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Vitamins, inositol and choline

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National Institute for Public Health
and the Environment
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Summary

Adequate methods with known performance characteristics used for the assessment of the amount of nutrients in formula are essential in the evaluation whether the composition deviates from the legal amounts. Many standardised analytical methods for the analysis of nutrients in formula are internationally available.

Currently, EU regulation regarding formula (commission directive 2006/141/EC) does not provide any indication on reference methods to be used in official controls for the analysis of vitamins, choline and inositol in formula. Therefore, an overview of the available standardised methods of analysis for the measurement of vitamins, inositol and choline in infant and follow-on formula is provided. 89 standardised analytical methods from both national and international organizations developing standard analytical methods have been collected and their performance characteristics (i.e. analytical technique, target compound, matrixes validated, LOD, LOQ, analytical range, repeatability, reproducibility, accuracy/spike recovery) are described.

For most nutrients studied, several standardised analytical methods are available, with a maximum of 12 for vitamin A. Especially for water-soluble vitamins some current standardised methods are more than 25 years old. However, methods of analysis based on more modern analytical technologies have been developed in the recent decades. 11 new official AOAC methods for the analysis of Vitamin A, D, E, B12, Folic acid and based on more advanced techniques have been adopted as First Action method in 2011 and new AOAC official methods of analysis are expected to be approved in the near future for other nutrients.

For several nutrients the overall activity is the sum of the activities of several forms of the compound. However, it is important to notice that the analytical methods available may not always measure the overall activity but only a limited proportion based on specific forms of the compound. In the EU legislation the compound forms that may be used in the manufacturing of formula are listed, however, it does not provide unambiguous specification about the forms of the vitamin to be assessed.

Moreover, the minimum and maximum values are provided per energy content, logic from a nutritional point of view. This means that both the uncertainty in the energy calculation and the uncertainty in the measurement of the target nutrient contribute to the total uncertainty in the assessment of the nutrient content when this is provided per energy content.

The existence of many different available methods with different performance characteristics and possibly different target forms of the nutrients is identified as a serious gap in the current harmonisation status. In absence of relevant European Union rules, the choice of the best method of analysis for each of the target nutrients in formula should be based on several factors, such as the capacity of the method to assess all the forms of the vitamin that are nutritionally relevant in formula and the analytical performances of the method (i.e. accuracy/spike recovery, reproducibility, lower and upper limit of quantification). As CODEX is the most internationally recognized organization publishing standardised analytical methods, the methods of analysis recommended by CODEX might, although arbitrarily chosen, be taken as guidance for the choice.

Keywords: infant formula, methods of analysis, vitamins, choline, inositol.

Contents

1 Introduction

Infants that are not fully breast-fed depend completely, or at least partly, on infant formula in the first months of life. Infant formula should therefore completely provide the nutritional need of infants in these first months. At six months of age, infants will start to consume suited complementary foods in addition to follow-on formula. In the period between 6 and 12 months of age the consumption of follow-on formula will decrease and the consumption of complementary foods will increase.

Because of the large dependence on infant formula and follow-on formula (henceforth together called formula) for the delivery of nutrients required for growth and development it is important that formula indeed deliver an adequate nutrient supply without the risk of excessive intake. Both too low and excessive intake of nutrients may result in adverse health effects. The type and seriousness of these adverse health effects depend on e.g. the type of nutrient, the extent of deviation, the duration, and the age of the infant.

The composition of formula is laid down by law (1). This legislation lays down which nutrients have to or may be present in formula and in which amounts. However, no reference to specific analytical methods or performance criteria of these methods are provided by law. To estimate and possibly avoid public health risks, the chemical analyses to determine the amount of a nutrient in formula is essential in the evaluation whether the composition deviates from the legal amounts. For the correct interpretation of the analytical results also some characteristics of the methods and how to apply the method correctly are important. For the chemical analyses of most nutrients in formula, multiple - but different - standardised analytical methods are internationally available, while for some nutrients such standardised methods are not present.

1.1 Aim of the report

This report provides an overview of the available standardised analytical methods for the determination of nutrients in formula. This report focusses on the vitamins, inositol and choline compounds. In addition, several analytical method characteristics are provided i.e. the target compound(s) being actually measured, the matrixes wherein the methods have been validated and the method performance characteristics.

2 Approach

In the present study, current and draft standardised methods of analysis for the measurement of vitamins, choline and inositol in formula have been collected from international and national organizations developing and setting standardised methods.

The sources that were used to search for standardised analytical methods are listed below:

International

- AOAC International (AOAC)
- International Organisation for Standardisation (ISO)
- Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN)
- American Association of Cereal Chemists (AACC)
- CODEX alimentarius (FAO/WHO)
- International Dairy Federation (IDF)

European

- European Committee for Standardisation (CEN)
- European Pharmacopoeia (EP)

USA

- Infant Formula Council (IFC)
- American Oil Chemists' Society (AOCS)

Other national

- Chinese National Standards (GB)
- Russian National Standards (GOST)
- Nordic Committee for food analysis (NMKL)
- Standards Australia (SA)
- Standards New Zealand (NZS)

With exception of CODEX alimentarius and SPIFAN, all are organizations developing standardised methods. CODEX alimentarius periodically publishes lists of recommended standardised methods of analysis and sampling. The SPIFAN is an international expert panel recently established on initiative of AOAC International to establish method requirements, collect and validate new methods of analysis for target nutrients in infant formula.

Information on the methods has been gathered by consulting standard organization official websites and/or official (on-line or hard copy) publications or by directly contacting the organizations. From this information analytical method characteristics were obtained and documented per nutrient. In addition to the method name (reference) and the organisation behind this standardised method, also information is provided on the status (current or draft), the analytical principle, the target compounds, the matrix in which the method is validated, and several performance characteristics (i.e. accuracy, spike recovery, Limit of Detection (LOD),

quantitation range, relative standard deviation of repeatability (RSD_r), and relative standard deviation of reproducibility (RSD_R).

3 Results and discussion

The standardised analytical methods collected for each nutrient including several characteristics are listed in Appendices II-XVI. In this section the findings will be described and discussed in a broad perspective. For detailed information regarding a specific nutrient or method we would like to refer to the Appendices.

Data sources

Standardised analytical methods have been published by the following international and national organizations developing standardised methods: International Organisation for Standardisation (ISO), European Committee for Standardisation (CEN), AOAC International (AOAC), American Association of Cereal Chemists (AACC), International Dairy Federation (IDF), Nordic Committee for food analysis (NMKL), Chinese National Standards (GB), Russian National Standards (GOST), and Infant Formula Council (IFC). In total, 89 methods have been collected for the analysis of vitamins, choline and inositol in formula (Appendices II-XVI).

The methods collected have been either validated in infant formulas or validated in similar matrixes, for instance milk powder and milk. Only one method, AOAC official method 974.29 for vitamin A, has not been validated in any of these matrixes but it has still been adopted by CODEX alimentarius for follow-up formulas (2). The activities of SPIFAN, established on initiative of AOAC International, have resulted in the selection of a number of new candidate methods for the analysis of vitamin A, vitamin E, vitamin D, vitamin B12, vitamin B11 (folate/folic acid) and inositol. The methods that proved to satisfactorily fulfil the performance requirements established by the expert panel have entered the AOAC official methodsSM program. Based on validation data available, 11 new methods have recently achieved AOAC official First Action status in 2011 and have been included in this report. In addition, four AOAC Performance-Tested MethodsTM, one AOAC peer-verifiedSM method and SPIFAN candidate methods which are currently under evaluation of the AOAC expert panel have been included as well. The authors are not aware that other standard organisations/panels are currently working on candidate methods to become standardised methods in the near future.

