CODEX ALIMENTARIUS COMMISSION

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World Health Organization

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Agenda Item 3.1 **CRD05**

ORIGINAL LANGUAGE

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CODEX COMMITTEE ON METHODS OF ANALYSIS SAMPLING

40th Session

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

Information and comments submitted by AOAC, ISO and IDF on infant formula and formulas for special medical purposes intended for infants

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 40th Session of the Codex Committee on Methods of Analysis and Sampling (CCMAS40).

Recommendations to CCMAS40

AOAC/ISO/IDF recommends CCMAS40 to take the following actions:

1. Endorse AOAC 2015.06 / ISO 21424 | IDF 243 as Type II for the determination of nine Minerals and Trace Elements (i.e., Calcium, Magnesium, Potassium, Phosphorus, Sodium, Copper, Iron, Manganese, Zinc) in infant formula and reclassify the following existing Type II methods as Type III:
    a. ISO 8070 | IDF 119 (Calcium, Magnesium, Potassium, Sodium)
    b. AOAC 999.11 / NMKL 139 (Iron)
    c. AOAC 986.24 (Phosphorus)
2. Endorse AOAC 2011.14 / ISO 15151 | IDF 229 as Type III for the determination of nine Minerals and Trace Elements (i.e., Calcium, Magnesium, Potassium, Phosphorus, Sodium, Copper, Iron, Manganese, Zinc) in infant formula.
3. Revoke AOAC 984.27 as a Type III method for the determination of nine Minerals and Trace Elements (i.e., Calcium, Magnesium, Potassium, Phosphorus, Sodium, Copper, Iron, Manganese, Zinc) in infant formula.
4. Endorse AOAC 2015.09 / ISO 21446 as Type II for the determination of Vitamin K_1 in infant formula.
5. Endorse AOAC 2011.06 as Type II for the determination of Folic Acid in infant formula, and reclassify AOAC 992.06 / EN 14131 as Type III.

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 (CCNFSDU40)

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

Minerals Methods

CCNFSDU40 agreed to submit AOAC 2015.06 / ISO 21424 | IDF 243, Minerals and Trace Elements in Milk, Milk Products, Infant Formula, and Adult/Pediatric Nutritional Formula, to CCMAS40 for technical review, typing, endorsement, and inclusion in the Recommended Methods of Analysis and Sampling (CODEX STAN 234-1999) in Part A, section "Foods for Special Dietary Uses," with the description "Infant Formula." CCNFSDU40 also asked CCMAS40 to re-type or revoke the related existing methods for these nine minerals and trace elements in CODEX STAN 234-1999.

AOAC 2015.06 / ISO 21424 | IDF 243 reflects the most recent scientific method of analysis for the nine major minerals and trace elements in infant formula (i.e., calcium, magnesium, phosphorus, potassium, sodium, copper, iron, manganese, zinc) and was fully validated in these products. AOAC Multi-Laboratory Testing (MLT) and reproducibility validation information for AOAC 2015.06 has been published.[1] This method has been published as AOAC Official Final Action, and has also been published as a joint International Standardization Organization (ISO) and International Dairy Federation Standard.[2,3]

AOAC 2015.06 / ISO 21424 | IDF 243, employs rapid closed vessel microwave digestion of samples in inert Teflon™ vessels with nitric acid and hydrogen peroxide to a final temperature of 200°C to completely digest fat and other organic material. An internal standard (germanium for these nine elements) is added into the microwave vessel before digestion (specifically not on-line, post digestion) to improve accuracy and robustness. An ICP-MS with a modern collision/reaction cell is employed to simultaneously determine all nine elements (chromium, molybdenum, and selenium are also determined at the same time; see AOAC 2011.19 / ISO 20649 | IDF 235).

The MLT study utilized 13 carefully selected infant, child and adult nutritional formulas covering a broad matrix range, five placebo formulas to which no mineral premixes had been added, one standard reference material (NIST SRM 1849a) as well as six representative milk products and milk ingredients. Blind duplicates of all samples were tested to reliably measure repeatability. Ten laboratories from seven countries, employing ICP-MS instruments from three major vendors and microwave ovens from five different vendors produced data with excellent precision and accuracy, as shown in the individual tables below. A separate publication was devoted specifically to characterization of this method at low analyte concentrations and a detailed evaluation of the limits of quantitation.[4] The analysis of placebos during the MLT provided direct evidence that those LOQs were met. In all cases, the method LOQs are sufficient to allow analysis of infant formula at the CODEX STAN 72 (1981) minimum levels. No current Type II method can make this claim.

Following is a review of each of the existing Type II methods for the nine minerals and trace elements, followed by rationale supporting AOAC 2015.06 / ISO 21424 | IDF 243 as the Type II and recommendations for re-typing/revoking related existing methods.

Calcium, Magnesium, Potassium and Sodium

The current Type II method for the determination of calcium, magnesium, potassium and sodium in infant formula is ISO 8070 | IDF 119. For this method, samples are either dry-ashed in silica crucibles at 550°C for six hours or digested in an open or closed vessel microwave system with nitric acid – the method allows for differences in final digestion temperature and time and the addition (or not) of hydrogen peroxide. Digested solutions are then diluted, or ashes reconstituted in nitric acid and diluted, lanthanum trichloride added to minimize interferences, and resulting samples are analyzed by flame atomic absorption. Although this is a long-standing and viable method for determination of these four elements, there are a few concerns in having it serve as the Type II dispute-resolution method for CODEX STAN 234-1999. Flame atomic absorption, by its nature, is a single element technique and so sample preparation cannot make use of internal standardization to reduce human error and improve robustness. The dry ash sample preparation is time consuming and cumbersome; possible low recoveries can occur due to physical or chemical interaction with the crucible surface or incomplete re-dissolution of the ash in the nitric acid. On the other hand, the microwave digestion procedure described in the method does not follow current manufacturers' guidance on heating to at least 190°C to completely break down organic material (especially fat), and the recommended addition of hydrogen peroxide is only an option in this method.

