CODEX ALIMENTARIUS COMMISSION

Food and Agriculture Organization of the United Nations

World Health Organization

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Agenda Item 3 **MAS-CRD/04**

ORIGINAL LANGUAGE ONLY

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON METHODS OF ANALYSIS SAMPLING

ENDORSEMENT OF METHODS OF ANALYSIS AND SAMPLING PLANS FOR PROVISIONS IN CODEX STANDARDS

Updated information submitted by AOAC, ISO and IDF

Executive Summary

This document presents recommendations and supporting information from AOAC INTERNATIONAL (AOAC), the International Standardization Organization (ISO), and the International Dairy Federation (IDF) regarding infant formula methods of analysis topics to be discussed during the 41st Session of the Codex Committee on Methods of Analysis and Sampling (CCMAS41).

Recommendations to CCMAS41

AOAC/ISO/IDF recommend CCMAS41 to take the following actions:

1. Endorse AOAC 2015.14 / ISO 21470 as Type II for the determination of thiamin, riboflavin, niacin, and pyridoxine in infant formula, and reclassify the following existing Type II methods as Type III:
    a. EN 14122 (thiamin)
    b. EN 14152 (riboflavin)
    c. EN 15652 (niacin)
    d. AOAC 2004.07 / EN 14164 (pyridoxine)
2. Endorse AOAC 2015.10 / ISO 21468 as Type II for the determination of choline and carnitine in infant formula, and reclassify AOAC 999.14 as Type III for the determination of choline.
3. Endorse AOAC 2016.13 / ISO 23443 as Type II for the determination of beta-carotene and lycopene in infant formula.
4. Endorse AOAC 2016.14 / ISO 22579 | IDF 241 as Type II for the determination of fructans in infant formula.
5. Endorse ISO 23305 as Type II for the determination of biotin in infant formula.
6. Confirm 2015.09 / ISO 21446 should replace AOAC 999.15 / EN 14148 Type II for the determination of vitamin K in follow-up formula and reclassify AOAC 999.15 / EN 14148 as Type III.

Agenda Item #3: Endorsement of Methods of Analysis and Sampling Plans for Provisions in Codex Standards

Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU41)

Methods of analysis for provisions in the Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (CODEX STAN 72-1981)

Thiamin, Riboflavin, Niacin and Pyridoxine

CCNFSDU41 agreed to submit AOAC 2015.14 / ISO 21470, "Simultaneous Determination of Total Vitamins B_1, B_2, B_3 and B_6 in Infant Formula and Related Nutritionals by Enzymatic Digestion and LC-MS/MS," to CCMAS41 for review, endorsement as Type II, and inclusion in CXS 234-1999. CCNFSDU41 also asked CCMAS41 to re-type the related existing methods for these vitamins in CXS 234-1999.

AOAC 2015.14[1] / ISO 21470[2] reflects the most up to date scientific method of analysis for thiamin, riboflavin, niacin and pyridoxine in infant formula and was fully validated in these products. AOAC 2015.14 / ISO 21470 prepares samples by enzymatic digestion with papain, α-amylase, and phosphatase to hydrolyze protein, complex carbohydrate, and free phosphorylated vitamin forms, respectively. Stable-isotope-labeled internal standards are incorporated into the sample preparation to correct for variability in both the sample preparation and instrument response. Prepared samples and working standard solutions are injected onto an ultra-high-pressure liquid chromatograph (UPLC), interfaced with a triple-quadrupole mass spectrometer (MS/MS). Analytes were separated on a C-18 column by reverse phase gradient chromatography using mobile phases of methanol and 20 mM ammonium formate in water. The MS/MS is configured to monitor precursor-fragment ion pairs for each analyte and internal standard. Vitamins B_1 (thiamin), B_2 (riboflavin), B_3 (niacin), and B_6 (pyridoxine) are quantitated by least squares regression using the response ratio of each analyte to its internal standard.