General observations

For most nutrients several standardised methods of analysis are available. As much as 12 standardised methods have been collected for vitamin A, 11 for vitamin D and 9 for vitamin E. Only for inositol, just one established standardised method is available (the Chinese standard GB 5143.25-2010).

According to article 11 of EC Regulation 882/2004 on official controls on foods, “Sampling and analysis methods used in the context of official controls shall comply with relevant Community rules or, (a) if no such rules exist, with internationally recognised rules or protocols, for example those that the European Committee for Standardisation (CEN) has accepted or those agreed in national legislation; or, (b) in the absence of the above, with other methods fit for the intended purpose or developed in accordance with scientific protocols” (2). Currently, there are no

European Community rules for chemical analysis of vitamins, choline and inositol in formula. The EU Commission Regulation 141/2006 on infant formula and follow-on formula does not provide any indication on reference methods to be used in official controls (1). This means that for many nutrients there is more than one standardised method available to choose from.

CODEX alimentarius is an intergovernmental body under the auspices of the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) of the United Nations. It can be regarded as the most internationally recognized standard organization. Periodically CODEX alimentarius does publish lists of international standards as recommended method of analysis for nutrients in formula. Methods of analysis are endorsed by CODEX as type II (methods to be recommended in cases of dispute and for calibration purposes), type III (alternative methods) or type IV (tentative methods). With the exception of method NMKL 167 for the analysis of vitamin D, all type II CODEX endorsed methods for the analysis of vitamins in formula are either European standard CEN methods or AOAC official methods (3,4).

However, it must be pointed out that the most recently approved standardised methods of analysis (AOAC official methods approved in 2011) have not been yet evaluated by CODEX alimentarius. It is thus likely that in the future, the list of CODEX endorsed methods of analysis will be changed and updated with the inclusion of newly approved standardised methods.

Analytical techniques

The standardised methods of analysis collected are based on a wide range of analytical techniques. The analysis of fat-soluble vitamins is mostly based on liquid chromatography (LC) techniques with either fluorescence or UV-Vis detection even though gas chromatographic (GC) techniques and spectrophotometric techniques were also reported. For vitamin D, three new methods have been approved as AOAC standardised method of analysis in 2011. They are based on Ultra Performance Liquid Chromatography (UPLC) with MS/MS detection. Mass spectrometry provides higher sensitivity and specificity compared to other detection systems. For vitamin E, the recently approved AOAC standardised method (2011.07) is based on UPLC with fluorescence detection.

For the analysis of water-soluble vitamins, it can be stated that several current international and national standard methods are based on microbiological assays which were established more than 25 years ago. Microbiological assays are available for vitamin B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B7 (biotin), B11 (folic acid) and vitamin B12 (cobalamins). Microbiological assays are very sensitive but generally suffer poor precision and are highly time-consuming. Furthermore, differences exist between the growth response of microorganisms to different forms of the same vitamin which exert biological activity (vitamers). Standardised methods of analysis based on chromatographic techniques have become available in the last decades. Most of them are HPLC based method with UV/VIS or fluorescence detection. HPLC methods are generally less sensitive than microbiological assays, but more reproducible. Even more recently, methods based on biosensor technology have been developed. Applications have been developed for vitamin B2, B5, B7, B11 and vitamin B12, specifically for formula and milk-based products. At present, four test kits for the determination of biotin, folic acid, pantothenic acid and vitamin B12 have achieved the AOAC Performance-Tested Methods TM status. Two official AOAC methods of analysis based

on biosensor technology are available, namely AOAC 2011.01 for determination of vitamin B12 and AOAC 2011.05 for the determination of folic acid.

Matrix and target compounds

The methods collected have been either validated in formula or validated in similar matrixes, like milk powder and milk. Only one method (AOAC official method 974.29 for vitamin A) has not been validated in formula or a similar matrix, but has still been adopted by CODEX alimentarius for follow-up formula (2).

The overall vitamin content of formula depends on the amount that is supplemented and the fraction naturally occurring in formula ingredients. The compounds that can be used to supplement formula according to European Commission regulation 2006/141/EC are listed in Appendix I. Depending on the nutrient, the contribution from natural ingredients varies to a different degree (5). It might be significant, for instance, for vitamin K, B2 and B11. However, for those nutrients for which the contribution of compounds naturally occurring in formula ingredients to the overall activity is negligible or marginal, the measurement of the supplemented forms of the nutrient will be sufficient to assess the overall nutrient activity.

In addition, for many vitamins, multiple forms exist which exhibit biological activities. These forms are known as vitamers. Further, nutrients can occur as free compounds or in a bound form (esters, glycosides). It appears that not all the methods of analysis are able to detect and quantify the multiple forms of many nutrients (i.e. both different vitamers and free and bound forms) in formula. Depending on the extraction protocol and the detection system, the overall vitamin activity may be assessed to a limited proportion of the vitamers. For instance, vitamin K present in formulas can include cis and/or trans vitamin K1, dihydro-K1, and the menaquinone series (vitamin K2). Of the three methods listed AOAC official methods, one is able to measure only both isomers of vitamin K1 (cis- and trans- phylloquinone), whereas two other methods can measure also dihydro-K1 and menaquinones. Another point in case is vitamin E which comprises α, β, γ and δ tocopherol and the corresponding tocotrienols. Most of vitamin E in formula is represented by supplemented α-tocopherol or tocopherol acetate. Most of the methods listed are able to measure α-tocopherol or tocopherol acetate, one methods can measure α, β, γ and δ tocopherol independently, but no methods are available to measure tocotrienols.

The same holds for water soluble vitamins that exist as different vitamers/homologues or that are present as free and bound compounds. For instance, the assessment of vitamin B11 in formula ranges from the determination of total free and bound folates (ISO EN 14131:2003) to free folic acid and free folates (AOAC 992.05) or only the monoglutamate forms of folic acid (AOAC 2011.06). For vitamin B6, The method EN 15663:2008 measures free and bound forms of the vitamin included the glycosylated forms whereas methods AOAC 2004.07 and EN 14164:2008 do not measure the glycosylated forms. As for vitamin C, both L-ascorbic and L-dehydroascrobic acids contribute to vitamin C activity. Method AOAC 985.33 is able to measure only ascorbic acid. In addition, the D- enantiomer of ascorbic and dehydroascrobic need to be discriminated from the L- counterparts because the D- forms are not biologically active.

It must also be pointed out that EU regulation is not unambiguous in specifying which forms of the vitamins have to be included in the calculation of vitamin activity. For instance, it provides no qualification on the definition of forms of vitamin K. Another notable example is vitamin D which

can be supplemented as vitamin D2 (colecalciferol) and D3 (ergocalciferol) in formula. EU regulation refers to colecalciferol which would exclude ergocalciferol (which is one of the supplemented forms of vitamin D accepted by EU regulation) from vitamin D assessment. Moreover, hydroxylated forms also show vitamin D activity but it is not clear from the regulation whether those forms have to be included in the assessment of the vitamin. Finally, EU regulation refers to folic acid thus excluding total folates (naturally occurring in formula ingredients) from the assessment of vitamin B11 activity.