[1] *J. AOAC Int*. 2018 Mar 1; 101(2):536-561. DOI: http://doi.org/10.5740/jaoacint.17-0318
[2] ISO 21424 | IDF 243: 2018 https://www.iso.org/standard/70901.html
[3] ISO 21424 | IDF 243: 2018 https://store.fil-idf.org/product/iso-21424i-idf-243/
[4] *J. AOAC Int*. 2017; 100 (2):522-531

Further, flame AA is not as sensitive as other more modern instrumental techniques, which is especially problematic for ingredients and products with lower sodium concentrations. Finally, the collaborative study for this method did not contain any infant formulas – only whole and skim milk powders and a milk powder reference material.

The tables below show how some figures of merit for the current Type II method (ISO 8070 | IDF 119) compare to those for the recommended Type II method (AOAC 2015.06 / ISO 21424 | IDF 243). For the reasons cited above, the accuracy, repeatability, reproducibility, and limit of quantitation of the current Type II method are mostly inferior to the recommended method. [Note: RTF refers to ready-to-feed infant formula levels. SLV refers to Single Laboratory Validation.[5] MLT refers to Multi-Laboratory Testing studies (i.e., collaborative studies).]

Parameters - Calcium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ISO 8070 | IDF 119**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	8 milk products only
Repeatability (RSD_r)	1.8 %	3.0 %
Reproducibility (RSD_R)	4.1 %	6.8 % (one outlier omitted)
Recovery range from SLV/MLT	90.2 – 104 %	Not available
Mean NIST SRM 1849a result from 10 labs or Mean BCR 063 result from 13 labs	5279 mg/kg (certified 5253 ± 51)	12.1 g/kg (certified 13.49 ± 0.10)
Limit of Quantitation	0.53 mg/100g RTF 0.88 mg/100 kcal	Lowest level tested was 38 mg/100g and was the outlier (HorRat = 6.3)
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	30 mg/100g RTF 50 mg/100 kcal	

Parameters - Magnesium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ISO 8070 | IDF 119**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	8 milk products only
Repeatability (RSD_r)	1.5 %	3.3 %
Reproducibility (RSD_R)	3.5 %	4.6 % (one outlier omitted)
Recovery range from SLV/MLT	92.8 – 104 %	Not available
Mean NIST SRM 1849a result from 10 labs or Mean BCR 063 result from 13 labs	1626 mg/kg (certified 1648 ± 36	1.08 g/kg (certified 1.263 ± 0.024)
Limit of Quantitation	0.0049 mg/100g RTF 0.0082 mg/100 kcal	Lowest level tested was 5.4 mg/100g and was the outlier (HorRat = 1.8)
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	3.0 mg/100g RTF 5.0 mg/100 kcal	

[5] Gill, B.D.; Indyk, H.E.; Blake, C.J.; Konings, E.J.; Jacobs, W.A.; Sullivan, D. (2015) Evaluation protocol for the review of method validation data by the AOAC Stakeholder Panel for Infant Formula and Adult Nutritionals Expert Review Panel. *JAOAC* 98, 112-115. DOI: 10.5740/jaoacnt.14-158.
https://pdfs.semanticscholar.org/3099/504855165aa1bd61ce9240bad7ea7a0aea57.pdf

Parameters - Potassium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ISO 8070 | IDF 119**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	8 milk products only
Repeatability (RSD_r)	1.4 %	3.6% (one outlier omitted)
Reproducibility (RSD_R)	3.6 %	5.5 % (one outlier omitted)
Recovery range from SLV/MLT	92.4 – 110.2 %	Not available
Mean NIST SRM 1849a result from 10 labs or Mean BCR 063 result from 13 labs	9365 mg/kg (certified 9220 ± 110)	17.0 g/kg (certified 17.68 ± 0.19)
Limit of Quantitation	0.56 mg/100g RTF 0.92 mg/100 kcal	Lowest level tested was 2.6 mg/100g and was the outlier (HorRat = 8.7)
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	36 mg/100g RTF 60 mg/100 kcal	

Parameters - Sodium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ISO 8070 | IDF 119**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	8 milk products only
Repeatability (RSD_r)	1.5 %	4.6 % (one outlier omitted)
Reproducibility (RSD_R)	3.6 %	6.8 % (one outlier omitted)
Recovery range from SLV/MLT	99.8 – 106 %	Not available
Mean NIST SRM 1849a result from 10 labs or Mean BCR 063 result from 13 labs	4263 mg/kg (certified 4265 ± 83)	4.34 g/kg (certified 4.37 ± 0.031)
Limit of Quantitation	0.11 mg/100g RTF 0.18 mg/100 kcal	Lowest level tested was 4.0 mg/100g and was the outlier (HorRat = 12)
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	12 mg/100g RTF 20 mg/100 kcal	

Phosphorus

The current Type II method for the determination of phosphorus in infant formula is AOAC 986.24. For this method, samples are dry ashed at 600°C for 3-4 hours followed by resolubilization in HCl/HNO_3, complexation with molybdovanadate reagent and analysis by spectrophotometry at 400 nm. Because phosphorus cannot be determined by atomic absorption, this method has been one of the few choices for laboratories to test this element until the advent of ICP techniques. However, internal standardization cannot be used and dry ashing is likely to be less robust than wet digestion. For the reasons discussed above for ISO 8070 | IDF 119, this method demonstrates inferior reproducibility compared to the proposed Type II method AOAC 2015.06 / ISO 21424 | IDF 243. Also, the limit of quantitation is unknown and the collaborative study was conducted using a limited number of infant formula matrices. See the table below.

