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Food products — Guidance on how to express vitamins and their vitamers content

Produits alimentaires — Lignes directrices pour exprimer les teneurs en vitamines et en leurs vitamères

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Vitamins can be naturally found in foods in different molecular forms. In more elaborated food products, vitamins can be used for fortification by adding several molecular forms with different levels of vitamin activity. There are regulations governing the addition of vitamins in food products. The authorized compounds for fortification depend on the type of food. Regulation deals with, for instance baby food or food supplements. The main problem is that the vitamin activities of the authorized compounds are not clearly described.

At the same time, it is not mandatory to list the chemical name of the compound used for food fortification purposes according to food labelling regulations. For example, vitamin E can be written in the list of ingredients without knowing if it is D-alpha tocopherol or D,L-alpha tocopherol, even though each molecular form presents different vitamin E activity.

ISO and CEN analytical standards express results in mass units related to the vitamin standard used for quantification. As expression in specific units of vitamin activity can be linked to regional/national regulatory requirements, analytical methods do not give guidance for this conversion. This document proposes ways to express the vitamin content in order to facilitate harmonization between different laboratories and also to reduce misunderstanding of the results expressed in vitamin content and in some cases in the vitamin activity of vitamers.

Food products — Guidance on how to express vitamins and their vitamers content

1 Scope

This document provides guidelines on:

— how to express vitamin quantity,

— the expression of different molecular forms in appropriate units,

— and in some cases, vitamin activity, according to vitamers present or used in food products, in addition to the quantitative content determination obtained from ISO and CEN analytical standards.

It provides information to be used as a basis for discussion between stakeholders and food control laboratories. It is not intended to be prescriptive or exhaustive.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
vitamer
any of a number of chemical compounds of a particular vitamin, generally having a similar molecular structure, each of which shows vitamin activity in a vitamin-deficient biological system

Note 1 to entry: See Reference [1].

4 Guidelines per vitamins

4.1 General

This document begins by addressing vitamin E, as this vitamin is considered to be the most complex in terms of variation of possible units of measurement. Vitamin E is followed by the other fat-soluble vitamins and water-soluble vitamins respectively.

To include the diversity of available conversion factors, regulations in different countries/regions are evaluated, including the European Union (EU), Regulations of the United States Food and Drug Administration (USFDA), United States of America (USA), National Standard (GB) of China, Food Standards Australia/New Zealand (FSANZ). In addition, standards of global organizations (CODEX[2]) are included.

4.2 Vitamin E

Vitamin E is a fat-soluble vitamin. Vitamin E is mainly found in the fat of the different food products as tocopherols and tocotrienols esters.

Food regulations of many countries authorize the fortification of different kind of food products with molecules having a vitamin E activity. The names of the authorized molecular forms to add are generally linked to the type of food product. Table 1 gives an example of vitamin E compounds permitted for use for food fortification according to EU regulations, depending on the product type.

Table 1 — Example of authorized vitamin E compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	D-alpha-tocopherol	430,7	Regulation (EU) n° 609/2013[3]
	DL-alpha-tocopherol	430,7	
	D-alpha-tocopheryl acetate	472,8	
	DL-alpha-tocopheryl acetate	472,8	
	D-alpha-tocopheryl polyethylene glycol 1000 succinate		
Supplemented food	D-alpha-tocopherol	430,7	Regulation (EC) n°1925/2006[4]
	DL-alpha-tocopherol	430,7	
	D-alpha-tocopheryl acetate	472,8	
	DL-alpha-tocopheryl acetate	472,8	
	D-alpha-tocopheryl acid succinate	530,8	
Food supplements	D-alpha-tocopherol	430,7	Directive n°2002/46/EC[5]
	DL-alpha-tocopherol	430,7	
	D-alpha-tocopheryl acetate	472,8	
	DL-alpha-tocopheryl acetate	472,8	
	D-alpha-tocopheryl acid succinate	530,8	
	Mix of tocopherols and tocotrienols		

There is no global convention on how the amount of vitamin E should be expressed for labelling purposes. In Table 2 an overview of units of measurement for vitamin E is given according to several national/regional regulations and a CODEX standard.

Table 2 — Definition of vitamin E for labelling purposes in several standards/regulations

US (until 2019-12-31)	US (as from 2020-01-01)	EU	CODEX	China/Australia/New Zealand
IU[a]	mg alpha-tocopherol	mg alpha – TE[b]	mg alpha - TE	mg alpha – TE

[a] International Unit, a measure of biological activity, different for each substance.

[b] Tocopherol equivalent.

Table 2 *(continued)*

US (until 2019-12-31)	US (as from 2020-01-01)	EU	CODEX	China/Australia/New Zealand
1 IU = 0,67 mg for D-alpha tocopherol (natural)	1 mg vitamin E (as alpha-tocopherol) label claim = 1 mg of natural alpha-tocopherol (D-alpha-tocopherol)	1 mg alpha – TE = 1 mg D-alpha tocopherol	1 mg alpha – TE = 1 mg D-alpha tocopherol	1 mg alpha – TE = 1 mg D-alpha tocopherol
1 IU = 0,9 mg for D, L -alpha tocopherol (synthetic)	1 mg vitamin E (as alpha-tocopherol) label claim = 2 mg of synthetic alpha-tocopherol (D,L-alpha-tocopherol			

[a] International Unit, a measure of biological activity, different for each substance.

