CODEX ALIMENTARIUS COMMISSION

Food and Agriculture Organization of the United Nations

World Health Organization

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Agenda Item 3 **CRD06**

ORIGINAL LANGUAGE ONLY

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING

42nd Session
Budapest, Hungary

13 – 16 June 2023
with report adoption on 20 June 2023 (virtual)

SUBMISSION OF METHOD FOR ENDORSEMENT OF METHODS OF ANALYSIS AND SAMPLING

(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 42nd Session of the Codex Committee on Methods of Analysis and Sampling (CCMAS42).

Recommendations to CCMAS42

AOAC/ISO/IDF recommends CCMAS42 to take the following actions:

1. Endorse AOAC 2018.06 / ISO 4214 | IDF 254 / AACC 07-50.01 as Type II for the determination of Total Amino Acids (minus Taurine and Tryptophan) in Infant Formula and Adult/Pediatric Nutritional Formula.
2. Endorse AOAC 2017.03 as Type II for the determination of Total Tryptophan in Infant Formula and Adult/Pediatric Nutritional Formula.
3. Endorse AOAC 2014.02 as Type III for the determination of Vitamin B12 (Cyanocobalamin) in Infant Formula and Adult/Pediatric Nutritional Formula.

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

Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU43)

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

Total Amino Acids (minus Taurine and Tryptophan)

CCNFSDU43 agreed to submit AOAC 2018.06 / ISO 4214 | IDF 254 / AACC 07-50.01 as Type II for the determination of Total Amino Acids (minus Taurine and Tryptophan) in Infant Formula and Adult/Pediatric Nutritional Formula to CCMAS42 for review, endorsement as Type II, and inclusion in CXS 234-1999.

The provision for Protein in the Codex standard for infant formula and formula for special medical purposes intended for infants (CXS 72-1981, Section 3.1.3a, footnotes 3 and 4) states that for an equal energy value the formula must contain an available quantity of each essential and semi-essential amino acid at least equal to that contained in the reference protein of breast milk as defined in Annex I of the standard.

AOAC 2018.06[1,2] / ISO 4214 | IDF 254[3] / AACC 07-50.01[4] specifies a method for the determination in one single analysis of the following amino acids: alanine, arginine, aspartic acid (combined with asparagine), cystine (dimer of cysteine, combined with cysteine), glutamic acid (combined with glutamine), glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. This method does not apply to the determination of tryptophan and taurine.

The method hydrolyses proteins in 6 mol/l hydrochloric acid (HCl) for 24 h at 110 °C in the presence of phenol, 3-3′-dithiodipropionic acid (DDP) and norvaline. Phenol is added to prevent halogenation of tyrosine. Norvaline is added as an internal standard. DDP is added to convert cystine and cysteine to S-2-carboxyethylthiocysteine (XCys), and the resulting derivative can be separated from other amino acids for quantification.

After neutralization, amino acids and converted XCys are derivatized with 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (AQC). Derivatized amino acids are separated using reversed-phase ultra-high-performance liquid chromatography (UHPLC) with UV detection at 260 nm.

During acid hydrolysis, glutamine (Gln) and asparagine (Asn) are converted to glutamic acid (Glu) and aspartic acid (Asp), respectively. Thus, Glu values represent the combined values of Glu and Gln, and Asp values represent the combined values of Asp and Asn. Cys2 values represent the combined values of cysteine and cystine since both are converted to XCys by DDP.

This method is applicable to infant and adult/pediatric nutritional formulas, dairy products and other matrices such as cereals. It was validated in 2022.

The performance of AOAC 2018.06 / ISO 4214 | IDF 254 / AACC 07-50.01 was evaluated extensively by both Single Lab Validation (SLV) and Multi-Laboratory Testing (MLT) studies that encompassed a broad range of matrices, including infant formulas (milk- and soy-based, including partially hydrolyzed and elemental products), toddler formula, adult nutritional powder, UHT skimmed milk, whey powder, sodium caseinate, whole milk powder, bran pet food, dry pet food and breakfast cereal. Fifteen laboratories from six countries participated in the MLT study.