Parameters - Phosphorus	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 986.24**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	7
Repeatability (RSD_r)	1.9 %	3.0, 2.4%
Reproducibility (RSD_R)	4.3 %	5.4 % (one outlier omitted)
Recovery range from SLV/MLT	98.1 – 109 %	93 – 108 %
Mean NIST SRM 1849a result from 10 labs	4015 mg/kg (certified 3990 ± 140)	No SRM data available
Limit of Quantitation	0.13 mg/100g RTF 0.22 mg/100 kcal	Not available, lowest level tested was 45 mg/100g
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	15 mg/100g RTF 25 mg/100 kcal	

Copper, Manganese and Zinc

The current Type II method for the determination of copper, manganese and zinc in infant formula is AOAC 985.35. For this method, similar to ISO 8070 | IDF 119 for calcium, sodium, potassium and magnesium, samples are prepared by dry ashing in a muffle furnace followed by determination by flame atomic absorption spectrophotometry. Dry ashing is of greater concern for the trace elements vs. the alkali metals because crucible adsorption is more likely and insoluble oxide formation with transition metals can lead to poor recovery. The other concern for this method is the relative lack of sensitivity for copper and manganese, which may be present at very low levels in infant formula. The collaborative study data, summarized in the following tables, confirms that recovery (accuracy) data for AOAC 985.35 is inferior to the recommended Type II method and has higher repeatability and reproducibility.

Parameters - Copper	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 985.35**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	7
Repeatability (RSD_r)	2.3 %	4.8, 7.0 %
Reproducibility (RSD_R)	6.3 %	9.7%
Recovery range from SLV/MLT	91.3 – 106 %	68 – 123 %
Mean NIST SRM 1849a result from 10 labs	19.60 mg/kg (certified 19.78 ± 0.26)	No SRM data available
Limit of Quantitation	0.00048 mg/100g RTF 0.00080 mg/100 kcal	Not available, lowest level tested was 0.056 mg/100g
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.021 mg/100g RTF 0.035 mg/100 kcal	

Parameters - Manganese	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 985.35**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	7
Repeatability (RSD_r)	2.1 %	4.8, 11.0 %
Reproducibility (RSD_R)	3.8 %	10.6 %
Recovery range from SLV/MLT	93.5 – 101 %	86 – 119 %
Mean NIST SRM 1849a result from 10 labs	48.71 mg/kg (certified 49.59 ± 0.97)	No SRM data available
Limit of Quantitation	0.0006 mg/100g RTF 0.0010 mg/100 kcal	Not available, lowest level tested was 0.030 mg/100g
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.0006 mg/100g RTF 0.0010 mg/100 kcal	

Parameters - Zinc	AOAC 2015.06 / ISO 21424 | IDF 243	AOAC 985.35
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	7
Repeatability (RSD_r)	1.6 %	2.0, 6.7 %
Reproducibility (RSD_R)	4.3%	7.3 %
Recovery range from SLV/MLT	93.0 – 108 %	90 – 122 %
Mean NIST SRM 1849a result from 10 labs	151.8 mg/kg (certified 151.0 ± 5.6)	No SRM data available
Limit of Quantitation	0.056 mg/100g RTF 0.093 mg/100 kcal	Not available, lowest level tested was 0.064 mg/100g
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.30 mg/100g RTF 0.50 mg/100 kcal	

Iron

The current Type II method for the determination of iron in infant formula is AOAC 999.11 | NMKL 139. For this method, samples are dry ashed slowly to a final temperature of 450°C and then dissolved in nitric acid and iron determined by flame atomic absorption spectrophotometry. This is similar to the other current Type II methods described above. As shown in the table below, repeatability and reproducibility are relatively poor for the current Type II method, and accuracy was not demonstrated, which is critical for a dry-ash method. Additionally, the collaborative study for the existing Type II method did not include any infant formulas, even though the testing of iron in infant formulas is undoubtedly an important consideration.

Parameters - Iron	AOAC 2015.06 / ISO 21424 | IDF 243	AOAC 999.11 | NMKL 139
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	1 milk powder + 6 other foods
Repeatability (RSD_r)	2.9%	10%
Reproducibility (RSD_R)	6.1%	12%
Recovery range from SLV/MLT	92.8 – 101 %	Not available
Mean NIST SRM 1849a result from 10 labs	172.7 mg/kg (certified 175.6 ± 2.9)	Agreement of two total diet CRMs was “generally very good” but not tabulated
Limit of Quantitation	0.0073 mg/100g RTF 0.012 mg/100 kcal	0.08 mg/100g
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.27 mg/100g RTF 0.45 mg/100 kcal	

AOAC 2011.14 / ISO 15151 | IDF 229

AOAC 2011.14 / ISO 15151 | IDF 229 is another recently validated, collaboratively studied and published method for mineral analysis. This method uses microwave digestion of the sample followed by determination of elemental concentrations by ICP-AES detection.[6,7,8] It is a "sister" technique to AOAC 2015.06 / ISO 21424 | IDF 243 in that many laboratories employ one or the other (or both) of these kinds of techniques for routine elemental analysis. For this reason, the AOAC SPIFAN stakeholders wanted to see comparative data on infant formula. The MLTs for both methods were of similar size and scope, employing different laboratories but the same 25 sample matrices, including placebos. The table below shows the excellent agreement of the overall results (averaged over all the matrices, with most individual means falling within 2-3% of each other).