[b] Tocopherol equivalent.

The vitamin E unit of measurement is generally defined as 1 mg of D-alpha-tocopherol (natural form). There is currently no standard or official analytical method able to quantify this natural form specifically.

There are two main analytical approaches for quantifying vitamin E:

1) Saponification of tocopheryl esters to liberate them as free tocopherols. Solvent extraction and separation of the individual tocopherols by liquid chromatography. Quantification by fluorescence detection.

   An example is EN 12822[6], which includes a saponification and an extraction step. It enables the separation by a normal phase HPLC, and the quantification of the free alpha, beta, gamma and delta tocopherols using fluorescence detection. Tocotrienols are also separated but not quantified. Tocotrienols are not taken into account for the expression of the vitamin result. The D-tocopherols are not distinguished from the D,L-tocopherols.

2) Direct solvent extraction and separation of different esters and free tocopherols by liquid chromatography. Quantification by fluorescence detection.

   An example is ISO 20633, where the hydrophilic coating of fat micelles is hydrolysed by an enzyme. The hydrophobic contents of the micelles are then extracted into iso-octane. The extract is analysed by normal phase HPLC. Alpha-tocopherol and alpha-tocopheryl acetate are quantified using fluorescence detection.

By taking into account the molecular mass and the activity ratio of the D and the D,L the following values can be found[8]:

— 1 mg of vitamin E = 1 mg of D-alpha-tocopherol = 1 mg alpha-TE;

— 1 mg of vitamin E = 1,35 mg of D,L-alpha-tocopherol;

— 1 mg of vitamin E = 1,1 mg of D-alpha-tocopheryl acetate;

— 1 mg of vitamin E = 1,49 mg of D,L-alpha-tocopheryl acetate;

— 1 mg of vitamin E = 1,23 mg of D-alpha-tocopheryl acid succinate.

As mentioned above, there is no specific detection at the quantification step for the D-alpha tocopherol (natural form) and the D,L-alpha tocopherol (synthetic form) respectively. So, when a sample is analysed, and there is no specific information about the added form (D or D,L) on the label of the product, there can be a misunderstanding about the true value of the vitamin E content. The difference can be a factor of 1,35 or higher.

Regarding the nutritional aspect, there is no general agreement about the activities of the other forms of tocopherols and tocotrienols.

When in the presence of certain amounts of beta and/or gamma and/or delta tocopherols, it is often underlined that the relative vitamin E activities should be included in the final result. The more common coefficients[8] are:

— 1 mg of D-beta-tocopherol = 0,5 mg of vitamin E;

— 1 mg of D-gamma-tocopherol = 0,1 mg of vitamin E;

— 1 mg of D-delta-tocopherol = 0,03 mg of vitamin E.

Tocotrienols are sometimes also taken into account by certain guidelines, such as the FAO/INFOODS guideline[9] as in the following example:

Vitamin E (mg/100 g) = alpha-tocopherol (mg/100 g) + 0,4 beta-tocopherol (mg/100 g) + 0,1 gamma-tocopherol (mg/100 g) + 0,01 delta-tocopherol (mg/100 g) + 0,3 alpha-tocotrienol (mg/100 g) + 0,05 beta-tocotrienol (mg/100 g) + 0,01 gamma-tocotrienol (mg/100 g)

Despite this fact, in most of the cases, only the alpha-tocopherol is considered.

At the session of the Codex Committee on Nutrition and Foods for Special Dietary Uses (40th Session, 5th - 9th December 2016, Hamburg, Germany), it was proposed to take only the alpha-tocopherol into account for labelling purposes [10]. In this case, the expression of vitamin E is very simple:

vitamin E = 1 mg of alpha-D-tocopherol = 1 mg RRR-alpha-tocopherol.

Taking into account most of these parameters, the vitamin E content can be calculated as follows:

a) If only D-alpha tocopherol (natural) is present in the product, the vitamin E content (expressed in D-alpha tocopherol equivalent) can be calculated as vitamin E (mg/100 g) = alpha-D-tocopherol

   or in some cases, e.g. for vegetable oils:

   vitamin E (mg/100 g) = alpha-tocopherol + 0,5 beta-tocopherol + 0,1 gamma-tocopherol +

   0,03 delta-tocopherol.

b) If D,L alpha tocopherol (synthetic) is present in the product, the alpha-tocopherol result is divided by 1,35 and the vitamin E content (expressed in D-alpha tocopherol equivalent) can be calculated as vitamin E (mg/100 g) = 0,74 alpha-tocopherol (mg/100 g)

   or in some cases:

   vitamin E (mg/100 g) = 0,74 alpha-tocopherol (mg/100 g) + 0,5 beta-tocopherol (mg/100 g) + 0,1 gamma-tocopherol (mg/100 g) + 0,03 delta-tocopherol (mg/100 g).

c) If the vitamin E molecular form is not known, it is recommended that the two former possibilities be studied. Follow-up action with the producer of the product can be necessary before a conclusion can be drawn.

d) Some methods do not involve a saponification step, like in ISO 20633. In these cases, the calculation is adapted as follows:

   vitamin E (mg/100 g) = D-alpha tocopherol (mg/100 g) + 0,909 D-alpha tocopherol acetate (mg/100 g).