Method performances

Methods performance characteristics that have been collected comprise the accuracy, spike recovery, limit of detection (LOD), limit of quantification (LOQ), range of applicability, relative standard deviation of repeatability (RSD_r) and relative standard deviation of reproducibility (RSD_R). These method performance characteristics are reported in Appendices II-XVI. Unfortunately not all the method performance characteristics could be retrieved for each method. Values for LOD, LOQ and the analytical range, for instance, are often not reported in the official protocols. In some cases only the instrumental analytical range or instrumental LOD, LOQ were available. In some cases, it was noticed that the reported analytical range does not comply with the provisions given in EU regulation on infant formula. For instance, the range of applicability of method AOAC 992.27 for the analysis of vitamin K (75 to 130 µg/L trans-vitamin K1) and the range of applicability of method 992.26 (12.2 to 13.3 µg/L) for the analysis of vitamin D do not comply with the provisions for the minimum amount of vitamin K1 in formula according to EU regulation (25µg/L) and the provisions for minimum and maximum amount of vitamin D in infant formula (6.25 µg/L and 19.2 µg/L, respectively). In addition, the values of LOD, LOQ are given in different units in the official protocols (i.e. /g, /L, /g of solids), whereas the compositional provisions are specified in EU regulation on infant formula per energy content (i.e. per 100kcal or 100kJ). This makes the direct comparison of the provisions with the method performance characteristics not straightforward. Moreover, the error in energy determination should be added to the analytical error for the determination of the target nutrient thus contributing to the overall uncertainty of the measurement.

Data on accuracy/spike recovery have been collected for most of the methods. Accuracy/spike recovery values ranges from 90% to 110% for most of the methods with few exceptions (i.e. method AOAC 2001.13 on vitamin A, recovery=79.5%). Data on methods reproducibility (RSD_R) have been collected for almost all the methods. The majority of the established methods of analysis collected have been validated in international collaborative studies, and in those cases reproducibility data are reported. For a few of the recently approved AOAC methods, reproducibility data are not yet available pending the results of collaborative studies. In the Chinese standards, data on reproducibility are not reported in the official protocols. Reproducibility values (RSD_R) are in most of the cases <20% even though higher values can also be found. For many methods a range of RSD_R is reported when methods have been validated in differently formulated formulas (for example milk- or soy-based infant formula).

4 Conclusions and recommendations

Currently, EU regulation does not provide any indication on reference methods to be used in official controls for the analysis of vitamins, choline and inositol in formula. Therefore, it is recommended to fill this gap in the near future and EU regulation could give provisions on reference methods of analysis for vitamins and other nutrients in formula for official controls.

The survey has shown that for each vitamin and choline several standardised methods are available. For inositol one standardised method is available. Several standardised methods are more than 20 years old, but also standardised methods using more modern analytical approaches are available. Setting standardised methods is an ongoing process, and new standardised methods will be approved in future.

Several standardised methods are not able to assess the total vitamin content due to the different forms nutrients may appear in formula and differences in extraction and detection protocols. In this respect it is recommended that unambiguous specification is provided by nutritionists and followed by EU regulation about which forms of the vitamins have to be included in the calculation of vitamin activity.

The lower and the upper limits of quantification of some methods do not comply with compositional provisions given in EU regulation for the target nutrients in formula. These provisions are usually given per energy content. From a mere analytical perspective this diminishes the accuracy of nutrients assessment, although there are good reasons to do so from a nutritional point of view.

In absence of relevant EU rules and of specific protocols/procedures, the choice of the best method for the analysis of each of the target nutrients in infant formula should take into account multiple factors such as the capacity of the method to assess all the forms of the vitamin that are nutritionally relevant in formula and method analytical performances (accuracy/spike recovery, reproducibility, lower and upper limit of quantification). CODEX alimentarius recommended methods of analysis might be taken as a, arbitrarily chosen, guidance in the choice. CODEX is the most internationally recognized organization which publishes lists of recommended methods for food analysis.

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Annex I
List of substances that may be used in the manufacture of infant formula and follow-on formula in order to satisfy the requirements on vitamins, choline and inositol. From Annex III of commission directive 2006/141/EC (1)

Compound	Formulation
Vitamins	
Vitamin A	Retinyl acetate
	Retinyl palmitate
	Retinol
Vitamin D	Vitamin D2 (ergocalciferol)
	Vitamin D3 (cholecalciferol)
Vitamin B1	Thiamin Hydrochloride
	Thiamin mononitrate
Vitamin B2	Riboflavin
	Riboflavin-5'-phosphate, sodium
Niacin	Nicotinamide
	Nicotinic acid
Vitamin B6	Pyridoxine hydrochloride
	Pyridoxine-5'-phosphate
Folate	Folic acid
Pantothenic acid	D-pantothenate, calcium
	D-pantothenate, sodium
	Dexpanthenol
Vitamin B12	Cyanocobalamin
	Hydroxocobalamin
Biotin	D-biotin

Compound	Formulation
Vitamin C	L-ascorbic acid
	Sodium L-ascorbate
	Calcium L-ascorbate
	6-palmityl-L_ascorbic acid (ascorbyl palmitate)
	Potassium ascorbate
Vitamin E	D-alpha tocopherol
	DL-alpha tocopherol
	D-alpha tocopherol acetate
	DL-alpha tocopherol acetate
Vitamin K	Phylloquinone (Phytomenadione)
Other nutritional substances	
	Choline
	Choline chloride
	Choline citrate
	Choline bitartrate
	Inositol

Annex II
EU compositional provisions and overview of the standardised methods of analysis for Vitamin A in infant and follow-on formula

Definition of compound (2006/141/EC): Vitamin A

Potential forms of the nutrient: all-trans-Retinol, 13-cis-retinol, other cis-retinol isomers, retynil acetate, retynil palmitate, other retynil esters.

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg RE*/ 100kJ		µg RE* /L**			µg RE*/ 100kJ		µg RE* /L**	
14	43	350	1268		14	43	350	1268

* *RE = all-trans-retinol equivalent. 1 µg RE = 3.33 IU Vitamin A = 1 µg all-trans retinol, 1.15 µg retynil acetate, 1.83 µg retynil palmitate, 6 µg all-trans-beta-carotene. Carotenoids are not included in the calculation and declaration of vitamin A activity.*

** *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 1. Overview of the standardised methods of analysis for vitamin A in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 992.06	AOAC official method. Codex type III for IF and follow-on formula	Current	HPLC with Visible detection	A	Milk-based infant formula (Liquid, ready-to-feed)
AOAC 992.04	AOAC official method. Codex type II for IF and follow-on formula	Current	HPLC with Visible detection	A	Milk and milk-based infant formula (powdered and liquid)
AOAC 974.29[a]	AOAC official method. Codex type IV for follow-on formula	Current	Colorimetric	B	Mixed feeds, premixes, human and pet food
EN 12823-1:2000	European standard. Codex type III for IF	Current	HPLC with UV or fluorometric detection	A	Margarine, milk powder (CRM 421) and lyophilized Brussels sprouts
AOAC 2001.13	AOAC official method	Current	HPLC with UV detection	B	Infant formula (powdered)
AOAC 2002.06	AOAC official method	Current	HPLC with UV detection	Retinyl palmitate	Fortified skim, low fat and reduced fat fluid milk, chocolate milk
ISO 12080-2: 2009/ IDF 142-2	-	Current	HPLC with UV or fluorometric detection	A	Skimmed milk powder
ISO 12080-1:2009/ IDF 142-1	-	Current	Colorimetry	A	Skimmed milk powder
AACC 86.06	-	Current	HPLC with UV detection	A	Infant formula (powdered)
AOAC 2011.07	AOAC official method	Current	UPLC with UV detection	A	Infant, adult, pediatric formula (powders, ready-to-feed liquids, liquid concentrates)
GOST 30627.1:1998	Russian	Current	Colorimetry	B	Infant formula
GB 5413.9-2010	Chinese	Current	HPLC with UV detection	A	Foods for infant and young children, milk and milk products

A= Free and bound individual retinol isomers (13-cis and all-trans): B= Free and bound retinol.