AOAC 2015.14 / ISO 21470 would replace EN 14122, EN 14152, EN 15652, and AOAC 2004.07 for vitamins B_1, B_2, B_3, and B_6, respectively. Neither EN 14152 nor EN 15652 included infant formula or adult nutritionals in their multi-lab testing studies (MLTs). EN 14122 included one tube feed nutritional and one infant formula, but the limited data showed inferior repeatability and reproducibility as compared to AOAC 2015.14 / ISO 21470. AOAC 2004.07 included eight liquid infant formulas in the MLT but showed lower recovery and had significantly higher repeatability and reproducibility. The robust sample sets used for the Single Laboratory Validation (SLV) and MLT studies, good recoveries, low quantitation limits, and good repeatability and reproducibility demonstrate that AOAC 2015.14 / ISO 21470 is fit for purpose to test complex infant formula and adult nutritional matrices.

The performance of AOAC 2015.14 / ISO 21470 was evaluated extensively by both SLV and MLT studies, which encompassed a broad range of infant formulas covering a wide nutrient fortification range. The SLV was completed using 12 SPIFAN I and 3 SPIFAN II matrices and MLT using 15 SPIFAN II matrices. 10 laboratories from 8 countries participated in the MLT study and produced data with excellent precision and accuracy for all samples in the SPIFAN II Kit.

The SLV and MLT data provide systematic, scientific evidence for a simple, selective, accurate, and precise method for the purpose of dispute resolution for the determination of thiamin, riboflavin, niacin and pyridoxine in all forms of infant, adult, and pediatric formulas. Following is a review of each existing Type II method, comparison of performance parameters of each method and AOAC 2015.14 / ISO 21470, and recommendations for re-typing any existing Type II methods.

Thiamin

The current Type II method for the determination of thiamin in infant formula is EN 14122. This European Standard specifies a method for the determination of vitamin B_1, which is extracted from food after acid

[1] McClure. Simultaneous Determination of Total Vitamins B_1, B_2, B_3, and B_6 in Infant Formula and Related Nutritionals by Enzymatic Digestion and LC-MS/MS – A Multi-Laboratory Testing Study Final Action: AOAC Method 2015.14. *Journal of AOAC INTERNATIONAL,* Volume 103, Issue 4, July-August 2020, Pages 1060-1072, https://doi.org/10.1093/jaoacint/qsaa012

[2] ISO 21470:2020. Infant formula and adult nutritionals – Simultaneous determination of total vitamins B1, B2, B3 and B6 – Enzymatic digestion and LC-MS/MS. https://www.iso.org/standard/70946.html

hydrolysis followed by dephosphorylation using an enzymatic treatment and quantified by high-pressure liquid chromatography (HPLC) with pre- or post-column derivatization to thiochrome. Vitamin B_1 is the mass fraction of total thiamin including its phosphorylated derivatives. The performance parameters of the current Type II method and AOAC 2015.14 / ISO 21470 are summarized below. [Note: RTF refers to ready-to-feed infant formula levels.]

Parameters – Thiamin	**AOAC 2015.14 / ISO 21470**	**EN 14122**
Infant/adult/placebo formula matrices used in MLT study	15 (4 liquid, 11 powder)	2
Repeatability (RSD_r)	2.3%	7.5%
Reproducibility (RSD_R)	8.2%	25.5%
Recovery	92.4 – 102.5%	n/a
NIST SRM 1849a (Certified value 12.57±0.98 mg/kg)	13.1 mg/kg	n/a
Limit of Quantitation	0.0062 mg/100 g RTF 0.010 mg/100 kcal	n/a
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.036 mg/100 g RTF 0.06 mg/100 kcal	

Riboflavin

The current Type II method for the determination of riboflavin in infant formula is EN 14152. This European Standard specifies a method for the determination of riboflavin after acid hydrolysis followed by dephosphorylation using an enzymatic treatment by HPLC separation with fluorometric detection. The performance parameters of the current Type II method and AOAC 2015.14 / ISO 21470 are summarized below.