In addition, it is important to consider that apart from adding vitamin E for food fortification purposes, it is common practice and permitted to add tocopherols as food additives (antioxidants) to food products, including:

— E 306: rich tocopherols extracts;

— E 307: alpha tocopherol;

— E 308: gamma tocopherol;

— E 309: delta tocopherol.

At the analysis step, this can lead to a difference between the vitamin E quantified value and the vitamin E labelled value because no test is capable of identifying the part of vitamin E added for nutritional purposes from the part added for antioxidation. In any case, the labelled vitamin E value should not include the tocopherol forms added as antioxidants.

4.3 Vitamin A

Vitamin A is a fat-soluble vitamin. Vitamin A is mainly found in the fat of the different food products as retinol esters.

The food regulations of many countries authorize the fortification of different kinds of food products with molecules having a vitamin A activity. The names of the authorized molecular forms to add in are generally linked to the type of food product. Table 3 gives an example of vitamin A compounds permitted for use for food fortification according to EU regulations, depending on the product type.

Table 3 — Example of authorized vitamin A compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	Retinol	286,5	Regulation (EU) n° 609/2013
	Retinyl acetate	328,5	
	Retinyl palmitate	524,9	
	Beta carotene	536,9	
Supplemented food	Retinol	286,5	Regulation (EC) n° 1925/2006
	Retinyl acetate	328,5	
	Retinyl palmitate	524,9	
	Beta carotene	536,9	
Food supplements	Retinol	286,5	Directive n°2002/46/EC
	Retinyl acetate	328,5	
	Retinyl palmitate	524,9	
	Beta carotene	536,9	

There is no global convention on how the amount of vitamin A should be expressed for labelling purposes. In Table 4 an overview of units of measurement for vitamin A is given according to several national/regional regulations and a CODEX standard.

Table 4 — Definition of vitamin A for labelling purposes in several standards/regulations

US (until 2019-12-31)	US (as from 2020-01-01)	EU	CODEX	China/Australia/New Zealand
IU	µg RAE[a]	µg RE	µg RE	µg RE
1 IU = 0,3 µg retinol	1 µg RAE = 1 µg retinol	1 µg RE = 1 µg retinol	1 µg RE = 1 µg retinol 1 µg retinol = 3,33 IU	1 µg RE = 1 µg retinol 1 µg retinol = 3,33 IU
1 IU = 0,6 µg of beta-carotene	1 µg RAE = 2 µg supplemental beta-carotene	6 µg beta-carotene = 1 µg RE	Carotenoids excluded	Carotenoids excluded
	1 µg RAE = 12 µg beta-carotene			
	1 µg RAE = 24 µg alpha-carotene			
	1 µg RAE = 24 µg beta-cryptoxanthin			

[a] Retinol activity equivalent.

[b] Retinol equivalent.

In general, the unit of measurement for vitamin A is µg RE and defined as 1 µg of retinol. The natural form is the trans retinol.

There are two main analytical approaches for quantifying vitamin A:

1) Saponification of retinol esters to liberate them as free retinol. Solvent extraction and separation of the cis and trans retinol by liquid chromatography. Quantification by UV or fluorescence detection.

   An example is EN 12823-1[11], which includes a saponification step and enables the separation and the quantification of all E-retinol (trans-retinol) and 13-Z-retinol (13-cis-retinol). EN 12823-2[12] enables the quantification of beta-carotene.

2) Direct solvent extraction and separation of different esters of retinol and retinol by liquid chromatography. Quantification by UV or fluorimetric detection.

   An example is ISO 20633, where the hydrophilic coating of fat micelles is hydrolysed by an enzyme. The hydrophobic contents of the micelles are then extracted into iso-octane. The extract is analysed by normal phase HPLC. Vitamin A palmitate (cis and trans) and vitamin A acetate (cis and trans) are quantified using UV detection.

By taking into account the molecular mass, the following values for retinyl esters can be found:

— 1 µg of all trans retinyl acetate = 0,872 µg RE (vitamin A);

— 1 µg of all trans retinyl palmitate = 0,546 µg RE (vitamin A).

In some regulations, beta-carotene can be included as vitamin A. In that case the beta-carotene content value should be transformed in RE. The correction factor is in most of the cases: 1 µg of retinol = 6 µg of beta-carotene.

In the literature, different factors for alpha-carotene and/or some other carotenoids can be taken into account like in the Souci-Fachmann-Kraut food composition and nutrition tables[13]:

vitamin A = retinol + 1/6 beta-carotene + 1/12 (other vitamin A active carotenoids).

Other vitamin A active carotenoids include alpha-carotene, gamma-carotene, cryptoxanthin and mutatochrome.

These carotenoids are usually not taken into account because of their low activity in comparison to beta-carotene and because of the need for additional assays.

To take into account most of these parameters, the vitamin A content can be calculated as:

vitamin A (µg/100 g) = retinol (µg/100 g) [all E-retinol (trans-retinol) + 13-Z-retinol (13-cis-retinol)]

or in some cases, depending on the regulation:

vitamin A (µg/100 g) = retinol (µg/100 g) + 0,167 beta-carotene (µg/100 g).

Some methods do not involve a saponification step, like in ISO 20633. In these cases, the calculation is adapted as follows:

vitamin A as retinol (µg/100 g) = 0,55 x retinyl palmitate (µg/100g) + 0,87 x retinyl acetate (µg/100 g).