[a] *= Not applicable to products containing provitamin A (carotene) as predominant source of vitamin A activity nor to high potency vita min A concentrates used for feed, premix, and food manufacture.*

Continued Table 1. Overview of the standardised methods of analysis for vitamin A in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	**Spike recovery**	**LOD**	**Quantitation range**	**RSD_r**	**RSD_R**
AOAC 992.06	-	99%	-	>158.56 µg retinol equivalent/L	4.9%	10.5%
AOAC 992.04	-	93.4%	-	-	3.62%	9.72%
AOAC 974.29	-	-	15 µg retinol equivalent/L	-	-	3.9-11.1%
EN 12823-1:2000	-	-	-	-	2.1% (all-trans-), 5.0% (cis)	3.4 % (all-trans), 24% (cis)
AOAC 2001.13	79.5%	-	-	15 µg/100g to 1g/ g powder	13.85-14.35%	10.57-16.43%
AOAC 2002.06	-	-	128 µg/L	440-3030 µg/L	2.3%	7.1-7.9%
ISO 12080-2: 2009/IDF 142-2	-	-	-	>3 µg RE/g	5%	15%
ISO 12080-1:2009/IDF 142-1	-	-	-	>3 µg RE/g	5%	15%
AACC 86.06	79.5%	-	-	15 µg/100g to 1g/ g powder	13.85-14.35%	10.57-16.43%
AOAC 2011.07	-	101%	-	9-450 µg/ 100 g ready-to-feed infant formula	6.0%	13.4%
GOST 30627.1:1998	-	-	-	-	3.5%	7%
GB 5413.9-2010	-	-	1 µg/100g	-	3.5%	-

LOD = limit of detection; RSD_r =Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation;
RSD_{IR} = Intermediate reproducibility relative standard deviation.

Annex III
EU compositional provisions and overview of the standardised methods of analysis for Vitamin D in infant and follow-on formula

Definition of compound (2006/141/EC): Vitamin D*

Potential forms of the nutrient: Cholecalciferol (Vit D3) and ergocalciferol (Vit D2), 25-OH-VitD3, 25-OH-VitD2; previtamin D3.

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg/ 100kJ		µg /L**			µg / 100kJ		µg / L**	
0.25	0.65	6.25	19.2		0.25	0.75	6.25	22.1

* *As cholecalciferol.*

** *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 2. Overview of the standardised methods of analysis for vitamin D in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 992.26	AOAC official method. Codex type III for IF and follow-on formula	Current	HPLC with UV detection	Vitamin D2 or D3	Ready-to-feed milk-based infant formula
AOAC 995.05	AOAC official method. Codex type III for IF and follow-on formula	Current	HPLC with UV detection	Vitamin D3 and D2	infant formula (liquid and powdered) and enteral products
EN 12821:2009	European standard. Codex type II for follow-on formula	Current	HPLC with UV detection	Vitamin D2 or D3	Margarine, porridge, milk powder (SRM 421), milk, cooking oil, infant formula
ISO 14892:2002, IDF 177:2002	-	Current	HPLC with UV detection	Vitamin D2 or D3	Skimmed milk powder
AOAC 2002.05	AOAC official method	Current	HPLC with UV detection	Vitamin D3	Infant formula (liquid and powdered milk products) products and vitamins premixes
AOAC 981.17	AOAC official method	Current	HPLC with UV detection	Vitamin D	Fortified milk and milk powder
GB 5413.9-2010	Chinese	Current	LC with UV detection	Vitamin D2 or D3	Foods for infant and young children, milk and milk products
NMKL 167, 2000	Nordic committee on food analysis standard. Codex type II for infant formula	Current	HPLC with UV detection	Vitamin D2 or D3	milk, gruel, gruel powder, margarine, cocking oil and fish oil.
AOAC 2011.11	AOAC official method	Current	UPLC with MS/MS detection	Vitamin D2 and D3. Previtamin D	Infant formula
AOAC 2011.12	AOAC official method	Current	UPLC with MS/MS detection	Vitamin D3 and D2	Infant formula, eggs, milk, bread, cereal, cheese, mushroom, yogurth liquids, liquid concentrates)
AOAC 2011.13	AOAC official method	Current	UPLC with MS/MS detection	Vitamin D2 and D3. Previtamin D	Infant, adult, pediatric formula (powders, ready-to-feed liquids, liquid concentrates)

Continued Table 2. Overview of the standardised methods of analysis for vitamin D in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	**Spike recovery**	**LOD**	**Quantitation range**	**RSD_r**	**RSD_R**
AOAC 992.26	-	98%;	-	12.2-13.3 µg/L	12.2-20.5%	20.7-36.2%
AOAC 995.05	-	99.1%	-	0.21-68.7 µg/L	4.4-13.71%	7.28-19.44%
EN 12821:2009	-	-	2.5 µg/100 g powder	0.4–14 µg/100 g powder	2.4%	7.1%;
ISO 14892:2002, IDF 177:2002	-	-	-	>10 µg/100 g powder	5%	6%
AOAC 2002.05	-	93.9%	-	0.4-12 µg/100 g powder	2.4%	7.1%;
AOAC 981.17	-	-	0.20 µg/100g	≥0.025 µg/g powder	21%	28%
GB 5413.9-2010	-	-	-	-	3.5%	-
NMKL 167, 2000	-	93.9%	0.020 µg/100g powder (vit D3), 0.025 µg/100g (D2)	0.4-12 µg/100 g powder	2.4%	7.1%;
AOAC 2011.11	-	105-108%	0.205 µg/100g powder (vit D3), 0.617 µg/100g (D2)	>0.065 µg/100g powder (vit D3), >0.083 µg/100g (D2)	3.7-7.0% for D3 and 3.7-8.2 for D2	3.82%
AOAC 2011.12	-	105.5%	-	>0.472 µg/100g powder (vit D3), >1.435 µg/100g (D2)	6.42%	-
AOAC 2011.13	98.4-110% (vit D3). 96.4-104% (vit D2)	-	-	16-480 µg/100 g ready-to-feed infant formula	2.87-5.07%	2.37-8.45% RSD_{IR}

LOD = limit of detection; RSD_r =Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation;
RSD_{IR} = Intermediate reproducibility relative standard deviation.

Annex IV
EU compositional provisions and overview of the standardised methods of analysis for Thiamin (vitamin B1) in infant and follow-on formula

Definition of compound: thiamin

Potential forms of the nutrient: Thiamin, thiamin monophosphate (TMP), Thiamin piroposphate (TPP)

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg/ 100kJ		mg /L**			µg / 100kJ		mg / L**	
14	72	0.35	2.12		14	72	0.35	2.12

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 3. Overview of the standardised methods of analysis for thiamin in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
EN 14122:2003/AC 2005	European standard. Codex type II for IF	Current. Under revision	HPLC with fluorometric detection	Total thiamin	Many foods including baby foods and milk powder.
AOAC 986.27[a]	AOAC official method. Codex type III for IF	Current	Fluorometry	Total thiamin	Milk-Based Infant Formula, Powdered Infant Formula, Milk/Powdered Milk
GB 5413.11-2010	Chinese	Current	HPLC with fluorometric detection	Total thiamin	Foods for infant and young children, milk and milk products
GOST 30627.5:1998	Russian	Current	Spectrophotometric	Thiamin	Infant formula

[a] *Subjected to significant spectral influence.*

Continued Table 3. Overview of the standardised methods of analysis for thiamin in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	Spike recovery	LOD	Quantitation range	RSD_r	RSD_R
EN 14122:2003/AC 2005	-	-	-	0.11 – 486 mg/100g	3.2-4.2%; Milk powder=3.8%	11.8-22.6%: Milk powder= 13.2%
AOAC 986.27[a]	-	-	-	-	3.6-5.6%	5.9-8.2%
GB 5413.12-2010	-	-	50 µg/100g	-	3.5%	-
GOST 30627.6	-	-	-	-	10.7%	10.7%

[a] *Subjected to significant spectral influence;*
LOD = Limit of Detection;
RSD_r = Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation.

