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# Limitations of current analytical reference methods to determine vitamins in foods: Challenges to support regulatory compliance and nutritional composition data

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## Abstract

**Background:** food composition data. Many international reference methods for the analysis of vitamins in foods originate from the 1990s. Advances in nutrition science and analytical technology and the continuing evolution of statutory regulations necessitate the need of new or supplementary regulatory standards. We have evaluated recent developments in these areas and conclude that most current international reference methods are no longer fit-for-purpose to accurately determine vitamin Content in foods and food supplements. We have made recommendations to consider new and/or updated reference methods and regulatory standards for the analysis of vitamins A, D, E, K, B1, B2, B3, B5, B6, B7, B9, B12, C and carotenoids in foods and food supplements. This area of nutrients may benefit from globally harmonised definitions specifying what compounds to include or exclude for analysis, and applicable bioactivity factors.

**Keywords:** Food, Analysis, Reference, Harmonisation, Methods, Vitamers

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## Introduction

Vitamins are critical for normal cell function, growth, and development, and since mammalian cells cannot, in general, biosynthesise them, they are essential components of the diet. The quantification of the total amounts of individual vitamins in foods, i.e., vitamins both naturally present and added to foods to improve or maintain their nutritional quality, is challenging. Developments in nutrition research, changes in regulations, ambiguity over which compounds to include or how to adequately quantify a vitamin contribute to these challenges. An ISO Technical Report (ISO, 2021) gives guidance on the multiple options to express vitamins on food product labels that accounts for different molecular forms and regulatory requirements.

There are generally three sectors interested in data on the vitamin content of foods and food supplements. Firstly, the food industry needs data to ensure safety and quality of their products in accordance with regulatory requirements and to ensure nutritional claims are met. Based on recipe calculations involving natural levels in ingredients and added amounts of vitamins, producers know the forms and quantities of vitamins in their products. Secondly, nutrition researchers and risk assessors use food consumption data to determine dietary intake (Castanheira, Saraiva, Rego, & Ollilainen, 2016). For this group it is important to know what value to assign to total vitamin amount, how it is determined, and how it should be expressed. This group relies heavily on available reference methods of analysis. Reliable analytical methods are essential for population-based policies related to Recommended Daily Allowances, total diet surveys, as well as supporting clinical testing. Thirdly, enforcers of regulations generally have no information on the composition of a food product. They need robust methods to be able to quantify vitamin content (natural + added) to verify label declarations and nutritional claims. A complicating factor is that some vitamins, such as riboflavin, ascorbic acid and  $\alpha$ -tocopherol, can be added to foods both as nutrients and as functional additives, which makes the verification of labelled nutritional amounts difficult, if not impossible.

Multiple horizontal European standards for the determination of vitamins in foods are published by the European Committee for Standardisation (CEN) and originate from the 1990s. Blake and Konings (2005) listed the available reference methods for vitamins in foods published by AOAC INTERNATIONAL, CEN and the International Organisation for Standardisation (ISO), which have not changed significantly since then. The scope of other more recently published reference methods for vitamins by AOAC INTERNATIONAL, ISO and the International Dairy Federation (IDF) is restricted to infant formula and adult nutritionals. Several of these CEN, AOAC, ISO and IDF reference methods are included in Codex standard CXS-234 on recommended methods of analysis and sampling (Codex, 1999) to verify Codex provisions.

Scientific knowledge on the presence and bioavailability of certain vitamin Compounds has evolved, and this may have an impact on the total vitamin Content quantified in a food product. Recently for example, Jakobsen, Melse-Boonstra, and Rychlik (2019) stated that there are no standardised methods for the quantification of the vitamers of vitamin A, vitamin D, and folate in foods. In addition, regulatory requirements, including newly approved compounds for some vitamins, have changed since the publication of the above-mentioned reference methods.

This paper elaborates on the most important recent and relevant developments in science, technology, and regulatory requirements that will necessitate updated or new analytical reference methods that will ensure accurate compositional data for regulatory compliance verification or support dietary recommendations.

## 2.0 Developments contributing to limitations in current reference methods

### 2.1. Vitamin A and carotenoids

Vitamin A is a fat-soluble vitamin obtained from the diet either as preformed vitamin A (mainly retinol and retinyl esters) in foods of animal origin or as provitamin A carotenoids in plant-derived foods, primarily in oils, fruits and vegetables (Meléndez-Martínez et al., 2022). The term vitamin A comprises retinol and a family of naturally occurring molecules associated with biological activity such as retinal, retinoic acid, retinyl esters, as well as provitamin A carotenoids that are dietary precursors of retinol (EFSA, 2015a).