Method performance data in Infant Formula and Adult/Pediatric Nutritional Formula

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 alanine, arginine, aspartic acid (combined with asparagine), cystine (dimer of cysteine, combined with cysteine), glutamic acid (combined with glutamine), glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine in all forms of infant, adult, and pediatric formulas.

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[1] Jaudzems G, Guthrie J, Lahrichi S, Fuerer C. Total Amino Acids by UHPLC-UV in Infant Formulas and Adult Nutritionals, First Action 2018.06. J AOAC Int. 2019;102(5):1574-1588. https://doi.org/10.1093/jaoac/102.5.1574

[2] Jaudzems G, Fuerer C. Determination of Total Amino Acids in Infant Formulas, Adult Nutritionals, Dairy, and Cereal Matrixes by UHPLC-UV: Interlaboratory Validation Study, Final Action 2018.06. J AOAC Int. 2022;105(6):1625-1639. https://doi.org/10.1093/jaoacint/qsac083

[3] ISO 4214:2022 | IDF 254 Milk and milk products — Determination of amino acids in infant and adult/paediatric nutritional formulas and dairy products. https://www.iso.org/standard/79803.html

[4] AACC 07-50.01 Total Amino Acids by UHPLC-UV. https://www.cerealsgrains.org/resources/Methods/Methods/07-50.pdf

	RSD_r %	RSD_R %	Rec. %	NIST1 Ref. value	NIST1 Meas. value	Method LOQ mg/100 g RTF	Codex STAN-72 mg/100 g RTF*	Method LOQ mg/100 kcal	Codex STAN-72 mg/100 kcal
Alanine	1.6 [1.1-2.3]	5.2 [4.2-8.9]	98 [90-109]	539.0	539.7 (100%)	4.1	-	6.8	-
Arginine	2.7 [1.8-4.5]	5.1 [3.8-8.2]	99 [94-110]	571.0	565.3 (99%)	7.9	-	13.2	-
Aspartic acid	2.4 [0.9-5.0]	4.8 [3.8-6.9]	96 [80-109]	1300.0	1288.1 (99%)	6.1	-	10.1	-
Cysteine & Cystine	3.4 [1.3-5.8]	11.6 [6.5-15.5]	95 [90-104]	149.0	163.1 (109%)	10.9	22.8	18.2	38
Glutamic acid	1.3 [0.7-1.9]	3.5 [2.7-4.4]	100 [94-107]	2936.0	3030.6 (103%)	6.7	-	11.1	-
Glycine	2.0 [1.3-3.3]	5.7 [4.1-8.2]	107 [101-114]	325.1	325.9 (100%)	3.4	-	5.7	-
Histidine	2.1 [0.7-6.0]	6.4 [4.0-9.2]	103 [94-110]	365.1	362.9 (99%)	7.1	24.6	11.8	41
Isoleucine	1.6 [0.7-2.8]	4.0 [2.9-5.4]	97 [90-105]	778.0	774.1 (100%)	6.0	55.2	9.9	92
Leucine	1.4 [0.5-3.0]	3.1 [2.4-4.1]	101 [96-108]	1394.0	1376.7 (99%)	6.0	101.4	9.9	169
Lysine	2.5 [1.4-4.4]	5.4 [3.7-9.4]	99 [94-106]	1184.0	1182.9 (100%)	6.6	68.4	11.1	114
Methionine	2.1 [1.0-4.1]	5.1 [4.2-7.3]	103 [97-113]	474.0	464.1 (98%)	6.8	14.4	11.3	24
Phenylalanine	2.6 [1.3-3.7]	4.2 [2.8-5.7]	97 [93-102]	682.3	661.2 (97%)	7.5	48.6	12.5	81
Proline	1.5 [0.9-2.3]	3.3 [2.1-4.6]	100 [94-103]	1260.0	1226.1 (97%)	5.2	-	8.7	-
Serine	1.8 [1.2-2.6]	5.8 [5.2-6.5]	93 [88-102]	805.0	806 (100%)	4.8	-	8.0	-
Threonine	1.4 [0.7-2.6]	4.4 [3.9-5.2]	98 [85-108]	696.0	692 (99%)	5.4	46.2	9.0	77
Tyrosine	2.6 [0.9-4.4]	5.1 [4.3-6.2]	98 [87-108]	610.0	654.9 (107%)	8.2	45.0	13.7	75
Valine	1.4 [0.6-3.0]	4.2 [3.2-5.3]	99 [91-108]	861.0	863.4 (100%)	5.3	54.0	8.9	90