Parameters – Riboflavin	**AOAC 2015.14 / ISO 21470**	**EN 14152**
Infant/adult/placebo formula matrices used in MLT study	15 (4 liquid, 11 powder)	0
Repeatability (RSD_r)	3.9%	n/a
Reproducibility (RSD_R)	6.9%	n/a
Recovery	95.4 – 99.7%	n/a
NIST SRM 1849a (Certified value 20.37±0.52 mg/kg)	21.4 mg/kg	n/a
Limit of Quantitation	0.0043 mg/100 g RTF 0.0072 mg/100 kcal	n/a
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.048 mg/100 g RTF 0.08 mg/100 kcal	

Niacin

The current Type II method for the determination of niacin in infant formula is EN 15652. This European Standard specifies a method for the determination of the mass fraction of niacin in foodstuffs by HPLC by three different processes of hydrolysis: 1) acid hydrolysis; 2) enzymatic hydrolysis; or 3) acid/alkaline hydrolysis. Niacin vitamers are quantified by HPLC with fluorimetric detection after a post-column derivatization with UV irradiation. The performance parameters of the current Type II method and AOAC 2015.14 / ISO 21470 are summarized below.

Parameters – Niacin	AOAC 2015.14 / ISO 21470	EN 15652
Infant/adult/placebo formula matrices used in MLT study	15 (4 liquid, 11 powder)	0
Repeatability (RSD_r)	2.7%	n/a
Reproducibility (RSD_R)	6.7% %	n/a
Recovery	100.3 – 110.7%	n/a
NIST SRM 1849a (Certified value 109±10 mg/kg)	105 mg/kg	n/a
Limit of Quantitation	0.044 mg/100 g RTF 0.074 mg/100 kcal	n/a
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.18 mg/100 g RTF 0.3 mg/100 kcal	

Pyridoxine

The current Type II method for the determination of pyridoxine in infant formula is AOAC 2004.07. Total pyridoxine is quantified by converting phosphorylated and free vitamers into pyridoxine and using ion pair reversed-phase liquid chromatography with fluorescence detection. The performance parameters of the current Type II method and AOAC 2015.14 / ISO 21470 are summarized below.

Parameters – Pyridoxine	AOAC 2015.14 / ISO 21470	AOAC 2004.07
Infant/adult/placebo formula matrices used in MLT study	15 (4 liquid, 11 powder)	8 liquid
Repeatability (RSD_r)	2.2%	4.7%
Reproducibility (RSD_R)	5.8%	9.7%
Recovery	97.1 – 100.2%	81.4 – 98.0 %
NIST SRM 1849a (Certified value 13.46±0.93 mg/kg)	14.0 mg/kg	n/a
Limit of Quantitation	0.0056 mg/100 g RTF 0.0094 mg/100 kcal	0.005 mg/100 g RTF
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.021 mg/100 g RTF 0.035 mg/100 kcal	

Choline and Carnitine

CCNFSDU41 agreed to submit AOAC 2015.10 / ISO 21468, "Determination of Free and Total Choline and Free and Total Carnitine in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography/Tandem Mass Spectrometry (HPLC-MS/MS): A Multilaboratory Testing Study," to CCMAS41 for review, endorsement as Type II, and inclusion in CXS 234-1999. CCNFSDU41 also asked CCMAS41 to re-type the related existing methods for these nutrients in CXS 234-1999.

AOAC 2015.10[3] / ISO 21468[4] reflects the most up-to-date scientific method of analysis for choline and carnitine in infant formula and was fully validated in these products. AOAC 2015.10 / ISO 21468 is applicable to the measurement of both free and total carnitine in infant formula. Both variations of the

[3] Shippar J et al. 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): A Multi-Laboratory Testing Study, Final Action 2015.10. *Journal of AOAC INTERNATIONAL*, qsaa073. https://doi.org/10.1093/jaoacint/qsaa073
[4] ISO 21468:2020. Infant formula and adult nutritionals – Determination of free and total choline and free and total carnitine – Liquid chromatography tandem mass spectrometry (HPLC-MS/MS). https://www.iso.org/standard/70945.html

method utilize a common HPLC and LC/MS configuration and a stable isotope-labeled carnitine internal standard. This internal standard is spiked into the samples from initial sample weighing to correct for any extraction or instrumental analysis variance. The free and total methods differ in extraction steps only: the free analysis employs simple dilution with water while the total extraction uses microwave digestion of samples with nitric acid. The primary advantage of LC/MS detection is superior specificity for the compounds of interest than most other methods. In addition, sensitivity and linear range of an analysis with LC/MS is typically far greater than most other HPLC detection methods. Microwave digestion is an efficient, effective, and robust technique to digest complex matrices.