Among approximately 600 carotenoids in nature, about 50 possess provitamin A activity in varying degrees of potency, but food composition data are only readily available for  $\beta$ -carotene,  $\alpha$ -carotene and  $\beta$ -cryptoxanthin (Ball, 2006; NHMRC, 2017). The predominant isomer of retinol is *all-trans*-retinol, which possesses 100% vitamin A bioactivity, while other isomers exhibit lower bioactivity. In foods, vitamin A can exist in several isomeric forms and as esters. Fresh and processed fruits and vegetables are the main contributors to the dietary intake, with  $\beta$ -carotene,  $\alpha$ -carotene and  $\beta$ -cryptoxanthin being the most abundant provitamin A carotenoids (Ball, 2006). Dairy products may also have an important contribution to the total dietary  $\beta$ -carotene intake. Currently, there is no globally harmonised way to express vitamin A activity of carotenoids. Some regulations include  $\beta$ -carotene and other provitamin A carotenoids to express a total amount of vitamin A. Some regulations require the calculation of Retinol Equivalents (RE) with the inclusion of carotenoid contents for labelling purposes of vitamin A. Recently, the U.S Food and Drug Administration (USFDA) introduced a new unit to express vitamin A activity: retinol activity equivalents (RAE), which includes the major provitamin A carotenoids (USFDA, 2016; USFDA, 2019). In addition, a differentiation in bioactivity of supplemental and dietary  $\beta$ -carotene is made.

Table 1 includes the conversion factors used in global standards and regulations including Codex standards (Codex Stan 72–1981; Codex Stan 2–1985 and Codex Stan 156–1987: Codex, 1981, 1985 and Codex, 1987, respectively) and regional regulations in the USA (USFDA, 2016; USFDA, 2019), European Union (EU) regulations (regulations 2002/46, 1169/2011, 2016/127: EU, 2002, 2011, 2016, respectively), China national standards (GB) (GB/Z 21922–2008, GB 25596–2010, GB 28050–2011, GB 14880–2012, GB 29922–2013, GB 10765–2021, GB 10766–2021, GB 10767–2021: GB, 2008, 2010, 2011, 2012, 2013, 2021a, 2021b, 2021c, respectively), Food Standards Australia New Zealand (FSANZ) Schedules (FSANZ, 2018a, 2018b, 2018c, 2018d) and Nutrient Reference Values (NRV) for Australia and New Zealand (NHMRC, 2017).

The retinol equivalent factors in the FSANZ Schedules are specific for carotenoids that are being added to foods. The retinol activities of carotene forms like  $\beta$ -apo-8'-carotenal and  $\beta$ -apo-8'-carotenoic acid ethyl ester that are listed in general pharmacopoeias are included in FSANZ Schedules, whereas these forms are excluded in NHMRC NRV. The factors used in the NHMRC NRV are for carotenoids naturally occurring or fortified in foods and are in line with the most recent decision of the FAO (FAO/WHO, 2001).

It should be noted that there are no carotenoid conversion factors to express vitamin A for European labelling requirements (EU 1169/2011: EU, 2011). The physiological equivalence between provitamin A carotenoids and retinol is an issue on which a scientific consensus has not yet been established (Dias et al., 2021). However, the European Food Safety Authority (EFSA) concluded in 2015 that 1  $\mu$ g of retinol, or 6  $\mu$ g of  $\beta$ -carotene, or 12  $\mu$ g of other provitamin A carotenoids is equal to 1  $\mu$ g RE (EFSA, 2015a).

In natural foodstuffs, the all-trans retinol form is frequently accompanied by smaller amounts of the less potent *13-cis*-retinol that has a 75% vitamin A activity (Ames, 1966; Weiser & Somorjai, 1992). Some current global reference methods for the determination of vitamin A in foods include both all-trans and the *13-cis*-retinol forms in the quantification of vitamin A (AOAC INTERNATIONAL, 2023a; CEN, 2009a; ISO, 2015a). However, historically the *13-cis* form was not always considered for inclusion, which may result in differences in reported vitamin A amounts, dependent on the analytical method used. In addition, lack of harmonised definitions of vitamin A adds to confusion whether the *13-cis* form should be included, and which bioactivity factor should be applied. Codex standard CXS-72 on infant formulas and formulas for special medical purposes intended for infants expresses 1  $\mu$ g RE as 1  $\mu$ g *all-trans*-retinol and specifies that retinol contents shall be provided by preformed retinol, while any contents of carotenoids should not be included in the calculation and declaration of vitamin A activity (Codex, 1981). China national standards for infant formula and formula for older infants and young children (GB, 2010, 2013, 2021a, 2021b, 2021c) refer to all-trans retinol as the compound to be considered. However, newer China standards for all foods including infant formula (GB 5009.82–2016: GB, 2016a) do not distinguish between the *all-trans* and *13-cis* form. Depending on fortification and processing conditions, the amount of *13-cis*-retinol can be 10–30% of

total retinol (Panfili, Manzi, & Pizzoferrato, 1998). AOAC has published Standard Method Performance Requirements (SMPR®) where *all-trans* and *13-cis*-retinol are included in the applicability statement for an AOAC Official Method for the determination of vitamin A in infant formula and adult nutritionals (AOAC INTERNATIONAL, 2023b).