Bias of ICP-AES/ICP-MS: ratio of respective MLT means for each element:

Element	Bias (%)
Calcium (Ca)	99.5
Copper (Cu)	101.7
Iron (Fe)	98.4
Magnesium (Mg)	97.3
Manganese (Mn)	98.9
Phosphorus (P)	99.1
Potassium (K)	98.3
Sodium (Na)	99.2
Zinc (Zn)	101.4

The figures of merit for AOAC 2015.06 (ICP-MS) and 2011.14 (ICP-AES) are compared in Appendix 1. Note, although both methods generate equivalent results on the average over many samples and generally meet CODEX STAN 72-1981 requirements, AOAC 2015.06 / ISO 21424 | IDF 243 has distinctly better repeatability and reproducibility statistics for all elements and particularly for the trace elements, due to the inherent sensitivity of the ICP-MS platform. This makes it the clear choice to be the Type II dispute resolution method and AOAC, ISO, and IDF all support this endorsement.

Minerals Methods Summary

Together, all these data provide systematic scientific evidence that AOAC 2015.06 / ISO 21424 | IDF 243 offers a simple, selective, accurate and precise method for the purposes of dispute resolution for the determination of the nine major minerals and trace elements in all forms of infant, adult, and/or pediatric formula.

The existing Type II methods for calcium, magnesium, phosphorus, potassium, sodium, copper, iron, manganese, zinc all rely on a dry ash sample preparation, which is inferior to the rapid, complete and unbiased sample digestions that can be realized with modern microwave ovens. The LOQs demonstrated by the ICP-MS method at the typical 1 g to 50 mL dilution are better than those for the current Type II methods, even after accounting for the large pre-concentration of the sample that occurs when large sample sizes are dry ashed and reconstituted to a relatively small final volume. None of the existing Type II methods can employ internal standardization because their mode of determination (flame atomic absorption or spectrophotometry) is not multielemental. In general, the ICP-AES platform (AOAC 2011.14 / ISO 15151 | IDF 229) is also preferred to the existing Type II methods for these nine elements; even though the LOQs will be roughly similar, the power of internal standardization to add robustness is an overriding consideration. Note that the existing Type III ICP-AES method in CODEX STAN 234-1999, AOAC 984.27, does not mention internal standardization and also mandates using the outdated and potentially dangerous technique of digestion in perchloric acid, which exposes the analyst to unnecessary risk. Thus, we recommend removal of this Type III method in preference to its modern version, AOAC 2011.14 / ISO 15151 | IDF 229.

AOAC/ISO/IDF recommends CCMAS40 to endorse AOAC 2015.06 / ISO 21424 | IDF 243 as Type II. AOAC/ISO/IDF also recommends CCMAS40 to endorse AOAC 2011.14 / ISO 15151 | IDF 229 as Type III. In addition, we recommend revoking AOAC 984.27 as a Type III method for all nine minerals and trace elements, and re-typing all other existing methods for the nine minerals and trace elements as indicated in the below table.

[6] In publication
[7] ISO 15151 | IDF 229: 2018 https://www.iso.org/standard/70900.html
[8] ISO 15151 | IDF 229: 2018 https://store.fil-idf.org/product/iso-15151-i-idf-229/

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

Commodity	Provision	Method	Principle	Proposed Type
Infant Formula	Calcium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ICP mass spectrometry**	**II**
		AOAC 2011.14 / ISO 15151 | IDF 229	**ICP emission spectroscopy**	**III**
		ISO 8070 | IDF 119	Flame atomic absorption spectrophotometry	~~II~~ **III**
		AOAC 985.35	Flame atomic absorption spectrometry	III
		~~AOAC 984.27~~	~~ICP emission spectroscopy~~	~~III~~
	Copper	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ICP mass spectrometry**	**II**
		AOAC 2011.14 / ISO 15151 | IDF 229	**ICP emission spectroscopy**	**III**
		AOAC 985.35	Flame atomic absorption spectrophotometry	~~II~~ **III**
		~~AOAC 984.27~~	~~ICP emission spectroscopy~~	~~III~~
	Iron	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ICP mass spectrometry**	**II**
		AOAC 2011.14 / ISO 15151 | IDF 229	**ICP emission spectroscopy**	**III**
		AOAC 985.35	Flame atomic absorption spectrometry	III
		~~AOAC 984.27~~	~~ICP emission spectroscopy~~	~~III~~
		AOAC 999.11 | NMKL 139	AAS after dry ashing	~~II~~ **III**
	Magnesium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ICP mass spectrometry**	**II**
		AOAC 2011.14 / ISO 15151 | IDF 229	**ICP emission spectroscopy**	**III**
		ISO 8070 | IDF 119	Flame atomic absorption spectrophotometry	~~II~~ **III**
		AOAC 985.35	Flame atomic absorption spectrometry	III
		~~AOAC 984.27~~	~~ICP emission spectroscopy~~	~~III~~
	Manganese	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ICP mass spectrometry**	**II**
		AOAC 2011.14 / ISO 15151 | IDF 229	**ICP emission spectroscopy**	**III**
		AOAC 985.35	Flame atomic absorption spectrometry	~~II~~ **III**
		~~AOAC 984.27~~	~~ICP emission spectroscopy~~	~~III~~