The performance of AOAC 2015.10 / ISO 21468 was evaluated extensively by both SLV and MLT studies. Sample sets of 17 and 10 matrices (SPIFAN I Kit) were used during the SLV and MLT method validation stages, respectively. The SPIFAN I Kit was developed specifically for this purpose and encompasses a broad range of infant and follow-up formula covering a wide nutrient fortification range. 9 laboratories from 7 countries participated in the MLT study and produced data with excellent precision and accuracy for all samples.

The SLV and the MLT data provide systematic scientific evidence for a simple, selective, accurate and precise method for the purpose of dispute resolution for the determination of choline and carnitine in all forms of infant, adult, and pediatric formulas. Following is a review of each of the existing Type II methods (if applicable), followed by performance parameters of the current Type II method and AOAC 2015.10 / ISO 21468 and recommendations for re-typing existing methods.

Choline

The current Type II method for the determination of total choline in infant formula is AOAC 999.14, which is an enzymatic colorimetric method using UV spectrophotometric detection. The method involves acid hydrolysis and multiple enzymatic steps to generate peroxides relative to the choline concentration. The generated peroxide is used to oxidize phenol added to the sample and generate a derivative that can be measured at 505 nm. The performance parameters of the methods during MLT are summarized below. The requirements for testing in AOAC SMPR 2012.013 were for total choline only, but free choline data were also collected in the MLT.

Parameters – Total Choline	**AOAC 2015.10 / ISO 21468**	**AOAC 999.14**
Infant/adult/placebo formula matrices used in MLT study	10 (3 liquid, 7 powder)	8
Repeatability (RSD_r)	Free: 2.3% / Total: 1.9%	Total: 2.6%
Reproducibility (RSD_R)	Free: 6.7% / Total: 7.9%	5.1%
Recovery (2-250 mg/100g)	Free: 95.9 – 100.1% Total: 98.3 – 99.9%	n/a
NIST SRM 1869 (AOAC 2015.10) (certified value 109±11) NIST SRM 1849a (AOAC 999.14) (certified value 125±12)	105 mg/100 g	133 mg/100 g
Limit of Quantitation	2.0 mg/100 g RTF 3.3 mg/100 kcal	5.00 mg/100 g RTF 8.33 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	4.2 mg/100 g RTF 7.0 mg/100 kcal	

Carnitine

There is no current Type II method for the determination of carnitine in infant formula. However, the performance parameters of AOAC 2015.10 / ISO 21468 are summarized below.

Parameters – Carnitine	AOAC 2015.10 / ISO 21468
Infant/adult/placebo formula matrices used in MLT study	10 (3 liquid, 7 powder)
Repeatability (RSD_r)	Free: 2.6% / Total: 2.6%
Reproducibility (RSD_R)	Free: 4.7% / Total: 4.6%
Recovery (2-250 mg/100 g)	Free: 99.2 – 103.6% Total: 98.7 – 102.7%
NIST SRM 1849a (certified value 13.6±1.4)	13.0 mg/100 g
Limit of Quantitation	2.0 mg/100 g RTF 3.3mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.72 mg/100 g RTF 1.20 mg/100 kcal

Beta-Carotene and Lycopene

CCNFSDU41 agreed to submit AOAC 2016.13 / ISO 23443, "Determination of Lutein, β-Carotene, and Lycopene in Infant Formula and Adult Nutritionals – Reversed-Phase Ultra-High-Performance Liquid Chromatography (UHPLC) – Final Action 2019 (β-Carotene and Lycopene Only)," to CCMAS41 for review, endorsement as Type II and inclusion in CXS 234-1999.

AOAC 2016.13[5] / ISO 23443[6] reflects the most up-to-date scientific method of analysis for beta-carotene and lycopene in infant formula and was fully validated in these products. Test samples (reconstituted powders, RTF liquids, and liquid concentrates) are spiked with an internal standard and treated briefly with potassium hydroxide. This liberates carotenoids from the sample matrix without causing isomerization of the analytes, although it may not result in the complete hydrolysis of lipids. Samples are then extracted with MTBE and THF, followed by hexane. The supernatants from the liquid-liquid extraction are dried under nitrogen and reconstituted in 2-propanol. Separation is performed by reversed-phase chromatography on a C30 column. All-*trans* lutein, β-carotene, and lycopene are separated from their major *cis* isomers, as well as from zeaxanthin and α-carotene. Although this method does not involve high system backpressure normally associated with UHPLC, the low system volume is recommended for resolution with a 2.0 mm internal diameter (i.d.) column.