It is recommended that future reference methods that are developed and/or validated for vitamin A in foods shall include the quantification of both *all-trans* and *13-cis*-retinol. In addition, from a global perspective, a reference method for the determination of provitamin A carotenoids, including  $\alpha$ -carotene,  $\beta$ -carotene, and  $\beta$ -cryptoxanthin in foods should be considered.

In addition to provitamin A carotenoids, certain foods also provide carotenoids that are important in promoting and maintaining human health (e.g., lutein, zeaxanthin, astaxanthin, and cantaxanthin (Meléndez-Martínez et al., 2022)). From all the carotenoids present in the diet, only six major ones are found in human plasma and milk:  $\alpha$ -carotene,  $\beta$ -carotene, lycopene,  $\beta$ -cryptoxanthin, zeaxanthin, and lutein (Hostetler, 2017; Meléndez-Martínez et al., 2022) with lutein,  $\beta$ -carotene and lycopene the most common in human milk and lutein and  $\beta$ -carotene most commonly added to infant formula and adult nutritionals (Hostetler, 2020). This was the rationale for the development of AOAC and ISO standards for the determination of these carotenoids in these products (AOAC INTERNATIONAL, 2023c; ISO, 2020a).

With an increased awareness on health-based products it is expected that the market for carotenoid-based health supplements will grow in the coming years (Allied Market Research, 2023). Lutein,  $\beta$ -carotene, lycopene, astaxanthin and zeaxanthin and mixtures of these carotenoids are popular among consumers, with zeaxanthin supplements typically taken to support eye health. Two separate specifications for zeaxanthin (synthetic) and for zeaxanthin rich extract from *Tagetes erecta* are approved by the Joint Expert Committee on Food Additives (JECFA, 2006). Both sources can be used either as a nutritional supplement or as colouring agents in a wide range of foods. In 2013 the European Commission authorised the synthetic form as a novel food ingredient and needs as such to be labelled as “synthetic” (EU, 2013a). Obviously, there is a significant price difference between the synthetic and botanical source of zeaxanthin; requiring analytical methods developed for the purpose of confirming analyte origin. The botanical source of zeaxanthin is exclusively present as the (3R, 3'R) enantiomer, whereas synthetic zeaxanthin occurs as the meso or (3R, 3'S) isomer. To distinguish between the two sources, the use of chiral column chromatography is necessary. Because there were health concerns with the mesoform before its authorisation as a novel food ingredient, the French authorities developed an analytical method to separate and quantify the two zeaxanthin isomers (Regnard, Arella, & Deborde, 2014).

In the wild, the flesh of salmon and trout can have a red/pink colour due to the carotenoid astaxanthin. Astaxanthin is an essential nutrient for salmon and is produced in natural waterways by algae, yeast and bacteria, all of which are in the food chain of salmonids. The ratio of optical isomers of astaxanthin indicates if the astaxanthin comes from chemical synthesis or natural sources such as shrimp or krill. Astaxanthin synthesised in nature occurs as the *all-trans* (3S, 3S) form, whereas synthetic astaxanthin is a mixture of the two optical isomers and the meso form at a ratio of 1:2:1 (3R, 3'R), (3R, 3'S) and (3S, 3'S) and can be used to distinguish wild from farmed salmon. CEN has published a technical specification (CEN, 2011a, 2011b) that includes a method for the determination of the enantiomer ratio of astaxanthin. However, additional validation data are needed to advance this method to a full reference method.

The European Union authorised the use of an astaxanthin-rich oleoresin from *Haematococcus pluvialis* algae as a novel food. The European Regulation EU 2023/1581 (EU, 2023a) sets maximum levels of this naturally occurring carotenoid in food supplements used by children, adolescents, and the general population. Currently no official method exists to enforce these maximum levels. In 2022, CEN issued a call for methods to standardise the determination of carotenoids in algae in Technical Committee 454: Algae and algae products.

## 2.2. Vitamin E

Vitamin E as an antioxidant, protects many other biochemicals from damage by reactive oxygen and other free radicals. It works closely with vitamin C in this respect, particularly in cell membranes, where ascorbic acid regenerates the  $\alpha$ -tocopheryl radical to  $\alpha$ -tocopherol. Rich food sources of vitamin E are oils extracted from cereal grains, nuts, beans, and seeds. Cereal grain products, fish, meat, eggs, dairy products, and green leafy vegetables also provide significant amounts (Ball, 2006).