4.4 Vitamin D

Vitamin D is a fat-soluble vitamin. Vitamin D is found in the fat of many food products. Vitamin D_2 is found in vegetables and vitamin D_3 is found in food from animal origin (for example meat, fish and milk).

Food regulations of many countries authorize the fortification of different kinds of food products with molecules having a vitamin D activity. The names of the authorized molecular forms to add in are generally linked to the type of food product. Table 5 gives an example of vitamin D forms permitted for use for food fortification according to EU regulations, depending on the product type.

Table 5 — Example of authorized vitamin D compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	Vitamin D_2	396,7	Regulation (EU) n° 609/2013
	Vitamin D_3	384,6	
Supplemented food	Vitamin D_2	396,7	Regulation (EC) n° 1925/2006
	Vitamin D_3	384,6	
Food supplements	Vitamin D_2	396,7	Directive n°2002/46/EC
	Vitamin D_3	384,6	

There are several vitamers of vitamin D. The two major forms are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D without a subscript refers to either vitamin D_2 or vitamin D_3 or both and is known as calciferol. There is global agreement about the units and the conversion factors to be used. In Table 6 an overview of units of measurement for vitamin D is given according to several national/regional regulations and a CODEX standard.

Table 6 — Definition of vitamin D for labelling purposes in several standards/regulations

US (until 2019-12-31)	US (as from 2020-01-01)	EU	CODEX	China / Australia/ New Zealand
IU	µg	µg	µg	µg
1 IU = 0,025 µg ergocalciferol or cholecalciferol	1 µg = 40 IU	1 µg vitamin D_2 or 1 µg vitamin D_3 (or 1 µg vitamin D_2 + vitamin D_3)	1 µg vitamin D_3	1 µg vitamin D_2 or 1 µg vitamin D_3 (or 1 µg vitamin D_2+vitamin D_3)
vitamin D_2 and/or vitamin D_3	vitamin D_2 and/or vitamin D_3	vitamin D_2 and/or vitamin D_3	vitamin D_3	vitamin D_2 and/or vitamin D_3

In general, the unit of measurement for vitamin D is µg cholecalciferol or ergocalciferol.

It is considered that for vitamin D, 1 µg of ergocalciferol (vitamin D_2) is equivalent to 1 µg cholecalciferol (vitamin D_3) because both have the same vitamin D activity.

According to former US regulation, the International Unit (IU) of vitamin D is equal to 0,025 µg of vitamin D_2 or vitamin D3. The IU can be still be found on labels.

There are two main analytical approaches for quantifying vitamin D:

1) Saponification of the matrix to liberate vitamin D_2 and vitamin D_3 as free forms. Solvent extraction. A first HPLC step to purify and collect the vitamin D_2 and vitamin D_3 fraction. Then separation of the vitamin D_2 and vitamin D_3 by liquid chromatography. Quantification by UV detection.

   An example is EN 12821[14], which includes a saponification step. A first HPLC step enables the purification and collection of vitamins D_2 and D_3. Then vitamin D_2 and vitamin D_3 are separated by a second chromatography. Both are quantified by UV detection. The drawback of this method is that vitamin D_2 and vitamin D_3 cannot be quantified simultaneously because one is used as an internal standard to quantify the other.

2) Saponification of the matrix to liberate vitamin D_2 and vitamin D_3 as free forms. Derivatization of the vitamin D to a higher molecular mass. Then separation by HPLC and quantification by mass spectrometry. The advantage of this principle is that vitamin D_2 and vitamin D_3 can be quantified simultaneously.

   An example is ISO 20636 where samples are saponified and vitamin D is extracted by isooctane. Vitamin D is derivatized and then quantified by mass spectrometry after a chromatographic step. Stable isotopes of vitamin D_2 and vitamin D_3 are used as internal standards.

To conclude, the vitamin D content can be calculated as:

vitamin D (µg/100 g) = ergocalciferol (µg/100 g) + cholecalciferol (µg/100 g).

4.5 Vitamin K

Vitamin K is a fat-soluble vitamin. Vitamin K can be found as vitamin K_1 in vegetables (spinach, lettuce, broccoli, Brussels sprouts) and as vitamin K_1 and vitamin K_2 in meat and liver.

Food regulations of many countries authorize the fortification of different kind of food products with molecules having a vitamin K activity. The names of the authorized molecular forms to add in are generally linked to the type of food product. Table 7 gives an example of vitamin K compounds permitted for use for food fortification according to EU regulations depending the product type.

Table 7 — Example of authorized vitamin K compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	Phylloquinone (phytomenadione – vitamin K_1)	450,7	Regulation (EU) n° 609/2013
	Menaquinone (vitamin K_2)		
	K_2-MK_4	444,6	
	K_2-MK_7	649,0	

Table 7 *(continued)*

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Supplemented food	Phylloquinone (phytomenadione – vitamin K_1)	450,7	Regulation (EC) n° 1925/2006
	Menaquinone (vitamin K_2)		
	K_2-MK_4	444,6	
	K_2-MK_7	649,0	
Food supplement products	Phylloquinone (phytomenadione – vitamin K_1)	450,7	Directive n°2002/46/EC
	Menaquinone (vitamin K_2)		
	K_2-MK_4	444,6	
	K_2-MK_7	649,0	

There are three main vitamers of vitamin K. The major form is vitamin K_1 (phylloquinone or phytomenadione or phytonadione). The other forms are vitamin K_2 (menaquinone) and vitamin K_3 (menadione).