There are currently no Type II methods listed for the determination of carotenes in foods for special medical purposes, including infant formula. In addition, while beta-carotene is referenced in CAC/GL 10-1979, lycopene is not included in CXS 72-1981 or CAC/GL 10-1979. However, infant formulas containing both beta carotene and lycopene are being traded globally so there is the possibility of regulatory disputes regarding the level of these nutrients in a particular product. To address potential regulatory disputes, it is appropriate to propose a Type II method for the determination of beta-carotene and lycopene in infant formula.

The performance of AOAC 2016.13 / ISO 23443 was evaluated extensively by both SLV and MLT studies, which encompassed a broad range of infant and follow-up formula covering a wide nutrient fortification range. The SLV and MLT studies were completed using 7 SPIFAN II matrices. 10 laboratories from 7 countries participated in the MLT study and produced data with excellent precision and accuracy for all samples in the SPIFAN II Kit.

[5] Hostetler, et al. Determination of Lutein, β-Carotene, and Lycopene in Infant Formula and Adult Nutritionals by Ultra-High Performance Liquid Chromatography: Collaborative Study, Final Action 2016.13 for β-Carotene and Lycopene Only. *Journal of AOAC INTERNATIONAL,* Volume 103, Issue 3, May-June 2020, Pages 818-832. https://doi.org/10.1093/jaocint/qsz017

[6] ISO 23443:2020. Infant formula and adult nutritionals – Determination of β-carotene , lycopene and lutein by reversed-phase ultra-high performance liquid chromatography (RP-UHPLC). https://www.iso.org/standard/75599.html

The SLV and the MLT data provide systematic scientific evidence for a simple, selective, accurate, and precise method for the purpose of dispute resolution for the determination of beta-carotene and lycopene in all forms of infant, adult, and pediatric formulas. Following is a review of the performance parameters of AOAC 2016.13 / ISO 23443.

Beta-Carotene

There is no current Type II method for the determination of beta-carotene in infant formula. The performance parameters of AOAC 2016.13 / ISO 23443 are summarized below.

Parameters – Beta Carotene	**AOAC 2016.13 / ISO 23443**
Infant/adult/placebo formula matrices used in MLT study	7 (1 liquid, 6 powder)
Repeatability (RSD_r)	≤6.0%
Reproducibility (RSD_R)	≤13.7%
Recovery (15 µg/kg spike)	98 – 104%
NIST SRM 1869 (Certified value 1.05±0.26 mg/kg)	0.98 mg/kg
Limit of Quantitation	0.27 µg/100 g RTF 0.53 µg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	No minimum levels set

Lycopene

There is no current Type II method for the determination of lycopene in infant formula. The performance parameters of AOAC 2016.13 / ISO 23443 are summarized below.

Parameters – Lycopene	**AOAC 2016.13 / ISO 23443**
Infant/adult/placebo formula matrices used in MLT study	1 (1 powder)
Repeatability (RSD_r)	1.6%
Reproducibility (RSD_R)	7.4%
Recovery (15 µg/kg spike)	99 – 104%
NIST SRM 1869 (Reference value 2.47±0.23 mg/kg)	2.71 mg/kg
Limit of Quantitation	0.30 µg/100 g RTF 0.58 µg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	No minimum level set

Fructans

CCNFSDU41 agreed to submit AOAC 2016.14 / ISO 22579 | IDF 241, “Determination of Fructans – High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) After Enzymatic Treatment,” to CCMAS41 for review, endorsement as Type II, and inclusion in CXS 234-1999.