Annex V
EU compositional provisions and overview of the standardised methods of analysis for Riboflavin (vitamin B2) in infant and follow-on formula

Definition of compound (2006/141/EC): Riboflavin

Potential forms of the nutrient: Riboflavin, riboflavin-5'-phosphate and other phosphorilated forms, FAD, FMN

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg / 100kJ		mg/L*			µg / 100kJ		mg/L*	
19	95	0.48	2.80		19	95	0.48	2.80

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 4. Overview of the standardised methods of analysis for riboflavin in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
EN 14152:2003/AC:2005	European standard. Codex type II for infant formula	Current. Under revision	HPLC with fluorometric detection	Total riboflavin	Milk powder, pig's liver
AOAC 985.31[a]	AOAC official method. Codex type III for Infabt formula	Current	Fluorometry	Total riboflavin. Uncertain whether phosphorylated forms captured	Ready-to-feed Infant formula
GB 5413.12-2010	Chinese	Current	HPLC with fluorometric detection	Total riboflavin	Foods for infant and young children, milk and milk products
GOST 30627.6	Russian	Current	Fluorometry	Vitamin B2	Infant formula

[a] *Subjected to significant spectral influence. Uncertain whether phosphorylated forms captured.*

Continued Table 4. Overview of the standardised methods of analysis for riboflavin in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	Spike recovery	LOD	Quantitation range	RSD_r	RSD_R
EN 14152:2003/ AC 2005	-	-	-	0.11 – 486 mg/ 100g	1.17-3.17%; Milk powder=3.17%	7.31-7.89%; Milk powder= 7.31%
AOAC 9.31[a]	-	-	-	-0.05-0.2 µg/mL of riboflavin containing solutions	4.2-8.5%	4.8-10.4%
GB 5413.12-2010	-	-	-	->50 µg/100g	3.5%	-
GOST 30627.6	-	-	-	-	10.7%	10.7%

[a] *Subjected to significant spectral influence. Uncertain whether phosphorylated forms captured;*
LOD = Limit of Detection;
RSD_r = Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation.

Annex VI
EU compositional provisions and overview of the standardised methods of analysis for Niacin (vitamin B3) in infant and follow-on formula

Definition of compound (2006/141/EC): Preformed niacin

Potential forms of the nutrient: Nicotinamide, nicotinic acid, and related phosphorilated forms, NAD, NADH, NADH, NADPH

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg/100kJ		mg/L*			µg/100kJ		mg/L*	
72	375	1.8	11.06		72	375	1.8	11.06

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 5. Overview of the standardised methods of analysis for niacin in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
EN 15652:2009[a]	European. Codex type II for IF	Current	HPLC with fluorometric detection	Total niacin	breakfast cereal powder, Chocolate cereals, cooked ham, green peas, lyophilized green peas with ham, lyophilized soup, nutritive orange juice, milk powder and wheat flour
AOAC 985.34	AOAC official method. Codex type III for IF	Current	Microbiological Method	Free and bound forms	Ready-To-Feed Milk-Based Infant Formula
AOAC PVM 1:2000	AOAC peer validated method	Current	Anion exchange LC with UV detection	Free and bound forms	Orange milk-based and soy-based infant formula
GB 5413.15-2010	Chinese	Current	Fluorometry	Niacin and niacinamide	Foods for Infants and Young Children, Raw Milk, and Dairy Products
GOST 30627.4:1998	Russian	Current	Colorimetry	Vitamin PP	Infant formula

[a] *The standard specifies three different ways of hydrolysis : Acid (A), Enzymatic (B) and acid/alkaline (C).*

Continued Table 5. Overview of the standardised methods of analysis for niacin in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	**Spike recovery**	**LOD**	**Quantitation range**	**RSD_r**	**RSD_R**
EN 15652:2009[a]	-	90-107%	2 mg/kg	0.5-24 mg/100 g	Milk powder= 2.8% (A), 2.9% (B), 3.4% (C)	Milk powder= 4.3% (A), 4.3% (B), 17.2% (C)
AOAC 985.34	-	-	-	-	3.3-5.5%	9.2-10.8%
AOAC PVM 1:2000	89.4-94.3%	98-105%	0.2 µg/mL for the solution introduced in LC	>0.7 µg/mL for the solution introduced in LC	6.7-11.7%	-
GB 5413.15-2010	-	94%	0.1 mg/100g	-	-	-
GOST 30627.4:1998	-	-	-	-	10.7%	10.7%

[a] *Subjected to significant spectral influence. Uncertain whether phosphorylated forms captured;*
LOD = Limit of Detection;
RSD_r = Repeatability relative standard deviation;
RSDR = Riproducibility relative standard deviation.

Annex VII
EU compositional provisions and overview of the standardised methods of analysis for Pantothenic acid (vitamin B5) in infant and follow-on formula

Definition of compound (2006/141/EC): Pantothenic acid

Potential forms of the nutrient: Pantothenic acid, CoA, dexpanthenol

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg/100kJ		mg /L*			µg/100kJ		mg /L*	
95	475	2.375	14		95	475	2.375	14

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 6. Overview of the standardised methods of analysis for pantothenic acid in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 992.07	AOAC official method. Codex type II for IF and follow-on formula	Current	Microbiological method	Total Pantothenate (free + ACP- and CoA-bound)	Milk-Based liquid ready-to-eat Infant Formula
GB 5413.17-2010: part 1	Chinese	Current	Microbiological method	Total Pantothenate	Foods for infant and young children, milk and milk products
GB 5413.17-2010: part 2	Chinese	Current	HPLC with UV detection	Total Pantothenate	Foods for infant and young children, milk and milk products
Qflex kit test pantothenic acid PI[a]	AOAC Performance Tested Methods[sm]	Current	Optical Biosensor immunoassay	Total Pantothenate[a]	cereals, milk powder, milk based infant formula, soy-based infant formula, pet foods, fortified beverages, dietary vitamin supplement tablets and premixes

[a] *Cross reactivity with other compounds.*

Continued Table 6. Overview of the standardised methods of analysis for pantothenic acid in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	Spike recovery	LOD	Quantitation range	RSD_r	RSD_R
AOAC 992.07	-	-	0.4 µg/kg	-	4.59%	10.23%
GB 5413.17-2010: part 1	-	-	-	-	3.5%	-
GB 5413.17-2010: part 2	-	-	0.1 mg/100 g	-	3.5%	-
Qflex kit test pantothenic acid PI[a]	114.7%	-	4.4 ng/mL[b]	>18.9 ng/mL[b]	0.9-6.9%	7.2-25.2%

[a] *Subjected to significant spectral influence. Uncertain whether phosphorylated forms captured*
[b] *Instrumental LOD and LOQ*
LOD = *Limit of Detection*
RSD_r = *Repeatability relative standard deviation*
RSD_R = *Riproducibility relative standard deviation*

Annex VIII
EU compositional provisions and overview of the standardised methods of analysis for Vitamin B6 in infant and follow-on formula

Definition of compound (2006/141/EC): Vitamin B6

Potential forms of the nutrient: pyridoxine, pyridoxal, pyridoxamine and the related phosphorylated forms.