In general, the unit of measurement for vitamin K is µg vitamin K_1 (phylloquinone) or vitamin K_2 (menaquinone).

Menaquinone (vitamin K_2) can also be added to different kinds of food in different molecular forms (Table 7). The difference in molecular form is linked to the length of the isoprenoid chain. The number of isoprenoid groups gives the name of the menaquinone. With the 4 isoprenoid group the name is vitamin K_2-MK4 and with the 7 isoprenoid group the name is vitamin K_2-MK7.

To conclude, the vitamin K content can be calculated as:

Vitamin K (µg/100 g) = vitamin K_1 (µg/100 g), or vitamin K_2 (µg/100 g).

It should be noted that a significant level of cis isomer can be found in synthetic vitamin K_1. Cis-vitamin K_1 is considered less bioavailable. However, food regulations do not distinguish between cis- and trans-vitamin K_1. From a food composition perspective, it can be more relevant to quantify trans-vitamin K_1, whereas from a regulatory/food labelling perspective total vitamin K_1 should be considered.

Most of methods quantify total vitamin K_1, but the recent IS ISO 21446 enables the quantification of the cis and the trans isomers separately.

In ISO 21446, vitamin K_1 is extracted from products with iso-octane after precipitation of proteins and release of lipids with methanol. Prepared samples are injected on a direct phase HPLC column where cis and trans vitamin K_1 are separated isocratically with an iso-octane-isopropanol mobile phase. The column eluent is mixed with a dilute ethanolic solution of zinc chloride, sodium acetate and acetic acid, and vitamin K_1 is reduced to a fluorescent derivative in a zinc reactor column. The resulting fluorescent compound is quantified with fluorimetric detection.

EN 14148[17], for example, includes the destruction of lipids by an enzymatic treatment and a post-column reduction before a fluorimetric quantification. It enables the quantification of total vitamin K_1. The two isomers (trans and cis) of vitamin K_1 are not separated. Vitamin K_2-MK4 and vitamin K_2-MK7 can be separated from vitamin K_1 by this method but are not yet included in the scope.

4.6 Thiamine (vitamin B_1)

Thiamine is a water-soluble vitamin. Thiamine can be found in a lot of food products mainly as phosphorylated forms.

Food regulations of many countries authorize the fortification of different kind of food products with molecules having a vitamin B_1 activity. The names of the authorized molecular forms to add in are generally linked to the type of food product. Table 8 gives an example of vitamin B_1 compounds permitted for use for food fortification according to EU regulations, depending on the product type.

Table 8 — Example of authorized vitamin B_1 compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	Thiamine hydrochloride	337,3	Regulation (EU) n° 609/2013
	Thiamine mononitrate	327,4	
Supplemented food	Thiamine hydrochloride	337,3	Regulation (EC) n° 1925/2006
	Thiamine mononitrate	327,4	
Food supplements	Thiamine hydrochloride	337,3	Directive n°2002/46/EC
	Thiamine mononitrate	327,4	
	Thiamine chloride monophosphate	380,8	
	Thiamine chloride pyrophosphate	460,8	

The commonly used unit of measurement for vitamin B_1 was 1 mg of thiamine hydrochloride, but nowadays it is considered to be 1 mg of thiamine base. The molecular mass of the thiamine cation is 265,3.

For expressing the different vitamin B_1 compounds with the different molecular masses, a correction factor is used:

— 1 mg of vitamin B_1 = 1 mg of thiamine base (M = 300,8);

— 1 mg of thiamine mononitrate (M = 327,4) = 0,919 mg of thiamine base (M = 300,8);

— 1 mg of thiamine hydrochloride (M = 337,3) = 0,892 mg of thiamine base (M = 300,8);

— 1 mg of thiamine ion (M = 265,3) = 1,134 mg of thiamine base (M = 300,8).

To conclude, the vitamin B_1 content can be calculated as:

vitamin B_1 (mg/100 g) = thiamine base (mg/100 g).

There are several analytical approaches for quantifying vitamin B_1. Vitamin B_1 should be liberated as a free form. Then it can be analysed by HPLC and quantified directly by mass spectrometry, or by fluorimetry after a derivatization step.

EN 14122[18], for example, includes an extraction of thiamine after an acid hydrolysis and an enzymatic treatment. Thiamine is separated by HPLC and quantified by fluorimetry after a pre- or post-derivatization.

ISO 21470, for example, specifies a method for the simultaneous quantitative determination of four water-soluble vitamins in infant formula and related nutritional products, including relevant forms of vitamins B_1, -B_2, -B_3 and -B_6 by enzymatic digestion and UHPLC-MS/MS quantification. ISO 21470 is not intended to be used on products where vitamins have not been added.

4.7 Riboflavin (vitamin B_2)

Riboflavin is a water-soluble vitamin. Riboflavin can be found in a lot of food products, mainly as phosphorylated forms.

Food regulations of many countries authorize the fortification of different kinds of food products with molecules having a vitamin B_2 activity. The names of the authorized molecular forms to add in are generally linked to the type of food product. Table 9 gives an example of vitamin B_2 compounds permitted for use for food fortification according to EU regulations, depending on the product type.