Commodity	Provision	Method	Principle	Proposed Type
	Phosphorus	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ICP mass spectrometry**	**II**
		AOAC 2011.14 / ISO 15151 | IDF 229	**ICP emission spectroscopy**	**III**
		~~AOAC 984.27~~	~~ICP emission spectroscopy~~	~~III~~
		AOAC 986.24	Spectrophotometry (molybdovanadate)	~~II~~ **III**
	Potassium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ICP mass spectrometry**	**II**
		AOAC 2011.14 / ISO 15151 | IDF 229	**ICP emission spectroscopy**	**III**
		ISO 8070 | IDF 119	Flame atomic absorption spectrophotometry	~~II~~ **III**
		~~AOAC 984.27~~	~~ICP emission spectroscopy~~	~~III~~
	Sodium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ICP mass spectrometry**	**II**
		AOAC 2011.14 / ISO 15151 | IDF 229	**ICP emission spectroscopy**	**III**
		ISO 8070 | IDF 119	Flame atomic absorption spectrophotometry	~~II~~ III
		~~AOAC 984.27~~	~~ICP emission spectroscopy~~	~~III~~
	Zinc	**AOAC 2015.06 / ISO 21424 | IDF 243**	**ICP mass spectrometry**	**II**
		AOAC 2011.14 / ISO 15151 | IDF 229	**ICP emission spectroscopy**	**III**
		AOAC 985.35	Flame atomic absorption spectrometry	~~II~~ **III**
		~~AOAC 984.27~~	~~ICP emission spectroscopy~~	~~III~~

Vitamin K Methods

CCNFSDU40 agreed to submit AOAC 2015.09 / ISO 21446, Determination of *trans* and Total Vitamin K_1 in infant, pediatric, and adult nutritionals by HPLC with post-column reduction and fluorescence detection, to CCMAS40 for technical review, typing, endorsement, and inclusion in the Recommended Methods of Analysis and Sampling (CODEX STAN 234-1999) in Part A, section "Foods for Special Dietary Uses," with the description "Infant Formula." In making this decision, CCNFSDU confirmed that the forms of the analytes determined by AOAC 2015.09 / ISO 21446 are consistent with those specified in relevant Codex texts (CXS 72-1981; CXG 10). CCNFSDU40 also asked CCMAS40 to re-type the related existing methods for vitamin K in CXS 234-1999.

AOAC 2015.09 / ISO 21446 reflects the most recent scientific method of analysis for vitamin K in infant formula and was fully validated in these products. AOAC SLV and MLT data for AOAC 2015.09 has been published.[9] This method has also been published as an ISO Standard.[10] This method demonstrates improved extraction efficiency of vitamin K_1 from more complex infant formula matrices using a less labor intensive sample preparation procedure, allows for separation of the biologically inactive cis isomer from the biologically active trans isomer, and allows for the determination of total (cis + trans) vitamin K_1, if required. For this method, vitamin K_1 is extracted from products with iso-octane after proteins are precipitated and lipids are released with methanol.

[9] *J. AOAC Int*. 2018 Jul 25. DOI: https://doi.org/10.5740/jaoacint.18-0155
[10] ISO 21446 https://www.iso.org/standard/70938.html

Prepared samples are injected onto a silica HPLC column where cis and trans vitamin K_1 are separated with an iso-octane-isopropanol mobile phase. Column eluant is mixed with a dilute ethanolic solution of zinc chloride, sodium acetate, and acetic acid before it passes through a zinc reactor column where cis and trans vitamin are chemically reduced. The resulting hydroquinones are then detected by fluorescence at an excitation wavelength of 245 nm and an emission wavelength of 440 nm.

The performance of AOAC 2015.09 / ISO 21446 was evaluated extensively by both SLV and MLT studies. Sample sets of 17 and 19 matrices (SPIFAN I and SPIFAN II Kits) used during both SLV and MLT method validation stages were developed specifically for this purpose and encompasses a broad range of infant and follow-on formula covering a wide nutrient fortification range. Eight laboratories from six 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 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 vitamin K in all forms of infant, adult, and pediatric formulas.

The performance parameters of the methods are summarized below. [Note: RTF refers to ready-to-feed infant formula levels. SLV refers to Single Laboratory Validation.[11] MLT refers to Multi-Laboratory Testing studies (i.e., collaborative studies).]

Parameters	**AOAC 2015.09 / ISO 21446**	
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + SRM 1849a	
Analyte	*trans* Vitamin K_1	Total Vitamin K_1
Repeatability (RSD_r)	SLV: 2.0% / MLT: 3.06%	MLT: 3.15%
Reproducibility (RSD_R)	6.36%	6.11%
Recovery (100 µg/kg)	SLV: Mean: 97.8% Range 91.9-106%	NAP
NIST SRM 1849a (Certified value 1060±170 µg/kg)	MLT: 1080 µg/kg	MLT: 1150 µg/kg
Limit of Quantitation	0.09 µg/100 g RTF 0.15 µg/100 kcal	
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	2.4 µg/100 g RTF 4 µg/100 kcal	

AOAC/ISO/IDF recommends CCMAS40 to endorse AOAC 2015.09 | ISO 21446 as Type II. There are no current Type II methods for the determination of vitamin K in infant formula, so there is no need to re-type existing methods.