Vitamin E is represented traditionally as a group of 8 molecules in two classes: tocopherols and tocotrienols, which in food all act as antioxidants. Of these 8 molecules, however, only  $\alpha$ -tocopherol is considered as a vitamin E source for nutritional labelling (Institute of Medicine Panel, 2000). Thus, for labelling of vitamin E, the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) agreed in 2016 to include only the  $\alpha$ -tocopherol form (REP17/NFSDU, 2016), where 1 mg  $\alpha$ -tocopherol is equivalent to 1 mg RRR- $\alpha$ -tocopherol (Codex, 2017).  $\alpha$ -Tocopherol contains three asymmetric carbon atoms, allowing for 8 optical isomers (Jensen & Lauridsen, 2007). While only RRR- $\alpha$ -tocopherol is present in nature, synthetic  $\alpha$ -tocopherol occurs as a racemic mixture (*all-rac*- $\alpha$ -tocopherol). In its 2015 scientific opinion, EFSA stated that only the naturally occurring RRR- $\alpha$ -tocopherol should be considered as vitamin E (EFSA, 2015b). EFSA endorsed the Institute of Medicine Panel definition that the difference in relative activity of *all-rac*- $\alpha$ -tocopherol compared with RRR- $\alpha$ -tocopherol is 50% and defined 1 mg *all*-

*rac*- $\alpha$ -tocopherol equal to 0.5 mg RRR- $\alpha$ -tocopherol and that 1 IU *all-rac*- $\alpha$ -tocopherol (or corresponding esters) as equal to 0.45 mg 2R stereoisomeric forms of  $\alpha$ -tocopherol and 1 IU RRR- $\alpha$ -tocopherol (or corresponding esters) as equal to 0.67 mg 2R- $\alpha$ -tocopherol (Institute of Medicine Panel, 2000). EFSA's opinion has been implemented in specific European regulations 2002/46/EC (EU, 2002) and EU 2016/127 (EU, 2016), which authorise the fortification of different kinds of food products with vitamins. An overview of units of expression and their definition of vitamin E as per Codex standards (Codex, 1981, 1985, 1987) and regional regulations in the US (USFDA, 2016, 2019), Europe (EFSA, 2015b; EU, 2002, 2011, 2016), China (GB, 2008, 2010, 2011, 2012, 2013, 2021a, 2021b, 2021c) and Australia/New Zealand (FSANZ, 2018a, 2018b, 2018c, 2018d) is shown in Table 2.

The definitions for all the units of expression of vitamin E as shown in Table 2 mean that compounds to be included as vitamin E need to be corrected for their biological activity. If a product contains both natural tocopherol sources and synthetic tocopherol or its esters, it is necessary to apply the correct factors to yield an accurate result. For fortification purposes, manufacturers usually include *all-rac*- $\alpha$ -tocopheryl acetate or *all-rac*- $\alpha$ -tocopherol in their formulations. Additionally, RRR- $\alpha$ -tocopherol is present originating from natural oils as part of product ingredients. It should be noted that Codex guideline CXG 10–1979 (Codex, 1979) and European regulations EC 1170/2009 (EC, 2009) and EU 609/ 2013 (EU, 2013b), allow the use of D- and DL- $\alpha$ -tocopheryl acid succinate in addition to D- and DL- $\alpha$ -tocopherol, D- and DL- $\alpha$ -tocopheryl acetate and DL- $\alpha$ -tocopheryl polyethylene glycol-1000 succinate, mixed tocopherols and tocotrienols for fortification of foods and food supplements. Unfortunately, the detailed compositional information of a processed food is not typically available to analytical laboratories since only manufacturers possess recipe information.

Available international reference methods to determine vitamin E in foods are usually based on two approaches: (i) saponification of tocopheryl esters to liberate the free tocopherols followed by solvent extraction and separation of the individual tocopherols by HPLC and quantification by fluorescence detection, with an example of such a reference method being EN 12822:2014 (CEN, 2014a), and (ii) direct solvent extraction and simultaneous quantitative determination of vitamin E as  $\alpha$ -tocopherol and  $\alpha$ -tocopheryl acetate (e.g., AOAC and ISO methods for infant formula and adult nutritionals: AOAC INTERNATIONAL, 2023a; ISO, 2015a). With this approach it is assumed that  $\alpha$ -tocopherol originates from a natural source, such as vegetable oils added as an ingredient, and  $\alpha$ -tocopheryl acetate from a synthetic source used for fortification.

While not commonly used, the  $\alpha$ -tocopherol succinate ester is a permitted form of vitamin E for fortification; however, it is not measured and/or validated by these methods.