Table 9 — Example of authorized vitamin B_2 compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific dietary purpose	Riboflavin	376,4	Regulation (EU) n° 609/2013
	Riboflavin 5'phosphate sodium	478,3	
Supplemented food	Riboflavin	376,4	Regulation (EC) n° 1925/2006
	Riboflavin 5'phosphate sodium	478,3	
Food supplement products	Riboflavin	376,4	Directive n°2002/46/EC
	Riboflavin 5'phosphate sodium	478,3	

Nowadays, the commonly used unit of measurement for vitamin B_2 is 1 mg of riboflavin.

The vitamin B_2 content can be calculated as:

vitamin B_2 (mg/100 g) = riboflavin (mg/100 g).

It should be noted that food regulations allow the addition of riboflavin as a food additive (colouring agent) in food products with the following compounds:

— E 101 (i): Riboflavin;

— E 101 (ii): Riboflavin 5'phosphate;

— E 101 (iii): Riboflavin, from *Bacillus Subtilis*.

At the analysis step this can lead to a difference between the vitamin B_2 quantified value and the vitamin B_2 labelled value because no test is capable to identify the part of vitamin B_2 added for nutritional purpose from the part for colouring. In any case, the labelled vitamin B_2 value should not include the riboflavin added as a colouring agent.

There are several analytical approaches for quantifying vitamin B_2. Riboflavin should be liberated as a free form. Then it can be analysed by HPLC and quantified directly by mass spectrometry, or by fluorimetry. There is no need of a derivatization step because riboflavin is naturally fluorescent.

EN 14152[20], for example, includes an extraction of riboflavin after an acid hydrolysis and an enzymatic treatment. Riboflavin is separated by HPLC and quantified by fluorimetry.

ISO 21470, for example, specifies a method for the simultaneous quantitative determination of four water-soluble vitamins in infant formula and related nutritional products, including relevant forms of vitamins B_1, -B_2, -B_3 and -B_6 by enzymatic digestion and UHPLC-MS/MS quantification. ISO 21470 is not intended to be used on products where vitamins have not been added.

4.8 Niacin (vitamin B_3 or vitamin PP)

Niacin is a water-soluble vitamin. Niacin can be found in a lot of food products, mainly as phosphorylated forms. There are two free forms of niacin, nicotinamide and nicotinic acid.

Food regulations of many countries authorize the fortification of different kinds of food products with molecules having a niacin activity. The names of the authorized molecular forms to add in are generally linked to the type of food product. Table 10 gives an example of vitamin B_3 compounds permitted for use for food fortification according to EU regulations, depending on the product type.

Table 10 — Example of authorized vitamin B_3 compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific dietary purpose	Nicotinamide	122,1	Regulation (EU) n° 609/2013
	Nicotinic acid	123,1	
Supplemented food	Nicotinamide	122,1	Regulation (EC) n° 1925/2006
	Nicotinic acid	123,1	
Food supplement products	Nicotinamide	122,1	Directive n°2002/46/EC
	Nicotinic acid	123,1	
	Inositol hexanicotinate	810,7	

Nowadays, the commonly used unit of measurement for niacin is 1 mg of nicotinic acid.

Certain regulations (EU, US and Codex Alimentarius) consider the tryptophan content as being niacin equivalent. This ought to be taken into account if it is mandatory: 60 mg tryptophan = 1 mg niacin equivalent.

If it is not specified in the regulation, the niacin content can be calculated as:

niacin (mg/100 g) = nicotinic acid (mg/100 g) + 1,008 x nicotinamide (mg/100 g).

There are several analytical approaches to quantify vitamin B_3. Nicotinic acid and nicotinamide need to be liberated as a free form. Then they can be analysed by HPLC and quantified directly by mass spectrometry, or by fluorimetry after derivatization. Some methods involve a transformation step from one vitamer in the other. As such, the total niacin is directly quantified.

EN 14152[20], for example, includes an extraction of niacin after an acid hydrolysis or an enzymatic treatment. Nicotinamide and nicotinic acid are separated by HPLC and quantified by fluorimetry after a derivatization step by UV action. Inositol hexanicotinate is not quantified by this method.

ISO 21470, for example, specifies a method for the simultaneous quantitative determination of four water-soluble vitamins in infant formula and related nutritional products, including relevant forms of vitamins B_1, -B_2, -B_3 and -B_6 by enzymatic digestion and UHPLC-MS/MS quantification. ISO 21470 is not intended to be used on products where vitamins have not been added.

4.9 Vitamin B_6

Vitamin B_6 is a water-soluble vitamin. Vitamin B_6 can be found in a lot of food products free or as phosphorylated forms. There are three vitamers of vitamin B_6; pyridoxine (or pyridoxol), pyridoxal and pyridoxamine.

Food regulations of many countries authorize the fortification of different kinds of food products with molecules having a vitamin B_6 activity. The names of the authorized molecular forms to add in are generally linked to the type of food product. Table 11 gives an example of vitamin B_6 compounds permitted for use for food fortification according to EU regulations, depending on the product type.

Table 11 — Example of authorized vitamin B_6 compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	Pyridoxine hydrochloride	205,6	Regulation (EU) n° 609/2013
	Pyridoxine 5’ phosphate	249,6	
	Pyridoxine dipalmitate	646,0	
Supplemented food	Pyridoxine hydrochloride	205,6	Regulation (EC) n° 1925/2006
	Pyridoxine 5’ phosphate	249,6	
	Pyridoxine dipalmitate	646,0	
Food supplement products	Pyridoxine hydrochloride	205,6	Directive n° 2002/46/EC
	Pyridoxine 5’ phosphate	249,6	
	Pyridoxal 5’ phosphate	247,1	

Nowadays, the commonly used unit of measurement for vitamin B_6 is 1 mg of pyridoxin.