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg/100kJ		mg /L*			µg/100kJ		mg /L*	
9	42	0.225	1.24		9	42	0.225	1.24

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 7. Overview of the standardised methods of analysis for vitamin B6 in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 2004.07	AOAC official method. Codex type II for IF	Current	HPLC with fluorometric detection	Total vitamin B_6 except glycosilated forms	Milk and soy-based infant formula
EN 14164:2008	European standard. Codex type II for IF	Current. Under revision	HPLC with fluorometric detection	Total vitamin B_6 except glycosilated forms	Milk and soy-based infant formula
EN 14663:2005	European standard. Codex type III for IF	Current	HPLC with fluorometric detection	Total vitamin B6	Validated insemolina with milk, potato puree, vegetables with ham (baby food); multi vitamin drink
AOAC 985.32	AOAC official method. Codex type III for IF	Current	Microbiological method	Total vitamin B_6 except glycosilated forms	Ready-to-eat milk-based infant formula
EN 14166:2009	European standard. Codex type III for IF	Current	Microbiological method	Total vitamin B_6 except glycosilated forms	wholemeal flour, milk powder, mixed vegetables and pigs liver
GB 5413.13-2010	Chinese	Current	HPLC with fluorometric detection	Total vitamin B_6 except glycosilated forms	Foods for infant and young children, milk and milk products

Continued Table 7. Overview of the standardised methods of analysis for vitamin B6 in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	**Spike recovery**	**LOD**	**Quantitation range**	**RSD_r**	**RSD_R**
AOAC 2004.07	-	86.1-95.9%	-	4 µg - 1 mg/100g	2.0-16.4%	8.2-52.1%
EN 14164:2008	-	86.1-95.9%	-	4 µg - 1 mg/100g	2.0-16.4%	8.2-52.1%
EN 14663:2005	94-102%	-	-	0.034 mg/100 g to 1.21 mg/100 g	3.6-9.9% Milk powder: 6.3%	Semolina with
AOAC 985.32	-	-	-	-	2.4-5.9%	Milk, powder: 10.2%
EN 14166:2009	-	-	-	0,5 mg/100 g to 1,9 mg/100 g	5-9%; Milk powder=6%	13.0-17.7%
GB 5413.13-2010	-	-	1.5 µg/100 g, 1.3 µg/100 g , 1.6 µg/100 g for pyridoxine, pyridoxal and pyridoxamine respectively	-	3.5%	15-28%; Milk powder=15%

LOD = limit of detection;
RSD_r = Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation.

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Annex IX
EU compositional provisions and overview of the standardised methods of analysis for Biotin (vitamin B7) in infant and follow-on formula

Definition of compound (2006/141/EC): Biotin

Potential forms of the nutrient: biotin, biocytin (e-N-biotinyl-L-lysine), oxybiotin. Protein bound biotin

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg/100kJ		µg /L*			µg/100kJ		µg /L*	
0.4	1.8	10	53		0.4	1.8	10	53

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 8. Overview of the standardised methods of analysis for biotin in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
Infant formula council C-1, 1982	-	Current	Microbiological method	Biotin	Liquid and powdered infant formula
EN 15607:2009	European standard. Codex type II for IF	Current	HPLC with fluorometric detection	Total Biotin (free+biocytin)	Cereal breakfast powder, infant milk powder, lyophilized green peas with ham, lyophilized chicken soup and on nutritive orange juice
Qflex kit test biotin[a]	AOAC Performance Tested Method[sm]	Current	Optical Biosensor immunoassay	Biotin[a]	Foods for infant and young children, milk and milk powder
GB 5413.19-2010	Chinese	Current	Microbiological method	Biotin	Cereals, milk powder, milk-based infant formula, soy-based infant formula, milk-free rice infant formula, vitamin premixes, and dietary vitamin supplements

[a] *Cross reactivity with other compounds.*

Continued Table 8. Overview of the standardised methods of analysis for biotin in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	Spike recovery	LOD	Quantitation range	RSD_r	RSD_R
Infant formula council C-1, 1982	-	-	-	-	-	-
EN 15607:2009	-	-	-	16-200 µg/100g powder	11.6%	29.8%
Qflex kit test biotin[a]	96%	89-102%	2 µg/100 g	8-280 µg/100 g powder	6.6%	9.1%
GB 5413.19-2010	-	-	2 µg/100 g	-	3.5%	-

[a] *Cross reactivity with other compounds;*
LOD = Limit of Detection;
RSD_r = Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation.

Annex X
EU compositional provisions and overview of the standardised methods of analysis for Folic acid (vitamin B11) in infant and follow-on formula

Definition of compound (2006/141/EC): Folic acid

Potential forms of the nutrient: Folic acid, tetrahydrofolic acid (THF), 5-methyl-, 10-methyl-, 5-formyl-, 10-formyl-THF as both monoglutammate and poliglutammate

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg/100kJ		µg /L*			µg/100kJ		µg /L*	
2.5	12	62.5	156.4		2.5	12	62.5	156.4

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 9. Overview of the standardised methods of analysis for folic acid in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 992.05	AOAC official method. Codex type II for IF	Current	Microbiological method	free folic acid + free, unbound natural folates	Milk-based liquid ready-to-feed infant formula
EN 14131:2003	European standard. Codex type II for IF	Current	Microbiological method	total folates (free+bound)	Milk powder, wholemeal flour, mixed vegs and pig liver
GB 5413.16-2010	Chinese	Current	Microbiological method	Folic acid	Foods for infant and young children, milk and milk products
Qflex test Kit FOLIC ACID	AOAC Performance Tested Methodsm	Current	Optical Biosensor immunoassay	free folic acid + proportion of free, natural folate	Milk and infant formula
AOAC 2011.05	AOAC official method	Current	Optical Biosensor immunoassay	Folate and 5-methyl-tetrahydrofolate polyglutamate	Milk powder, infant formula (NIST SRM 1846 AND 1849)
AOAC 2011.06	AOAC official method	Current	UPLC with MS/MS detection	all the monoglutamates forms of folic acid	Infant formula, baby foods, protein powder,

Continued Table 9. Overview of the standardised methods of analysis for folic acid in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	Spike recovery	LOD	Quantitation range	RSD_r	RSD_R
AOAC 992.05	-	96.9%	-	-	9.35%	25.44%
EN 14131:2003	-	-	-	-	6.7%	16.9%
GB 5413.16-2010	-	-	-	-	3.5%	-
Qflex test Kit FOLIC ACID	-	-	4 µg/100 g powder	8-280 µg/ 100 g powder	4.4%	8.1%
AOAC 2011.05	102.3% - 107.5%	-	2.5 µg/100g powder	10-1000 µg/ 100g powder	3.48%	4.63%
AOAC 2011.06	-	94.10%-101.34%	2.0 µg/100 g powder	5 µg-2g/100g powder	6.25%	-

LOD = Limit of Detection;
RSD_r = Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation.

Annex XI
EU compositional provisions and overview of the standardised methods of analysis for Vitamin B12 in infant and follow-on formula

Definition of compound (2006/141/EC): Vitamin B12

Potential forms of the nutrient: Cyano-, hydroxy-, acquo-, methyl- and adenosylcobalamin.