Of nutritional significance is that neither of these commonly used analytical approaches distinguishes between RRR- $\alpha$ -tocopherol and the *all-rac*- $\alpha$ -tocopherol respectively. Recently, Gill and Indyk, 2020 published a suitable application where both RRR- $\alpha$ -tocopherol content and total  $\alpha$ -tocopherol can be assayed for routine compliance testing. This method uses saponification as part of sample preparation, allowing for the measurement of endogenous  $\alpha$ -tocopherol and  $\alpha$ -tocopherol ester forms subsequent to HPLC separation utilising a chiral column and fluorescence detection. By calculating contributions of each  $\alpha$ -tocopherol stereoisomer, the method is capable of distinguishing between natural and synthetic sources. A future compendial reference method based on this analytical approach is therefore required that can distinguish between natural and synthetic sources of  $\alpha$ -tocopherol and thereby enable regulatory compliance verification.

### 2.3. Vitamin D

Vitamin D is represented by two fat-soluble compounds, vitamin D<sub>2</sub> (ergocalciferol) and vitamin D<sub>3</sub> (cholecalciferol) and their respective 25-hydroxyvitamin D metabolites. The major food sources for naturally occurring vitamin D<sub>3</sub> include flesh of fatty fish and fish liver oils, while smaller amounts are found in egg yolks, milk, dairy products and beef liver, with vitamin D<sub>2</sub> present predominantly in fungi. 25-Hydroxy metabolites of vitamin D are present in some foods of animal origin; however, information on amounts in most other types of food is lacking (Jakobsen et al., 209; Jakobsen & Christensen, 2021). EFSA has recently set a factor of 2.5 for the bioactivity of 25-hydroxyvitamin D<sub>3</sub> as compared to vitamin D<sub>3</sub> (EFSA, 2023). Additional sources of dietary vitamin D are fortified foods such as milk, margarine and/or butter, breakfast cereals and dietary supplements. Currently, vitamin D<sub>3</sub> and vitamin D<sub>2</sub> may be added to both foods and food supplements. Recent new European Union Regulation EU 2023/4 (EU, 2023b) authorised the use of vitamin D<sub>2</sub> mushroom powder as a novel food that stipulates its incorporation in a wide range of food categories compliant with maximum levels of vitamin D<sub>2</sub>. Before the introduction of this regulation, vitamin D<sub>3</sub> was mainly used in Europe to fortify foods or applied in supplements. It should be noted that with the current European Standard (CEN, 2009b) for the determination of vitamin D in foods, only vitamin D<sub>2</sub> or vitamin D<sub>3</sub> can be quantified, wherein one of the compounds is used as an analytical internal standard in the presence of the other compound. Consequently, this method cannot be applied if both compounds are present in a food, a limitation given that nutrition labelling provisions, as according to Codex (Codex, 1985) or European regulation (EU, 2011), shall be based on total vitamin D present when added to the food.

Recently, following an EFSA Scientific opinion (EFSA, 2018), new regulatory limits for vitamin D in infant formula were established by the European Commission with the Commission Delegated Regulation EU 2019/828 (EU, 2019) introducing a vitamin D interval range of 2 to 2.5  $\mu\text{g } 100\text{kcal}^{-1}$ . Available AOAC, CEN

and ISO global reference methods (AOAC INTERNATIONAL, 2023d; CEN, 2009b; ISO, 2018a), able to determine either vitamin D<sub>2</sub> and/or vitamin D<sub>3</sub>, have been assessed on their fitness for purpose to verify regulatory compliance (Gilliland et al., 2022; Konings et al., 2021). The assessment illustrates the high probability that a compliant product, with a true vitamin D level within the EU regulatory range, would fail to meet the revised regulatory requirements due to analytical method variability alone. Based on this assessment, it can be concluded that current, state-of-the-art methods cannot consistently verify product compliance for vitamin D with European regulatory requirements.

An additional challenge in vitamin D analysis in foods is that it exists in thermal equilibrium with the previtamin D conformer, and the simultaneous measurement of vitamin D and previtamin D is necessary to obtain an accurate and reliable estimate of its content in foods. Depending on storage conditions, foods containing vitamin D will have a significant contribution of previtamin D. Analytical methods typically use calciferol internal standards and are susceptible to analytical bias due to differences in the proportion of previtamin D in the sample and in the internal standard. To overcome this problem, a sufficiently high heat treatment during saponification is necessary to ensure that both the internal standard and sample analyte reach equilibrium prior to analysis (Gill et al., 2020).

If possible, a fit-for-purpose method applicable to the relatively narrow regulatory range for vitamin D in infant formula must be developed. Furthermore, to estimate the total vitamin D content in foods, a future reference method needs to include the 25-hydroxyvitamin D metabolites in addition to parent vitamin D<sub>2</sub> and vitamin D<sub>3</sub>.