Table 2. Recommended Methods of Analysis and Sampling (CXS 234-1999)

Commodity	**Provision**	**Method**	**Principle**	**Type**
Infant Formula	Vitamin K	**AOAC 2015.09 / ISO 21446**	**HPLC**	**II**

[11] Gill, B.D.; Indyk, H.E.; Blake, C.J.; Konings, E.J.; Jacobs, W.A.; Sullivan, D. (2015) Evaluation protocol for the review of method validation data by the AOAC Stakeholder Panel for Infant Formula and Adult Nutritionals Expert Review Panel. *JAOAC* 98, 112-115. DOI: 10.5740/jaoacnt.14-158.
https://pdfs.semanticscholar.org/3099/504855165aa1bd61ce9240bad7ea7a0aea57.pdf

Folic Acid Methods

CCNFSDU40 agreed to submit AOAC 2011.06, Total Folate (as folic acid) in Infant Formula and Adult Nutritionals by Trienzyme Extraction and LC-MS/MS Quantitation, to CCMAS40 for technical review, typing, endorsement, and inclusion in the Recommended Methods of Analysis and Sampling (CODEX STAN 234-1999) in Part A, section "Foods for Special Dietary Uses," with the description "Infant Formula." In making this decision, CCNFSDU confirmed that the forms of the analytes determined by AOAC 2011.06 are consistent with those specified in relevant Codex texts (CXS 72-1981; CXG 10). CCNFSDU40 also asked CCMAS40 to re-type the related existing methods for folic acid in CODEX STAN 234-1999.

AOAC 2011.06 reflects the most recent scientific method of analysis for folic acid in infant formula and was fully validated in these products. AOAC 2011.06 has been published as an Official Final Action Method[12] and is being published as an ISO Standard.

The current Codex Type II methods for the determination of total folate (as folic acid) in infant formula are AOAC 992.05 and EN 14131, Total Folate (Pteroylglutamic Acid) in Infant Formula. The AOAC method is not capable of estimating bound natural folates. EN 14131 has not been validated for infant formula matrices. Additionally, both methods are based on microbiological technique which is a lengthy and laborious and can easily be compromised by variable vitamer response. The microbiological assays are known to be non-specific and are very susceptible to matrix interferences as well have poor precision.

AOAC 2011.06 has extensive SLV and MLT data using 11 carefully selected infant and adult nutritional formulas covering the matrix range. 11 laboratories covering five countries produced data with excellent precision and accuracy. The SLV and the MLT data provide systematic scientific evidence for a simple, accurate and precise method for the purposes of dispute resolution for the determination of folic acid in all forms of infant, adult, and/or pediatric formula.

AOAC 2011.06 employs trienzyme extraction, solid phase extraction and liquid chromatography – tandem mass spectrometry to determine total folate as folic acid in infant formula. The performance parameters of the method are summarized below. [Note: RTF refers to ready-to-feed infant formula levels. SLV refers to Single Laboratory Validation.[13] MLT refers to Multi-Laboratory Testing studies (i.e., collaborative studies). N/a refers to not available.]

Parameters	**AOAC 2011.06**	**AOAC 992.05**	**EN 14131**
Infant/adult/placebo formula matrices used in MLT study	10 (3 liquid, 7 powder) + SRM 1869	3	0
Analyte	Total Folate (free + bound folic acid and natural folates) are measured, calculated as folic acid and summed up	Free folic acid + free, unbound natural folates, aggregated and measured as folic acid	Total folate (free+ bound) folic acid and natural folates aggregated and measured as folic acid
Repeatability (RSD_r)	3.5 -6.6	9.35	6.7-14.8
Reproducibility (RSD_R)	9.0-15.7	25.44	13.8-22.3
Recovery	94.4-107.7	n/a	n/a
NIST SRM 1849a (Reference value 2.15 ± 0.14 mg//kg)	2.11 mg/kg	n/a	n/a
Limit of Quantitation	0.4 µg/100 g RTF 0.7 µg/100 kcal	n/a	n/a
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	6 µg/100 g RTF 10 µg/100 kcal		

[12] *J. AOAC Int*. 2018 Nov 1; 101(6):1881-1894. DOI: http://dx.doi.org/10.5740/jaoacint.18-0114

[13] Gill, B.D.; Indyk, H.E.; Blake, C.J.; Konings, E.J.; Jacobs, W.A.; Sullivan, D. (2015) Evaluation protocol for the review of method validation data by the AOAC Stakeholder Panel for Infant Formula and Adult Nutritionals Expert Review Panel. *JAOAC* 98, 112-115. DOI: 10.5740/jaoacnt.14-158.
https://pdfs.semanticscholar.org/3099/504855165aa1bd61ce9240bad7ea7a0aea57.pdf

AOAC/ISO/IDF recommends CCMAS40 to endorse AOAC 2011.06 as Type II and re-type the existing methods for folic acid as indicated in the below table.