"RSD_r %" and "RSD_R %", the relative standard deviation of repeatability and reproducibility are expressed as the average and range (square brackets) of the values measured in nine samples during the MLT

"Rec. %", the recovery expressed as the average and range (square brackets) of the values measured by spiking experiments in nineteen samples during the SLV

"NIST1 Ref. value" and "NIST1 Meas. Value", the reference values listed in the certificate of analysis of NIST SRM 1869 (Infant/Adult Nutritional Formula II) and the average values measured during the MLT (values relative to the reference values are shown in brackets)

"Method LOQ" and "Codex STAN-72", the LOQ values are based on the lowest point of the calibration curve and are compared to the requirements of Codex STAN 72-1981 (the minimum level for infant formula is based on a minimum energy level of 60 kcal/100g RTF). All values are in mg/100 g or mg/100 kcal Ready-to-Feed (RTF). The LOQs can be lowered by adding supplementary levels in the calibration curve

Method performance data including dairy, and cereal matrices

The MLT data were extended beyond infant and adult/pediatric nutritional formulas to include dairy (UHT skimmed milk, whey powder, sodium caseinate, whole milk powder,) and cereal (bran pet food, dry pet food and breakfast cereal) matrices. Collectively, these data provide systematic, scientific evidence that the method is fit-for-purpose for the determination of alanine, arginine, aspartic acid (combined with asparagine), cystine (dimer of cysteine, combined with cysteine), glutamic acid (combined with glutamine), glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine in all the matrices listed above.

RSDr and RSDR values are comparable to those listed in the previous table. Spiking recovery experiments were not performed on dairy and cereal matrices. Method accuracy was established by comparison with NIST standard reference materials. LOQ values are calculated from the calibration curve and are expressed per 100g of cereal powder sample.

	RSD_r %	RSD_R %	NIST2 Ref. value	NIST2 Meas. value	NIST3 Ref. value	NIST3 Meas. value	Method LOQ mg/100g
Alanine	1.8 [1.1-2.6]	5.3 [4.0-8.9]	845.0	816.7 (97%)	317.5	271.5 (86%)	8.9
Arginine	3.0 [1.8-7.4]	5.8 [3.8-8.5]	890.0	850.7 (96%)	316.5	254.2 (80%)	17.4
Aspartic acid	2.4 [0.9-5.0]	5.5 [3.8-8.9]	1960.0	1950 (99%)	430.6	374.4 (87%)	13.3
Cysteine & Cystine	3.1 [1.3-5.8]	9.8 [3.8-15.5]	180.0	199.8 (111%)	151.4	125.6 (83%)	24.0
Glutamic acid	1.8 [0.7-3.7]	4.5 [2.7-9.1]	5340.0	5389.8 (101%)	2211.8	2010.7 (91%)	14.7
Glycine	2.4 [1.0-5.7]	5.6 [3.0-8.2]	460.0	471.3 (102%)	336.2	313 (93%)	7.5
Histidine	2.3 [0.7-6.0]	6.1 [3.8-9.2]	617.0	668.5 (108%)	159.2	147.8 (93%)	15.5
Isoleucine	1.9 [0.7-3.1]	4.8 [2.8-7.9]	1120.0	1296 (116%)	265.4	254.1 (96%)	13.1
Leucine	1.5 [0.5-3.0]	3.4 [2.4-4.5]	2410.0	2424.4 (101%)	540.7	484.8 (90%)	13.1
Lysine	3.1 [1.4-5.5]	6.0 [3.7-9.6]	2050.0	1953 (95%)	101.2	99.5 (98%)	14.6
Methionine	2.8 [1-5.9.0]	7.1 [4.2-19.1]	680.0	643.3 (95%)	136.6	101.9 (75%)	14.9
Phenylalanine	2.6 [1.2-4.0]	4.7 [2.8-8.0]	1210.0	1212.2 (100%)	366.7	323 (88%)	16.5
Proline	1.7 [0.8-3.1]	3.6 [2.1-5.3]	n/a	2404.1 (n/a)	n/a	671.9 (n/a)	11.5
Serine	1.9 [1.2-2.6]	6.3 [3.5-9.2]	1420.0	1400.8 (99%)	368.6	335.9 (91%)	10.5
Threonine	1.5 [0.7-2.6]	4.3 [3.0-6.1]	1090.0	1099.2 (101%)	236.9	217.2 (92%)	11.9
Tyrosine	3.0 [0.8-7.5]	5.9 [3.5-12.6]	1120.0	1229.6 (110%)	227.1	192.2 (85%)	18.1
Valine	1.6 [0.6-3.0]	4.6 [3.2-5.7]	1340.0	1555.5 (116%)	337.2	325.7 (97%)	11.7