## 2.4. Vitamin K

Vitamin K is represented by phylloquinone (vitamin K<sub>1</sub>), found in highest levels in certain green vegetables and vegetable oils, and the menaquinones (vitamin K<sub>2</sub>, MK-n) found in animal foods such as meat and eggs (MK-4), but primarily in fermented foods such as cheeses and natto that contain MK-5 to MK-10. Naturally occurring phylloquinone has the C-2' double bond in the trans configuration. Synthetic phylloquinone, used for fortification of predominantly infant formula, typically contains both the trans and cis isomers, but only the trans isomer is responsible for the vitamin's biological activity (Woollard, Indyk, Fong, & Cook, 2002). Differences between distribution of vitamin K vitamers following dietary intake have been observed in serum (Sato, Schurgers, & Uenishi, 2012; Schurgers & Vermeer, 2002).

Dihydro-K<sub>1</sub> is present in foods containing hydrogenated vegetable oils and contributes to physiologically active vitamin K in the diet (Dumont, Peterson, Haytowitz, & Booth, 2003). Fortification of foods with vitamin K is uncommon, except for infant formula to which synthetic preparations of phylloquinone are

added. There are several regulations allowing the fortification of foods and food supplements with vitamin K, primarily as phylloquinone, but menaquinone-7 (MK-7) and menaquinone-6 (MK-6) forms are also approved in foods for special medical purposes and total diet replacement for weight control by European regulations (EC, 2009; EU, 2013b).

The European standard for the analysis of vitamin K in foods (CEN, 2003a) enables the quantification of total cis- and trans-vitamin K1 and also enables the quantification of MK-4 and MK-7. However, an interlaboratory study for the quantification of MK-n is still needed to demonstrate that the method is fit for purpose to quantify these compounds. Recently, quantification of vitamin K1 and MK-4 to MK-10 in food achieved satisfactory results when comparing two detection methods, fluorescence and mass spectrometry (MS) (Jensen, Rød, Loznjak Svarc, Oveland, & Jakobsen, 2022). A method for the determination of total vitamin K1 in infant formula and adult nutritionals has been published by AOAC and ISO (AOAC INTERNATIONAL, 2023e; ISO, 2019). This method can distinguish between the bioactive trans- and non-bioactive cis-phyloquinone, but does not allow the measurement of MK-n. However, global regulatory requirements do not make any distinction between the geometric isomers. From the validation data of this method, based on 13 different fortified infant formula and adult nutritionals including four placebo materials, it became clear that approximately 12% of the total phyloquinone amount is cis-phyloquinone. Most of this amount (9%) comes from the premix used for fortification and the remaining fraction (3%) comes from the ingredients used for these products. The products analysed represent most matrices on the global market.

To enable verification of the regulatory requirements and information in food composition tables for vitamin K a reference method is needed to quantify the amount of phyloquinone and the menaquinones, as well as dihydro-K<sub>1</sub>. Future regulatory requirements should consider the different bioactivities of cis- and trans vitamin-K<sub>1</sub> and the individual menaquinones.

## 2.5. Vitamin B<sub>1</sub>

All plants contain primarily non-phosphorylated thiamine (vitamin B<sub>1</sub>), while animal products contain thiamine mainly as phosphorylated forms (Gropper, Smith, & Carr, 2021). Thiamine diphosphate is involved as a coenzyme for enzymes in carbohydrate and branch-chained amino acid metabolism.

There are several international reference methods for the quantification of vitamin B<sub>1</sub> in foods, although only a few international reference methods are validated for the determination of vitamin B<sub>1</sub> in infant formula. In general, these methods liberate thiamine in its free form prior to measurement. An early fluorometric AOAC Official Method 942.23 (AOAC INTERNATIONAL, 2023f) cannot now be considered as fit

for purpose. Currently, the most commonly applied procedure to determine vitamin B<sub>1</sub> uses HPLC with direct quantification either by mass spectrometry, or by fluorometry after a derivatisation step. A European standard, EN 14122:2014 (CEN, 2014b) for the determination of vitamin B<sub>1</sub> in foods provides an option to use either pre- or post-column derivatisation following enzymatic treatment. Usually, the vitamin B<sub>1</sub> content typically consists of thiamine and its phosphate derivatives. However, the use of a post-column derivatisation method to determine thiamine can show two peaks; one for thiamine and the other for the metabolite 2-(1-hydroxyethyl)thiamine (HET), which has equal bioactivity to thiamine (Jakobsen, 2008; Takashi, Yukiko, Kohei, Mari, & Kaname, 1990; Takashi, Yukiko, Kohei, Masako, & Kaname, 1991). If the pre-column derivatisation technique is used, the two compounds thiamine and HET result in two different derivatives that co-elute.