AOAC 2016.14[7] / ISO 22579[8] | IDF 241 reflects the most up to date scientific method of analysis for fructans in infant formula and was fully validated in these products. The method uses a mixture of highly purified enzymes to hydrolyze alpha-glucans and sucrose to their monomeric components (glucose and fructose). After hydrolysis the mixture is passed through a solid phase extraction cartridge containing graphitized carbon. Polymeric and oligomeric saccharides are trapped on the cartridge while the monosaccharides are washed away with water. The oligosaccharides are then eluted in a solution containing 25% acetonitrile. The eluted oligosaccharides are treated with a mixture of exo- and endo-inulinases, which convert the fructans to their monomeric components (glucose and fructose). The released monosaccharides are then quantified chromatographically using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The determined amount of fructose is multiplied by 0.9 to compensate for water uptake during hydrolysis. This is then summed with the determined amount of glucose to calculate the total fructan. (Note, since a typical fructan chain contains multiple fructose units and a single glucose unit, the combination of glucose + 0.9 × fructose results in the appropriate correction for water uptake. However, for fructans that do not have a terminal glucose, the method will underestimate the fructan content slightly depending on the chain length (e.g., 3.6% underestimation for a trisaccharide, 1.1% underestimation for a decasaccharide.)

The performance of AOAC 2016.14 / ISO 22579 | IDF 241 was evaluated extensively by both SLV and MLT studies. A sample set of 19 matrices from the SPIFAN II Kit (plus 2 additional fructan-containing samples) was used during the SLV. The 2 additional samples were added because the SPIFAN II Kit contained only 6 matrices containing fructans. For the MLT, a sample set of 8 matrices was designed specifically for the study (including a selection from the SPIFAN II kit plus additional samples). Both kits encompassed a range of infant formula, follow-up formula, and adult nutritionals covering a wide nutrient fortification range (i.e., 0.3 g/100 g – 2.7 g/100 g). 12 laboratories from 9 countries participated in the MLT study and produced data with excellent precision and accuracy for all samples in the Fructans MLT Kit.

The SLV and the MLT data provide systematic scientific evidence for a simple, selective, accurate, and precise method for the purpose of dispute resolution for the determination of fructans in all forms of infant, adult, and pediatric formulas.

There are no current Type II methods for the determination of fructans in infant formula. In addition, fructans/FOS is not included in CXS 72-1981 or CAC/GL 10-1979. However, infant formulas containing FOS are being traded globally so there is the possibility of regulatory disputes regarding the level of FOS in a particular product. Existing methods for fructans referenced in CXS 234-1999 (i.e., AOAC 997.08 and 999.03) have not been validated for application to infant formula or adult nutritionals. AOAC 997.08 is not optimal because the error in determination of free sugars in the matrix (a necessary part of the method) may be higher than the amount of fructan present and as such reliable results cannot be obtained. AOAC 999.03 is not suitable for the determination of short chain fructans (FOS) because of the conversion of terminal fructose residues of the FOS to sugar alcohols before measurement. That conversion results in a significant underestimation (~25%) of fructan when FOS is used in the product. To address potential regulatory disputes and because fiber is already referenced in CXS 234-1999, it is appropriate to propose a Type II method for the determination of fructans in infant formula. Following are the performance parameters demonstrating why AOAC 2016.14 / ISO 22579 | IDF 241 should become a Type II method.

Parameters	**AOAC 2016.14 / ISO 22579 | IDF 241**
Infant/adult/placebo formula matrices used in MLT study	8 (3 liquid, 5 powder)

[7] Spichtig et al. Determination of Fructans in Infant Formula and Adult/Pediatric Nutritional Formula by Anion-Exchange Chromatography with Pulsed Amperoetric Detection after Enzymatic Treatment: Collaborative Study, Final Action 2016.14. *Journal of AOAC INTERNATIONAL,* Volume 103, Issue 5, September 2020, Pages 1301-1317. https://doi.org/10.1093/jaoacint/qsaa064

[8] ISO 22579 | IDF 241:2020 - Infant formula and adult nutritionals — Determination of fructans — High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) after enzymatic treatment, www.iso.org/standard/73491.html or https://store.fil-idf.org/product/iso22579-i-idf-241-infant-formula-and-adult-nutritionals-determination-of-fructans-high-performance-anion-exchange-chromatography-with-pulsed-amperometric-detection-hpaec-pad-aft/

Analyte	Fructans
Repeatability (RSD_r)	2.27 – 7.65%
Reproducibility (RSD_R)	5.90 – 15.1%
Recovery (% spike recovery during SLV at multiple levels)	86 – 119% (80 – 104% when extreme concentrations* are excluded)
NIST SRM 1869 (no certified value)	0.204 g/100 g
Limit of Quantitation	0.018 g/100 g RTF 0.03 g/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	No minimum level set

*During SLV, the highest/lowest recoveries were obtained on only a few samples at the extremes of the spiking range (0.03 g/100 g and 5.0 g/100 g).