The three vitamers are considered to have the same vitamin B_6 activity:

— 1 mg of vitamin pyridoxine hydrochloride (M = 205,6) = 0,823 mg of vitamin B_6 (M = 169,1);

— 1 mg of pyridoxine 5’ phosphate (M = 249,6) = 0,679 of vitamin B_6 (M = 169,1).

The vitamin B_6 content can be calculated as:

vitamin B_6 (mg/100 g) = pyridoxin (mg/100 g) + 1,012 pyridoxal (mg/100 g) + 1,006 pyridoxamine (mg/100 g)

There are several analytical approaches for quantifying vitamin B_6. The three vitamers need to be liberated as free forms. Then they can be analysed by HPLC and quantified directly by mass spectrometry, or by fluorimetry (see for example EN 14663[21]). Some methods involve a transformation step from two vitamers into the third one. The total vitamin B6 is directly quantified (see for example EN 14164[22]).

EN 14663[21], for example, includes an extraction of vitamin B_6 after an acid hydrolysis or an enzymatic treatment. Pyridoxin, pyridoxamine and pyridoxal are separated by HPLC and quantified by fluorimetry. Pyridoxine dipalmitate is not quantified by this method.

EN 14164[22], for example, includes an extraction of vitamin B_6 after an acid hydrolysis or an enzymatic treatment. Pyridoxamine and pyridoxal are transformed to pyridoxin. Then pyridoxin is separated by HPLC and quantified by fluorimetry. Pyridoxine dipalmitate is not quantified by this method.

ISO 21470, for example, specifies a method for the simultaneous quantitative determination of four water-soluble vitamins in infant formula and related nutritional products, including relevant forms of vitamins B_1, -B_2, -B_3 and -B_6 by enzymatic digestion and UHPLC-MS/MS quantification. ISO 21470 is not intended to be used on products where vitamins have not been added.

4.10 Biotin (vitamin H or vitamin B_8)

Biotin is a water-soluble vitamin. Biotin can be found in a lot of food products free or bound to proteins or peptides. There are two main vitamers with a biotin activity, D-biotin and D-Biocytin. Biocytin is rarely detected. But as intermediate in the metabolism of biotin, biocytin occurs naturally in blood serum and can be detected in offal.

Food regulations of many countries authorize the fortification of different kinds of food products with biotin. The name of the authorized molecular form to add in is generally linked to the type of food product. Table 12 gives an example of biotin permitted for use for food fortification according to EU regulations, depending on the product type.

Table 12 — Example of authorized molecules in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	D-Biotin	244,3	Regulation (EU) n° 609/2013
Supplemented food	D-Biotin	244,3	Regulation (EC) n° 1925/2006
Food supplement products	D-Biotin	244,3	Directive n°2002/46/EC

Nowadays, the commonly used unit of measurement for biotin is 1 μg of biotin.

1 μg of D-biocytin = 0,656 μg of biotin.

The biotin content can be calculated as:

biotin (μg/100 g) = D-Biotin (μg/100 g) + 0,656 D-Biocytin (μg/100 g)

D-biocytin is rarely quantified, but should be taken in account in the calculation if it is the case.

There are several analytical approaches for quantifying biotin. Biotin should be liberated as a free form. Then it can be analysed by HPLC and quantified by fluorimetry after a derivatization step (see for example EN 15607[24]) or by UV. In this case, a preconcentration step should be added before the HPLC (see ISO 23305, for example).

In ISO 23305, for example, the sample is dispersed in a phosphate buffer and then autoclaved. Biotin is purified and concentrated with an immunoaffinity column. Then it is analysed by HPLC and UV quantified. Biocytin and biotin are separated.

EN 15607[24], for example, includes an enzymatic treatment. Then the extract is analysed by HPLC with a post-column derivatization. Biocytin and biotin are separated and quantified by fluorimetry.

4.11 Folates (vitamin B_9)

Folic acid is the reference compound of a group of different vitamers which have a vitamin B_9 activity. Folic acid is a water-soluble vitamin B_9. Folates can be found in a lot of food products, free or bound to chains of different numbers of glutamate units.

The main free forms are folic acid, 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate. But there are around ten compounds which are considered to have vitamin B_9 activity.

Food regulations of many countries authorize the fortification of different kinds of food products with folates. The names of the authorized molecular forms to add in are generally linked to the type of food product. Table 13 gives an example of folates permitted for use for food fortification according to EU regulations depending the product type.

Table 13 — Example of authorized molecules in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	Folic acid (pteroylmonoglutamate acid)	441,4	Regulation (EU) n° 609/2013
	Calcium L-methylfolate	459,5	
Supplemented food	Folic acid (pteroylmonoglutamate acid)	441,4	Regulation (EC) n° 1925/2006

Table 13 *(continued)*

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food supplements	Folic acid (pteroylmonoglutamate acid)	441,4	Directive n°2002/46/EC
	Calcium L-methylfolate	459,5	

The commonly used unit of measurement for folates used to be 1 µg of folic acid.

All folates quantified were expressed in folic acid. However, multiple regulations have now introduced Dietary Folate Equivalents (DFE) as the unit of measurement; see Table 14.