The relative content of HET compared with thiamine is dependent on the sample type. In meat and liver, the content of HET varied between 7 and 23%, in yeast the content was 3.8%, while in white cabbage, broccoli, oat flour, infant formula, milk powder and wheat the content was below 2% (Jakobsen, 2008). For the quantitative HPLC determination of vitamin B<sub>1</sub> in foods by post-column derivatisation, it is therefore recommended to include separate quantitative determination of thiamine and HET when analysing meat samples, and to monitor for HET for other foods. A future reference method should include the validation of the quantification of HET in addition to thiamine.

The largest component of vitamin B<sub>1</sub> in infant formula is from fortification, with thiamine chloride hydrochloride, thiamine hydrochloride and thiamine mononitrate as allowed sources (Codex, 1979; EC, 2009). In food supplements, thiamine monophosphate chloride and thiamine pyrophosphate chloride are also allowed as a vitamin B<sub>1</sub> source (EC, 2009). As some reference methods were developed prior to authorisation of certain vitamin B<sub>1</sub> sources, it is important to include these compounds in the validation of future reference methods. AOAC Official Method 986.27 for the determination of vitamin B<sub>1</sub> in milkbased infant formula (AOAC INTERNATIONAL, 2023g) originates from the 1980's and is based on fluorometry. This method was considered not fit-for-purpose and was superseded in 2015 with a multi Bgroup vitamin method (AOAC INTERNATIONAL, 2023h; ISO, 2020b) based on LC-MS/MS, which will be described in more detail in the next paragraph on vitamin B<sub>2</sub>.

A confusion with the expression of vitamin B<sub>1</sub> is related to the chemical form and related molecular weights (ISO, 2021). There is no globally harmonised convention how the amount of vitamin B<sub>1</sub> on the nutrition label is expressed. Since thiamine (C<sub>12</sub>H<sub>17</sub>N<sub>4</sub>OS, molecular weight: 265.37 g mol<sup>-1</sup>), thiamine chloride (C<sub>12</sub>H<sub>17</sub>ClN<sub>4</sub>OS, molecular weight: 300.82 g mol<sup>-1</sup>) and thiamine chloride hydrochloride (molecular weight: 337.27 g mol<sup>-1</sup>) are all considered as vitamin B<sub>1</sub>, this is problematic for enforcement laboratories verifying label declarations.

## 2.6. Vitamin B<sub>2</sub>

Vitamin B<sub>2</sub> (riboflavin) is water-soluble and is biologically active in the form of two flavin coenzymes, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), participating in oxidation-reduction reactions in numerous metabolic pathways. Riboflavin can be found in a wide range of food products, mainly in its phosphorylated forms, and rich sources include liver, kidney, eggs, meat, milk and cheese (Ball, 2006).

The forms of vitamin B<sub>2</sub> allowed for fortification of foods and food supplements include riboflavin and FMN sodium (Codex, 1979; EC, 2009). A horizontal European standard, originating from the 1980's to determine total vitamin B<sub>2</sub> in foods is EN 14152:2014 (CEN, 2014c) that includes an extraction of riboflavin after combined acid hydrolysis and enzymatic treatment, with riboflavin separated by HPLC and quantified by fluorometry. With current analytical technology, it is more efficient to determine multiple B-vitamins in a single analysis using MS/MS detection. AOAC 2015.14 or its equivalent ISO 21470:2020 (AOAC INTERNATIONAL, 2023h; ISO, 2020b), for example, specify 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<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub> and B<sub>6</sub> by enzymatic digestion and UHPLC-MS/MS quantification, with enhanced sensitivity and specificity. Although these reference methods are not intended to be used on products where these vitamins have not been added, with some adaptations and new validation rounds, they may form the basis as a future reference method for application to foods.

## 2.7. Vitamin B<sub>3</sub>

In the labelling of food products, vitamin B<sub>3</sub> is often described as niacin. The vitamin Activity is linked to two different molecules: nicotinic acid and nicotinamide (where an -NH<sub>2</sub> replaces the -OH of the carboxylic group) and both forms are allowed for fortification in foods and food supplements (Codex, 1979; EC, 2009). Some food standards and regulations express the amount in niacin equivalents (NE), which includes tryptophan, where 1 mg NE is 1 mg niacin or 60 mg tryptophan (Codex, 1985; USFDA, 2016).