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg/100kJ		µg /L*			µg/100kJ		µg /L*	
0.025	0.12	0.625	3.54		0.025	0.12	0.625	3.54

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 10. Overview of the standardised methods of analysis for vitamin B12 in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 986.23	AOAC official method. Codex type II for IF	Current	Microbiological method	Total vitamin B_{12}	Milk-based infant formula
GB 5413.14-2010	Chinese	Current	Microbiological method	Total vitamin B_{12}	Foods for infant and young children, milk and milk products
AOAC 2011.01	AOAC official method	Current	Optical Biosensor immunoassay	Total vitamin B_{12}	Fortified Bovine Milk-Based Infant Formula Powder, Fortified Soya-Based Infant formula powder, premixes, supplements
Qflex test kit vit B_{12} PI	AOAC Performance Tested Method^{sm}	Current	Optical Biosensor immunoassay	Total vitamin B_{12}	Cereals, milk powder, milk-based formula, fortified beverages, vitamin premixes, and dietary supplements
AOAC 2011.08	AOAC official method	Current	LC with UV detection	Total vitamin B_{12}	Milk- and soy-based infant formula, cereals, cocoa beverages, health care products and vitamins premixes
AOAC 2011.09	AOAC official method	Current	HPLC with UV detection	Total vitamin B_{12}	Milk-based infant formula powder
AOAC 2011.10	AOAC official method	Current	HPLC with visible detection	Total vitamin B_{12}	Milk- and soy-based infant formula

Continued Table 10. Overview of the standardised methods of analysis for vitamin B12 in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	**Spike recovery**	**LOD**	**Quantitation range**	**RSD_r**	**RSD_R**
AOAC 986.23	-	-	0.01 µg/100g powder	0.1-10 µg/ 100g powder	1.7-2.9%	12.2-17.1%
GB 5413.14-2010	-	-	-	-	-	-
AOAC 2011.01	-	98.6%	0.15µg/100 g powder	0.525 µg/ 100 g powder	1.59-6.82%	9.74-11.9%
Qflex test kit vit B_{12} PI	105%	-	0.15µg/100 g powder	0.525 µg/ 100 g powder	16.7%	25.8%
AOAC 2011.08	-	93-107%	0.1 µg/100g powder	0.30-13 µg/ 100g powder	2.1-3.0%	4.3% (RSD_{IR})
AOAC 2011.09	-	-	-	-	-	-
AOAC 2011.10	-	91.9-108%	-	>0.06 µg/ 100 g ready-to-feed infant formula	1.99-6.44%	0.80-5.71% (RSD_{IR})

LOD = *Limit of Detection;*
RSD_r = *Repeatability relative standard deviation;*
RSD_R = *Riproducibility relative standard deviation;*
RSD_{IR} = *Intermediate reproducibility relative standard deviation.*

Annex XII
EU compositional provisions and overview of the standardised methods of analysis for Vitamin C in infant and follow-on formula

Definition of compound: Vitamin C

Potential forms of the nutrient: L-ascorbic acid, L-dehydroascorbic acid, ascorbyl palmitate

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
mg/100kJ		mg /L*			mg/100kJ		mg /L*	
2.5	7.5	62.5	221.25		2.5	7.5	62.5	221.25

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 11. Overview of the standardised methods of analysis for vitamin C in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 984.26	AOAC official method	Current	Fluorometry	L- ascorbic acid, L-dehydroascorbic acid	All foods. Validated in dry soy-based and liquid milk-based infant formula
AOAC 985.33	AOAC official method	Current	Redox Titration	L- ascorbic acid	Ready to feed milk-based infant formula
EN 14130:2003	European standard. Codex type II for infant formula	Current	HPLC with UV detection	L- ascorbic acid, L-dehydroascorbic acid	Orange juice, liquid soup, powder milk, freeze-dried soup, breakfast cereals and fruits baby food
GB 5413.18-2010	Chinese	Current	Fluorometry	L- ascorbic acid, L-dehydroascorbic acid	Foods for infant and young children, milk and milk products
GOST 30627.2:1998	Russian	Current	Redox Titration	Ascorbic acid	Infant formula

Continued Table 11. overview of the standardised methods of analysis for vitamin C in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	Spike recovery	LOD	Quantitation range	RSD_r	RSD_R
AOAC 984.26	-	-	-	-	3.2%	3.61-12.58%
AOAC 985.33	-	-	-	-	4.5-10.4%	4.7-10.1%
EN 14130:2003	-	99.1-102%	-	-	3.6-9.9% Milk powder: 6.3%	11.4-21.6% Milk powder: 11.4%
GB 5413.18-2010	-	94%	0.1 mg/100g	-	3.8%	-
GOST 30627.2:1998	96-107%	-	-	-	2.5%	5.3%

LOD = *Limit of detection;*
RSD_r = *Repeatability relative standard deviation;*
RSD_R = *Riproducibility relative standard deviation.*

Annex XIII
EU compositional provisions and overview of the standardised methods of analysis for Vitamin K in infant and follow-on formula

Definition of compound (2006/141/EC): Vitamin K

Potential forms of the nutrient: Trans- and cis-Phylloquinone (Vit K1), menaquinones (Vit K2)

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
µg /100kJ		µg /L*			µg /100kJ		µg /L*	
1	6	25	177		1	6	25	177

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 12. Overview of the standardised methods of analysis for vitamin K in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 992.27[a]	AOAC official method	Current	LC with UV detection	cis- and trans-K_1	Fortified milk and Ready to feed milk-based infant formula
AOAC 999.15[b]	AOAC official method . Codex type II for infant formula	Current	HPLC with fluorometric detection	Either aggregated cis + trans K_1 or individual cis and trans forms depending on LC column. Dihydro- K_1 and Menaquinones	Ready-to-feed milk, whey-and soya-based infant formula powders, fluid and powdered milk
EN 14148:2003[b]	European standard. Codex type II for infant formula	Current	HPLC with fluorometric detection	Either aggregated cis + trans K_1 or individual cis and trans forms depending on LC column. Dihydro- K_1 and Menaquinones	Ready-to-feed milk, whey-and soya-based infant formula powders, fluid and powdered milk

[a] *In corn oil containing infant formula , only concentration of trans-vit K1 can be calculated; Poor specificity;*
[b] *both cis- and trans- K_1 are determined. The cis-form is inactive. To separate the two vitamers the C18 HPLC column must be replaced by a C30 HPLC column.*

Continued Table 12. Overview of the standardised methods of analysis for vitamin K in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	Spike recovery	LOD	Quantitation range	RSD_r	RSD_R
AOAC 992.27[a]	-	98±4% (trans), 95 ± 9% (cis)	-	75-130 µg/L trans vit K_1	15.3-24.3%	21.4-28.9%
AOAC 999.15[b]	-	98.3-98.6%	-	>1 µg Vitamin K_1/ 100 g solids	2.59-5.68%	5.98-18.19%
EN 14148:2003[b]	-	98.3-98.6%	-	>1 µg Vitamin K_1/ 100 g solids	2.59-5.68%	5.98-18.19%

[a] *In corn oil containing infant formula , only concentration of trans-vit K1 can be calculated; Poor specificity;*
[b] *both cis- and trans- K1 are determined. The cis-form is inactive. To separate the two vitamers the C18 HPLC column must be replaced by a C30 HPLC column;*
LOD = Limit of Detection;
RSD_r = Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation.

Annex XIV
EU compositional provisions and overview of the standardised methods of analysis for Vitamin E in infant and follow-on formula

Definition of compound (2006/141/EC): Vitamin E

Potential forms of the nutrient: D- and L- α, β, γ and δ tocopherol, tocotrienols and relative esters

Limits (2006/141/EC)							
Infant formula				Follow-up formula			
Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
mg*/ 100kJ		mg* /L**		mg*/ 100kJ		mg* /L**	
0,5/g of polyunsaturated fatty acids expressed as linoleic acid as corrected for the double bonds (***) but in no case less than 0,1 mg per 100 available kJ	1.2	2.5	35.4	0,5/g of polyunsaturated fatty acids expressed as linoleic acid as corrected for the double bonds (***) but in no case less than 0,1 mg per 100 available kJ	0.12	2.5	35.4

* *α-tocopherol equivalent;*
** *Minimum Values /L have been calculated taken into account the minimum and provision for energy as reported in annex I and II of EU Commission directive 2006/141/EC;*
*** *0,5 mg α-TE/1 g linoleic acid (18:2 n-6); 0,75 mg α-TE/1 g α-linolenic acid (18:3 n-3); 1,0 mg α-TE/1 g arachidonic acid (20:4 n-6); 1,25 mg α-TE/1 g eicosapentaenoic acid (20:5 n-3); 1,5 mg α-TE/1 g docosahexaenoic acid (22:6 n-3).*