"RSD_r %" and "RSD_R %", the relative standard deviation of repeatability and reproducibility are expressed as the average and range (square brackets) of the values measured in sixteen samples during the MLT

"NIST2 Ref. value" and "NIST2 Meas. Value", the reference values listed in the certificate of analysis of NIST SRM 1549a (Whole Milk Powder) and the average values measured during the MLT (values relative to the reference values are shown in brackets)

"NIST3 Ref. value" and "NIST3 Meas. Value", the reference values listed in the certificate of analysis of NIST SRM 3233 (Fortified Breakfast Cereal, values were converted from dry-mass to total mass basis) and the average values measured during the MLT (values relative to the reference values are shown in brackets)

"Method LOQ", the LOQ values based on the lowest point of the calibration curve and expressed in mg/100 g cereal powder sample. The LOQs can be lowered by adding supplementary levels in the calibration curve

Tryptophan

CCNFSDU43 agreed to submit AOAC 2017.03[5] for the determination of Total Tryptophan in Infant Formula and Adult/Pediatric Nutritional Formula to CCMAS42 for review, endorsement as Type II and inclusion in CXS 234-1999.

There are currently no Type II methods listed for the determination of tryptophan in foods for special medical purposes, including infant formula. However, the Codex standard for infant formula and formulas for special medical purposes intended for infants (CXS-72-1981, Section 3.1.3a, footnotes 3 and 4) requires each essential and semi-essential amino acid at least equal to that contained in the reference protein (breast milk as defined in Annex I). Tryptophan is an essential amino acid and cannot be quantified with AOAC 2018.06 / ISO 4214 | IDF 254 / AACC 07-50.01 for the determination of Total Amino Acids.

Tryptophan is released (hydrolyzed) from intact protein using a combination of proteolytic enzymes. Following proteolysis, tryptophan is quantitated by reversed-phase isocratic high-performance liquid chromatography (HPLC) and fluorescence detection, which provides for a selective and specific determination of tryptophan in nutritional products.

Ten laboratories from seven countries participated in a MLT study to collect method performance data. Results of this validation are summarized in the below table.

Parameters – Tryptophan	**AOAC 2017.10**
Infant/adult/placebo formula matrices used in MLT study	14 (3 liquid, 11 powder)
Repeatability (RSD_r)	2.1% (Range: 0.9-3.6%)
Reproducibility (RSD_R)	4.2% (Range: 3.0-9.9%)
Recovery	93.8 – 104.9%
NIST SRM 1849a (18.4 mg/100g)	18.14 mg/100g
Limit of Quantitation	0.18 mg/100 g RTF 0.3 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	19.8 mg/100 g RTF 33 mg/100 kcal

Vitamin B_{12}

CCNFSDU43 agreed to submit AOAC 2014.02[6] for the determination of Vitamin B_{12} (Cyanocobalamin) in Infant Formula and Adult/Pediatric Nutritional Formula to CCMAS42 for review, endorsement as Type III and inclusion in CXS 234-1999.

