.1 µg hg^{-1} RTF) of typical infant formula concentrations. Recoveries measured for vitamin D_2 were between 97–101% and for vitamin D_3 were between 94–101%, which are within the limits set in the SMPR of 90–110%.

The method does not have any bias when its results compared against the values generated using A std. AOAC reference method.
No bias between measured results and certified values for vitamin D3 in NIST SRM 1849a.

Precision (RSD_r):
Repeatability for the method in SPIFAN kit samples ranged between 1.5–5.2% which complies with the ≤ 11.0% limit set with the SMPR. The Intermediate precision ranged between 3.1–7.9% with a mean value of 5.5%

Reproducibility (RSD_R):

- System suitability:

Recommendation:

The method may be considered by the ERP for the First Action Method.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method: VitD-18 #3

Title: Analysis of Vitamin D2 and Vitamin D3 by LC-MS/MS in Milk Powders, Infant Formulas, and Adult Nutritionals

Author: Fonterra

Summary of Method:
Samples are saponified at high temperature then lipid soluble components are extracted into isooctane. A portion of the isooctane layer is transferred, washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione is added to derivatise vitamin D to form a high molecular mass, easily ionisable adduct. The vitamin D-adduct is then re-extracted into a small volume of acetonitrile and analysed by reverse-phase liquid chromatography. Detection is by triple quadrupole mass spectrometer using multiple reaction monitoring (MRM). Stable isotope labelled (SIL) vitamin D2 and vitamin D3 internal standards are used for quantitation to correct for losses in extraction and any variation in derivatisation and ionisation efficiencies.

Method Scope/Applicability:
This method is applicable for vitamin D_2 or vitamin D_3 supplemented milk powders, infant formulas, and adult nutritional products.

General comments about the method:
This method is a well written method. The procedure is easy to understand. The derivatization procedure is simple, use stable isotope labled vitamin D2 and D3 as internal standards. The detection is sensitive and selective. The chromatography is within 5 min.

The saponification was conducted at 70oC for 1 hour
The PTAD dirivatization was conducted for 5 min
SPIFAN samples and NIST SRM 1849a were used in the single lab validation.

Method Clarity:
The method was easy to follow.

Method Safety Concerns:
No particular safety concerns.

Pros/Strengths:
- Use of stable isotope labeled Vitamin D2 and D3 as internal standards.
- PTAD derivatization improves the method sensitivity and selectivity.

Cons/Weaknesses
- Concern about the previtamin determination.

Supporting Data

- General Comment:

Validation data obtained on SPIFAN kit and NIST SRM 1849a. The data include linearity, precision, sensitivity, recovery, accuracy against SRM and AOAC 2002.05 method.
Chromatograms of vitD2 and vitD3 in samples are provided as well as the product ion scan mass spectra of derivatized standards.

  - Method Optimization: No optimization data provided.

- Performance Characteristics:

Meet the SMPR requirements in the analytical range, LOQ, Accuracy/Recovery, precision,

Analytical Range:

LOQ:

Accuracy/Recovery:

Precision (RSD_r):

Reproducibility (RSD_R):

- System suitability:

System suitability criteria is not provided.

Recommendation:

One serious concern is the determination of the previtamin Ds. The author discussed about the pre-vitamin D determination in the method. Author made two major assumptions, one is that the pre-vitamin D and vitamin D in the sample after saponification have reached equilibrium; 2) the equilibrium constant and kinetics are the same for the analytes and the isotope labelled forms of vitamin D.

However, there are data shown that the conversion between the previtamin D and vitamin D is a slow process. One report showed that at 60oC the equilibrium time was 12 hours, and at 80oC the equilibrium time was 2.4 hours.
The saponification conditions and time ini this method seem can not ensure a equilibrium between the previtamin D and vitamin D is established.

Recommendation: Need more investigation on the previtamin D determination.

Expert Review Panel for Infant Formula and Adult Nutrition

Evaluation of Method VitD-18 #4

Title: Analysis of D_2+D_3 by LC-MS/MS in Milk powder, IF + Adult nutrition

Author: Fontern
Reviewer Name:

Summary of Method:

Sample saponified + extracted in Isooctane. Sample then derivatized (PTAD) and analyzed by LC-MS/MS

Method Scope/Applicability:

General comments about the method:
DOES NOT measure pre-d
But meets other SMPR requirements

NO samples w/ D_2 measured

Method Clarity:
Easy to follow

Method Safety Concerns:
NO

Pros/Strengths:
- Uses derivatization to avoid plant steroid interference

Cons/Weaknesses

- NO Pre-D (fails to Meet Sample Requirements)

Supporting Data

- General Comment:

  - Method Optimization:

- Performance Characteristics:

  Analytical Range:

  .04-11

  LOQ:

  < 0.02

  Accuracy/Recovery:

  94-101

  Precision (RSD_r):

  1.5-5%

  Reproducibility (RSD_R):

- System suitability:

Recommendation:

~~FIRST~~ If Pre-D is added 1st Action