Table 13. Overview of the standardised methods of analysis for vitamin E in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 992.03	AOAC official method. Codex type III for IF	Current	HPLC with UV detection	Free and bound All-rac-α-tocopherol	Milk-based liquid ready-to-feed infant formula
AOAC 971.30	AOAC official method. Codex type IV for special foods	Current	Colorimetric	α-tocopherol and α-tocopherol acetate	Milk and milk products, feed. Dry products
EN 12822:2000	European standard. Codex type II for IF	Current. Under revision	HPLC with UV or fluorometric detection	Free and bond α, β, γ and δ tocopherol	Margarine, milk powder and oat powder
AACC 86.06	-	Current	HPLC with fluorometric detection	Free and bound All-rac-α-tocopherol	Infant formula
GB 5413.9-2010	Chinese	Current	HPLC with UV detection	α-tocopherol	Foods for infant and young children, milk and milk products
GOST 30627.3:1998	Russian	Current	Redox titration	α-tocopherol	Infant formula
AOAC 2011.07	AOAC official method	Current	UPLC with fluorometric detection	Free and bound All-rac-α-tocopherol	Milk-based infant formula
Determination of α-tocopherol acetate by HPLC and column switching	-	Draft	HPLC with UV detection	A-tocopheryl acetate. No α-tocopherol	Infant, adult, pediatric formula (powders, ready-to-feed liquids, liquid concentrates)
AOAC 2001.13	AOAC official method	Current	HPLC with fluorometric detection	Free and bound All-rac-α-tocopherol	Infant formula (powdered)

Continued Table 13. Overview of the standardised methods of analysis for vitamin E in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	**Spike recovery**	**LOD**	**Quantitation range**	**RSD_r**	**RSD_R**
AOAC 992.03	-	100.4%	-	-	8.46%	11.69%
AOAC 971.30	-	100%	-	-	7.70-9.03%	4.8%
EN 12822:2000	-	-	-	-	<11.1% (milk powder)	<19.6% (milk powder)
AACC 86.06	-	118%	-	>100 µg/ 100g powder	1.5%	13.9%
GB 5413.9-2010	-	93-107%	10 µg/100g	-	1.8%	-
GOST 30627.3:1998	-	-	-	-	7.1%	5.3%
AOAC 2011.07	-	97.2%	-	0.22-150 mg/ 100 g powder	7.1%	8.7% (RSD_{IR})
Determination of α-tocopherol acetate by HPLC and column switching	97.3-102%	-	-	>250 µg/100g ready-to-feed	-	0.71-2.15% (RSD_{IR})
AOAC 2001.13	118.56%	-	-	>100 µg/ 100g powder	-	4.65-13.91%

LOD = Limit of Detection;
RSD_r = Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation.

Annex XV
EU compositional provisions and overview of the standardised methods of analysis for choline in infant and follow-on formula

Definition of compound (2006/141/EC): Choline

Potential forms of the nutrient: Choline, phosphatidylcholine, acetylcholine

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
mg /100kJ		mg/L*			mg /100kJ		mg/L*	
1.7	12	42.5	354		-	-	-	-

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 14. overview of the standardised methods of analysis for choline in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
AOAC 999.14[a]	AOAC official method	Current	Enzymatic colorimetric method	Total choline excluded acid-resistant choline	Foods for infant and young children, milk and milk products
GB 5143.20-1997: Part 1	Chinese	Current	Enzymatic colorimetric method	Total choline	Infant food, milk and dairy products
GB 5143.20-1997: Part 2	Chinese	Current	Colorimetry	Total choline	Infant food, milk and dairy products
GB 5143.20-1997: Part 3	Chinese	Current	Ion Chromatography with electric conductivity detection	Total choline	Infant food, milk and dairy products

[a] *Method does not apply to powdered infant formula/milk containing > 100 mg vitamin C/100 g solids because of ascorbate suppression of color development.*

Continued Table 14. overview of the standardised methods of analysis for choline in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	Spike recovery	LOD	Quantitation range	RSD_r	RSD_R
AOAC 999.14[a]	4.41-6.34%	94.8-100.8%	-	45-174 mg choline/100 g solids	2.24-3.46%	4.41-6.34%
GB 5143.20-1997: Part 1	-	-	2.0 mg/100g	-	2.8%	-
GB 5143.20-1997: Part 2	-	-	5 mg/100g	-	2.8%	-
GB 5143.20-1997: Part 3	-	-	0.2 mg/100g	-	-	-

[a] *Method does not apply to powdered infant formula/milk containing > 100 mg vitamin C/100 g solids because of ascorbate suppression of color development;*
LOD = Limit of Detectio;
RSD_r = Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation.

Annex XVI
EU compositional provisions and overview of the standardised methods of analysis for inositol in infant and follow-on formula

Definition of compound: Inositol

Potential forms of the nutrient: Free myo-inositol, phosphatidylinositol, methyl esters, glycosides, phosphorylated forms and phytate

Limits (2006/141/EC)								
Infant formula					Follow-up formula			
Minimum	Maximum	Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
mg /100kJ		mg /L*			mg /100kJ		mg /L*	
1	10	25.0	295		-	-	-	-

* *Minimum and Maximum Values /L have been calculated taken respectively into account the minimum and the maximum provisions for energy as reported in annex I and II of EU Commission directive 2006/141/EC.*

Table 15. overview of the standardised methods of analysis for inositol in infant and follow-on formula.

Method name	Type of standard	Status	Analytical principle	Target compounds	Matrix validated
GB 5413.25-2010: Part 1	Chinese	Current	GC-FID	inositol	Foods for infant and young children, milk and milk products
GB 5413.25-2010: Part 2	Chinese	Current	Microbiological method	Inositol	Foods for infant and young children, milk and milk products
Ion chromatographic determination of inositol in infant formula and clinical products for enteral feeding	-	Draft	Ion exchange HPLC-pulsed amperometric detection	Free myo-inositol	Infant formula and clinical products for enteral feeding
Ion chromatographic determination of inositol in infant formula and clinical products for enteral feeding	-	Draft	Ion exchange HPLC-pulsed amperometric detection	Total myo-inositol	Infant, paediatric and adult nutritional products
Determination of myo-inositol by GC-FID after hydrolysis of bound inositol[a]	-	Draft	GC-FID	Total myo-inositol (except phytate)	Infant, adult, pediatric formula (powders, ready-to-feed liquids, liquid concentrates)

[a] *This method will be splitted in two parts (the original GC methods that were combined to give birth to the present one) and each validated separately.*

Continued Table 15. overview of the standardised methods of analysis for inositol in infant and follow-on formula.

Method name	Method performance characteristics					
	Accuracy	**Spike recovery**	**LOD**	**Quantitation range**	**RSD_r**	**RSD_R**
GB 5413.25-2010: Part 1	-	-	2.0 mg/100g	-	3.5%	-
GB 5413.25-2010: Part 2	-	-	2.0 mg/100g	-	3.5%	-
Ion chromatographic determination of inositol in infant formula and clinical products for enteral feeding	-	99.1-102%	-	> 5 mg/L	1.43-4.02%	0.715-2.49% (RSD_{IR})
Ion chromatographic determination of inositol in infant formula and clinical products for enteral feeding	-	94%	-	> 5 mg/L	<5.8%	<15.6% (RSD_{IR})
Determination of myo-inositol by GC-FID after hydrolysis of bound inositol[a]	96-107%	-	1 mg/100g	>3 mg/100g	5.7%	12%

[a] *This method will be splitted in two parts (the original GC methods that were combined to give birth to the present one) and each validated separately;*
LOD = Limit of Detection;
RSD_r = Repeatability relative standard deviation;
RSD_R = Riproducibility relative standard deviation;
RSD_{IR} = Intermediate relative standard deviation.

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