The current Type II method for the determination of vitamin B_{12} in infant formula is AOAC 2011.10 / ISO 20634 using reversed phase HPLC. A type III method for the determination is AOAC 986.23, a turbidimetric method. To provide an alternative option for the existing Type II method, using a simplified analytical approach, AOAC 2014.02 status is proposed to be adopted as an additional Type III method. This method is validated on a similar set of matrices as AOAC 2011.10 / ISO 20634 and has similar performances. Sample extracts are purified and concentrated with an immunoaffinity column. Vitamin B_{12} is determined as cyanocobalamin by UHPLC or HPLC with UV detection at 361 nm. Separation is done on a C18 column using an acetonitrile gradient in water. The existing Type II method applies purified sample extracts on size-exclusion and reversed-phase chromatography using a column-switching valve which may not be available or remain unexperienced for some labs.

Ten laboratories from seven countries participated in a MLT study to collect method performance data for AOAC 2014.02. Results of this validation are summarized in the below table and compared with validation data of the current Type II method.

[5] Draher J. HPLC Determination of Total Tryptophan in Infant Formula and Adult/Pediatric Nutritional Formula Following Enzymatic Hydrolysis, Multilaboratory Testing Study: Final Action 2017.03. J AOAC Int. 2019;102(5):1567-1573. https://doi.org/10.1093/jaoac/102.5.1567

[6] Giménez EC, Martin F. Vitamin B12 (cyanocobalamin) in Infant Formula Adult/Pediatric Nutritional Formula by Liquid Chromatography with Ultraviolet Detection: Collaborative Study, Final Action 2014.02. J AOAC Int. 2018;101(4):1112-1118. https://doi.org/10.5740/jaoacint.17-0452

Parameter – vitamin B_{12}	**AOAC 2011.10 / ISO 20634 Codex Type II**	**AOAC 2014.02**
Infant/adult/placebo formula matrices used in MLT study	11 (2 liquid, 9 powder)	10 (2 liquid, 8 powder)
Repeatability (RSD_r)	3.0-9.8%	1.1-6.5%
Reproducibility (RSD_R)	3.5-19.5%	6.0-23.8%
Recovery	95.1-105%	89.8-98.3%
NIST SRM 1849a (Reference value: 48.2 ± 0.85 µg/Kg powder)	43.7 µg/Kg (powder)	40.5 µg/Kg (powder)
Limit of Quantitation	0.08 µg/100 g RTF	0.013 µg/100 g RTF
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.06 µg/100 g RTF 0.1 µg/100 kcal	

Recommendations to CCMAS42

AOAC/ISO/IDF recommends CCMAS42 to take the following actions:

1. Endorse AOAC 2018.06 / ISO 4214 | IDF 254 / AACC 07-50.01 as Type II for the determination of Total Amino Acids (minus Taurine and Tryptophan) in Infant Formulas and Adult Nutritionals.
2. Endorse AOAC 2017.03 as Type II for the determination of Total Tryptophan in Infant Formula and Adult/Pediatric Nutritional Formula.
3. Endorse AOAC 2014.02 as Type III for the determination of Vitamin B12 (Cyanocobalamin) in Infant Formula and Adult/Pediatric Nutritional Formula.

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

Commodity	**Provision**	**Method**	**Principle**	**Proposed Type**
Infant Formula	Total Amino Acids	**AOAC 2018.06 / ISO 4214 | IDF 254 / AACC 07-50.01**	UHPLC-UV	**II**
Infant Formula	Tryptophan	**AOAC 2017.03**	HPLC-FLD	**II**
Infant Formula	Vitamin B_{12}	**AOAC 2014.02**	HPLC-UV	**III**