Table 14 — USA unit conversion for folates

Nutrient	Former unit	Former conversion	New unit	New conversion
Folate	µg		µg DFE (Dietary Folate Equivalent)	1 µg DFE = 1 µg folates 1 µg DFE = 0,6 µg folic acid

Recently in the EU, two new regulations have introduced the same principle: one for food for special medical purpose[25] and another for infant formula[26].

The folates (vitamin B_9) content can be calculated as:

vitamin B_9 as DFE (µg/100 g) = 1/0,6 folic acid content + folates content (µg/100 g),

or if it is outlined by regulation, the unit of measurement of vitamin B_9 = µg DFE.

There are several analytical approaches for quantifying folates. Vitamin B_9 was historically quantified by microbiological methods. The calibration was made with folic acid. Some HPLC methods have been more recently developed. A detection and a quantification by mass spectrometry enable differentiation between the different folates. In any case, a trienzymatic digestion should be used beforehand to prepare the samples. Although the LC techniques are nowadays widely deployed for the analysis of vitamins, microbiological methods are still currently used for vitamin B_9 (for example EN 14131[32]). Having two completely different principles often means discrepancy in results.

In AOAC 2011.06[27] for example, the folates in a sample are extracted in a buffer (pH = 6,0) containing internal standards by treatments with protease, amylase and rat plasma conjugase (trienzyme digestion). The extract is purified and concentrated using a weak anion exchange (WAX) Solid Phase Extraction (SPE). Polyglutamate forms of folates in the sample are deconjugated to monoglutamates during the extraction and are analysed by LC-MS/MS. Folic acid, 5-methyl-tetrahydrofolate (5-CH_3-THF) and 5-formyl-tetrahydrofolate (5-CHO-THF) are quantified, and total folate is estimated and expressed as folic acid. Isotopically labelled folic acid (^{13}C-folic acid), 5-CH_3-THF (^{13}C-5-CH_3-THF) and 5-CHO-THF (^{13}C-5-CHO-THF) are used as the internal standards.

4.12 Vitamin B_{12}

Vitamin B_{12} is a water-soluble vitamin. Vitamin B_{12} can be found in all food from animal origin (offal, sea food, meat, fish, eggs and milk products).

Cobalamin is the reference compound of a group of different vitamers which have a vitamin B_{12} activity. There are four vitamers of vitamin B_{12}; cyanocobalamin, hydroxycobalamin, methylcobalamin and adenosylcobalamin.

Food regulations of many countries authorize the fortification of different kinds of food products with vitamin B_{12}. The names of the authorized molecular forms to add in is generally linked to the type of food product. Table 15 gives an example of the vitamin B_{12} compound permitted for use for food fortification according to European regulation depending the product type.

Table 15 — Example of authorized vitamin B_{12} compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	Cyanocobalamine	1 355,4	Regulation (EU) n° 609/2013
	Hydroxycobalamine	1 346,4	
Supplemented food	Cyanocobalamine	1 355,4	Regulation (EC) n° 1925/2006
	Hydroxycobalamine	1 346,4	
Food supplements	Cyanocobalamine	1 355,4	Directive n°2002/46/EC
	Hydroxycobalamine	1 346,4	

Nowadays, the commonly used unit of measurement for vitamin B_{12} is 1 µg of cyanocobalamin.

The vitamin B_{12} content can be calculated as:

vitamin B_{12} (µg/100 g) = sum of the different vitamers expressed as cyanocobalamin (µg/100 g).

There are several analytical approaches for quantifying vitamin B_{12}. Vitamin B_{12} was historically quantified by microbiological methods. The calibration was done with cyanocobalamin. More recently, some HPLC methods have been developed. Although LC techniques are nowadays widely deployed for the analysis of vitamins, microbiological methods are still currently used for vitamin B_{12}. Having two completely different principles often means discrepancy in results.

In ISO 20634, for example, cyanocobalamin and other cobalt-containing corrinoids are extracted from the sample using sodium acetate buffer (pH = 4,5) and the latter converted to cyanocobalamin using potassium cyanide at 105 °C. Extracts are purified and concentrated with C8 or C18 solid-phase extraction (SPE) cartridges and analysed with size-exclusion and reversed-phase chromatography. Determination of vitamin B_{12} is made by liquid chromatography with visible detection at 550 nm.

4.13 Pantothenic acid (vitamin B_5)

D-Pantothenic acid is a water-soluble vitamin. D-Pantothenic acid can be found in nearly all food, with higher amounts found in offal, eggs and poultry.

Dexpantothenol (D-pantothenol) is the alcohol analogue of D-Pantothenic acid. It is not naturally present in food but can be used for fortification.

Food regulations of many countries authorize the fortification of different kinds of food products with D-pantothenic acid. The names of the authorized molecular forms to add in are generally linked to the type of food product. Table 16 gives an example of the pantothenic acid compounds permitted for use for food fortification according to EU regulations, depending on the product type.

Table 16 — Example of authorized pantothenic acid compounds for food fortification in the EU according to products concerned

Product type	Molecular form	Molecular mass (g/mol)	Examples of regulations
Food with a specific nutritional purpose	Calcium D-pantothenate	476,5	Regulation (EU) n° 609/2013
	Sodium D-pantothenate	241,2	
	Dexpantothenol (D-pantoth-enol)	205,3	