Biotin

CCNFSDU41 agreed to submit ISO 23305, “Determination of Total Biotin by Liquid Chromatography Coupled with Immunoaffinity Column Clean-Up Extraction,” to CCMAS41 for review, endorsement as Type II, and inclusion in CXS 234-1999. AOAC 2016.02 has already been adopted by the Codex Alimentarius Commission as Type II for the determination of biotin in infant formula and this method is included in CXS 234-1999. CCMAS40 endorsed ISO 23305[9] as Type II pending review and agreement by CCNFSDU.

Vitamin K

CCNFSDU41 agreed to inform CCMAS to replace AOAC 999.15 / EN 14148 for vitamin K with AOAC 2015.09 / ISO 21446 as Type II for the determination of vitamin K in follow-up formula. AOAC/ISO/IDF agree with this recommendation. AOAC 2015.09 / ISO 21446 reflects the most recent scientific method of analysis for vitamin K in follow-up formula and was validated in relevant products.

[9] ISO 23305:2020. Fortified milk powders, infant formula and adult nutritionals – Determination of total biotin by liquid chromatography coupled with immunoaffinity column clean-up extraction. https://www.iso.org/standard/75198.html

Appendix 1. Recommended Methods of Analysis and Sampling (CODEX STAN 234-1999)

Commodity	Provision	Method	Principle	Proposed Type
Infant Formula	Thiamin	**AOAC 2015.14 / ISO 21470**	**Enzymatic digestion and LC-MS/MS**	**II**
		EN 14122	HPLC with pre- or post-column derivatization to thiochrom	~~II~~ **III**
		AOAC 986.27	Fluorimetry	III
	Riboflavin	**AOAC 2015.14 / ISO 21470**	**Enzymatic digestion and LC-MS/MS**	**II**
		EN 14152	HPLC	~~II~~ **III**
		AOAC 985.31	Fluorimetry	III
	Niacin	**AOAC 2015.14 / ISO 21470**	**Enzymatic digestion and LC-MS/MS**	**II**
		EN 15652	HPLC	~~II~~ **III**
		AOAC 985.34	Microbioassay and turbidimetry	III
	Pyridoxine	**AOAC 2015.14 / ISO 21470**	**Enzymatic digestion and LC-MS/MS**	**II**
		AOAC 2004.07 / EN 14164	HPLC	~~II~~ **III**
		AOAC 985.32	Microbioassay	III
		EN 14166	Microbioassay	III
	Choline	**AOAC 2015.10 / ISO 21468**	**LC-MS/MS**	**II**
		AOAC 999.14	Enzymatic Colorimetric Method with limitations on applicability due to choline and ascorbate concentration	~~II~~ **III**
	Carnitine	**AOAC 2015.10 / ISO 21468**	**LC-MS/MS**	**II**
	Fructans	**AOAC 2016.14 / ISO 22579 | IDF 241**	**Enzymatic digestion with HPAEC-PAD**	**II**
	Beta Carotene	**AOAC 2016.13 / ISO 23443**	**UHPLC-UV**	**II**
	Lycopene	**AOAC 2016.13 / ISO 23443**	**UHPLC-UV**	**II**
	Biotin	AOAC 2016.02 **/ ISO 23305**	HPLC-UV	II
		EN 15607	HPLC-fluorescence	III
Follow-Up Formula	Vitamin K	**AOAC 2015.09 / ISO 21446**	**HPLC**	**II**
		AOAC 999.15 / EN 14148	HPLC with C30 column to separate the cis- and the trans- K vitamins	~~II~~ **III**