Table 3. Recommended Method of Analysis and Sampling (CXS 234-1999)

Commodity	**Provision**	**Method**	**Principle**	**Type**
Infant Formula	Folic acid	**AOAC 2011.06**	**LC-MS/MS**	**II**
		AOAC 992.05 / EN 14131	Microbioassay	~~II~~ **III**
		J AOAC Int. 2000:83; 1141-1148	Optical Biosensor Immunoassay	IV
		J Chromatogr. A., 928, 77-90, 2001	HPLC, incorporating immunoaffinity clean-up and conversion to 5-methyltetrahydrofolate	IV

Recommendations to CCMAS40

AOAC/ISO/IDF recommends CCMAS40 to take the following actions:

1. Endorse AOAC 2015.06 / ISO 21424 | IDF 243 as Type II for the determination of nine Minerals and Trace Elements (i.e., Calcium, Magnesium, Potassium, Phosphorus, Sodium, Copper, Iron, Manganese, Zinc) in infant formula and reclassify the following existing Type II methods as Type III:
    a. ISO 8070 | IDF 119 (Calcium, Magnesium, Potassium, Sodium)
    b. AOAC 999.11 | NMKL 139 (Iron)
    c. AOAC 986.24 (Phosphorus)
2. Endorse AOAC 2011.14 / ISO 15151 | IDF 229 as Type III for the determination of nine Minerals and Trace Elements (i.e., Calcium, Magnesium, Potassium, Phosphorus, Sodium, Copper, Iron, Manganese, Zinc) in infant formula.
3. Revoke AOAC 984.27 as a Type III method for the determination of nine Minerals and Trace Elements (i.e., Calcium, Magnesium, Potassium, Phosphorus, Sodium, Copper, Iron, Manganese, Zinc) in infant formula.
4. Endorse AOAC 2015.09 / ISO 21446 as Type II for the determination of Vitamin K_1 in infant formula.
5. Endorse AOAC 2011.06 as Type II for the determination of Folic Acid in infant formula, and reclassify AOAC 992.06 / EN 14131 as Type III.

Appendix 1 – Comparison of Performance Parameters of AOAC 2015.06 / ISO 21424 | IDF 243 and AOAC 2011.14 / ISO 15151 | IDF 229

[Note: RTF refers to ready-to-feed infant formula levels. SLV refers to Single Laboratory Validation.[14] MLT refers to Multi-Laboratory Testing studies (i.e., collaborative studies).]

Parameters - Calcium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 2011.14 / ISO 15151 | IDF 229**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	18 (6 liquid, 12 powder) + 6 milk products
Repeatability (RSD_r)	1.8 %	3.9 %
Reproducibility (RSD_R)	4.1 %	8.7 %
Recovery range from SLV/MLT	90.2 – 104 %	97.5 – 99.9 %
Mean NIST SRM 1849a result from 10 labs (AOAC 2015.06 / ISO 21424 | IDF 243) or from 12 labs (AOAC 2011.14 / ISO 15151 | IDF229)	5279 mg/kg (certified 5253 ± 51)	5302 mg/kg (certified 5253 ± 51)
Limit of Quantitation	0.53 mg/100g RTF 0.88 mg/100 kcal	0.10 mg/100g RTF 0.17 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	30 mg/100g RTF 50 mg/100 kcal	
Parameters - Magnesium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 2011.14 / ISO 15151 | IDF 229**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	18 (6 liquid, 12 powder) + 6 milk products
Repeatability (RSD_r)	1.5 %	2.2 %
Reproducibility (RSD_R)	3.5 %	7.4 %
Recovery range from SLV/MLT	92.8 – 104 %	96.5 – 98.9 %
Mean NIST SRM 1849a result from 10 labs (AOAC 2015.06 / ISO 21424 | IDF 243) or from 12 labs (AOAC 2011.14 / ISO 15151 | IDF229)	1626 mg/kg (certified 1648 ± 36)	1634 mg/kg (certified 1648 ± 36)
Limit of Quantitation	0.0049 mg/100g RTF 0.0082 mg/100 kcal	0.020 mg/100g RTF 0.033 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	3.0 mg/100g RTF 5.0 mg/100 kcal	

[14] Gill, B.D.; Indyk, H.E.; Blake, C.J.; Konings, E.J.; Jacobs, W.A.; Sullivan, D. (2015) Evaluation protocol for the review of method validation data by the AOAC Stakeholder Panel for Infant Formula and Adult Nutritionals Expert Review Panel. *JAOAC* 98, 112-115. DOI: 10.5740/jaoacnt.14-158.
https://pdfs.semanticscholar.org/3099/504855165aa1bd61ce9240bad7ea7a0aea57.pdf

Parameters - Potassium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 2011.14 / ISO 15151 | IDF 229**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	18 (6 liquid, 12 powder) + 6 milk products
Repeatability (RSD_r)	1.4 %	2.3 %
Reproducibility (RSD_R)	3.6 %	6.0 %
Recovery range from SLV/MLT	92.4 – 110.2 %	93.9 – 102 %
Mean NIST SRM 1849a result from 10 labs (AOAC 2015.06 / ISO 21424 | IDF 243) or from 12 labs (AOAC 2011.14 / ISO 15151 | IDF229)	9365 mg/kg (certified 9220 ± 110)	9319 mg/kg (certified 9220 ± 110)
Limit of Quantitation	0.56 mg/100g RTF 0.92 mg/100 kcal	1.5 mg/100g RTF 2.5 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	36 mg/100g RTF 60 mg/100 kcal	
Parameters - Sodium	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 2011.14 / ISO 15151 | IDF 229**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	18 (6 liquid, 12 powder) + 6 milk products
Repeatability (RSD_r)	1.5 %	2.8 %
Reproducibility (RSD_R)	3.6 %	7.7 %
Recovery range from SLV/MLT	99.8 – 106 %	92.0 – 98.4 %
Mean NIST SRM 1849a result from 10 labs (AOAC 2015.06 / ISO 21424 | IDF 243) or from 12 labs (AOAC 2011.14 / ISO 15151 | IDF229)	4263 mg/kg (certified 4265 ± 83)	4269 mg/kg (certified 4265 ± 83)
Limit of Quantitation	0.11 mg/100g RTF 0.18 mg/100 kcal	0.10 mg/100g RTF 0.17 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	12 mg/100g RTF 20 mg/100 kcal	
Parameters - Phosphorus	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 2011.14 / ISO 15151 | IDF 229**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	18 (6 liquid, 12 powder) + 6 milk products
Repeatability (RSD_r)	1.9 %	2.6 %
Reproducibility (RSD_R)	4.3 %	7.5 %
Recovery range from SLV/MLT	98.1 – 109 %	101 – 107 %
Mean NIST SRM 1849a result from 10 labs (AOAC 2015.06 / ISO 21424 | IDF 243) or from 12 labs (AOAC 2011.14 / ISO 15151 | IDF229)	4015 mg/kg (certified 3990 ± 140)	4009 mg/kg (certified 3990 ± 140)
Limit of Quantitation	0.13 mg/100g RTF 0.22 mg/100 kcal	0.010 mg/100g RTF 0.017 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	15 mg/100g RTF 25 mg/100 kcal	

Parameters - Copper	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 2011.14 / ISO 15151 | IDF 229**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	18 (6 liquid, 12 powder) + 6 milk products
Repeatability (RSD_r)	2.3 %	3.9 %
Reproducibility (RSD_R)	6.3 %	9.3 %
Recovery range from SLV/MLT	91.3 – 106 %	93.4 – 109 %
Mean NIST SRM 1849a result from 10 labs (AOAC 2015.06 / ISO 21424 | IDF 243) or from 12 labs (AOAC 2011.14 / ISO 15151 | IDF229)	19.60 mg/kg (certified 19.78 ± 0.26)	19.47 mg/kg (certified 19.78 ± 0.26
Limit of Quantitation	0.00048 mg/100g RTF 0.00080 mg/100 kcal	0.0030 mg/100g RTF 0.0050 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.021 mg/100g RTF 0.035 mg/100 kcal	
Parameters - Manganese	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 2011.14 / ISO 15151 | IDF 229**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	18 (6 liquid, 12 powder) + 6 milk products
Repeatability (RSD_r)	2.1 %	3.3 %
Reproducibility (RSD_R)	3.8 %	12.2 %
Recovery range from SLV/MLT	93.5 – 101 %	94.6 – 100 %
Mean NIST SRM 1849a result from 10 labs (AOAC 2015.06 / ISO 21424 | IDF 243) or from 12 labs (AOAC 2011.14 / ISO 15151 | IDF229)	48.71 mg/kg (certified 49.59 ± 0.97)	48.87 mg/kg (certified 49.59 ± 0.97)
Limit of Quantitation	0.0006 mg/100g RTF 0.0010 mg/100 kcal	0.0010 mg/100g RTF 0.0017 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.0006 mg/100g RTF 0.0010 mg/100 kcal	
Parameters - Zinc	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 2011.14 / ISO 15151 | IDF 229**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	18 (6 liquid, 12 powder) + 6 milk products
Repeatability (RSD_r)	1.6 %	3.0 %
Reproducibility (RSD_R)	4.3 %	7.6 %
Recovery range from SLV/MLT	93.0 – 108 %	95.2 – 96.8 %
Mean NIST SRM 1849a result from 10 labs (AOAC 2015.06 / ISO 21424 | IDF 243) or from 12 labs (AOAC 2011.14 / ISO 15151 | IDF229)	151.8 mg/kg (certified 151.0 ± 5.6)	152.6 mg/kg (certified 151.0 ± 5.6)
Limit of Quantitation	0.056 mg/100g RTF 0.093 mg/100 kcal	0.0030 mg/100g RTF 0.0050 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.30 mg/100g RTF 0.50 mg/100 kcal	

Parameters - Iron	**AOAC 2015.06 / ISO 21424 | IDF 243**	**AOAC 2011.14 / ISO 15151 | IDF 229**
Infant/adult/placebo formula matrices used in MLT study	18 (6 liquid, 12 powder) + 6 milk products	18 (6 liquid, 12 powder) + 6 milk products
Repeatability (RSD_r)	2.9 %	3.6 %
Reproducibility (RSD_R)	6.1 %	9.5 %
Recovery range from SLV/MLT	92.8 – 101 %	90.5 – 102 %
Mean NIST SRM 1849a result from 10 labs (AOAC 2015.06 / ISO 21424 | IDF 243) or from 12 labs (AOAC 2011.14 / ISO 15151 | IDF229)	172.7 mg/kg (certified 175.6 ± 2.9)	174.0 mg/kg (certified 175.6 ± 2.9)
Limit of Quantitation	0.0073 mg/100g RTF 0.012 mg/100 kcal	0.050 mg/100g RTF 0.083 mg/100 kcal
CODEX STAN 72-1981 minimum level for infant formula based on minimum energy level of 60 kcal/100g RTF	0.27 mg/100g RTF 0.45 mg/100 kcal	