Rich sources of vitamin B<sub>3</sub> include animal tissues, particularly as liver, muscles, kidney as well as cereals. In animal tissues, vitamin B<sub>3</sub> is mainly present as nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), while in cereals vitamin B<sub>3</sub> is mainly present as esters with polysaccharides (niacytin) or is bound to polypeptides and glycopeptides (niacinogenes) (Chamlagain, Rautio, Edelmann, Ollilainen, & Piironen, 2020). This is important when considering the analysis of natural niacin present in food, as nicotinic acid and nicotinamide must both be released from NAD and NADH by the analytical procedure.

In the current European Standard EN 15652:2009 (CEN, 2009c) three method options are available. Option A is using an acid treatment in the sample preparation, option B uses an enzymatic treatment, and option C incorporates an alkaline treatment. In options A and B, nicotinic acid and nicotinamide are separated and quantified. For these two options, vitamin B<sub>3</sub> expressed as nicotinic acid and gives similar results, although it should be noted that a small amount of nicotinamide is transformed into nicotinic acid with the acid treatment. Typically, option A is faster than option B; however, if the exact amount of each form needs to be quantified, option B must be used. With option C, nicotinamide is transformed into nicotinic acid and the results are the same as with options A and B, except for cereals where the natural amount of vitamin B<sub>3</sub> is increased by the alkaline treatment. Chamlagain et al. (2020) challenged that this treatment may reflect total niacin rather than actual bioaccessible niacin.

With recent modifications of the European regulations, it seems important to test the applicability of the current CEN standard (CEN, 2009c) on two newly permitted forms of vitamin B<sub>3</sub> that can be used in food supplements and foods. European Regulation (EC, 2009) authorised the addition of inositol hexanicotinate in food supplements and more recently, European Regulation 2020/16 (EU, 2020) authorised the use of nicotinamide riboside chloride as a novel food in food supplements with maximum levels for the general population and pregnant and lactating women. European Regulation EU 2023/1065 (EU, 2023c) very recently also authorised the use of nicotinamide riboside chloride in foods as a source of niacin.

It may be possible that with sample preparation option A (acid hydrolysis) of EN 15652:2009 (CEN, 2009c), vitamin B<sub>3</sub> from these two new forms can be released when used in foods and food supplements. With option B, the nicotinamide is released from the riboside chloride with the action of the enzyme NAD<sup>+</sup> glycohydrolase by breaking the N-ribosidic bond (Apitz, Mickelson, Shriver, & Cordes, 1971). Where inositol hexanicotinate is used as vitamin B<sub>3</sub> source in fortified foods, it may be more difficult to release all nicotinate moieties during analysis.

A technical challenge when analysing vitamin B<sub>3</sub> in food supplements relates to the considerable difference in maximum amounts that are allowed to be used for nicotinamide and nicotinic acid, respectively. The tolerable upper intake level for nicotinamide is between 70 and 100 times higher as compared with nicotinic acid (EFSA, 2006). It is therefore important that nicotinamide and nicotinic acid forms are quantified and reported separately in food supplements.

## 2.8. Vitamin B<sub>5</sub>

Vitamin B<sub>5</sub>, pantothenic acid, a component of both coenzyme A and acyl carrier protein, is involved in multiple metabolic reactions related to the release of energy from carbohydrates, fats, and amino acids.

Pantothenic acid is readily available in foods from animal and plant origin with the highest amounts in liver, kidney, yeast, egg yolk, and broccoli. Although also available in foods in its free form, the release of the bound forms is required for the quantification of total pantothenic acid in foods. Acid or alkaline hydrolysis conditions are not suitable to release this compound due to its lability at either acid or basic pH. Therefore, a combination of enzymatic treatments is generally used to liberate pantothenic acid before further quantification (Andrieux, Fontannaz, Kilinc, & Campos-Giménez, 2012).

AOAC 992.07 (AOAC INTERNATIONAL, 2023i) for the determination of pantothenic acid is based on a microbiological turbidimetric method and validated for milk-based infant formula. This method was replaced with AOAC 2012.16 and ISO 20639:2015 using UHPLC-MS/MS (AOAC INTERNATIONAL, 2023j; ISO, 2015b). Comparison of results between total and free pantothenic acid showed that the analysis of free pantothenic acid alone gave a good estimation of total pantothenic acid in the range of fortified products analysed. The method provides reliable free pantothenic acid results in a wide range of fortified foods including infant and adult nutritionals, cereal products and beverages, and shows good correlation with the microbiological method AOAC 992.07 (Andrieux et al., 2012). LC-MS/MS methodology is a more selective, faster, and more robust alternative to microbiological determination and the development and validation of a reference LC-MS/MS method for foods is recommended (Andrieux et al., 2012). It should be noted however, that pantothenol, the alcohol analogue of pantothenic acid, is also allowed as source for the fortification of foods, food supplements, infant formula and follow on formula, processed cereal-based food and baby food, food for special medical purposes and total diet replacements for weight control (Codex, 1979; EC, 2009; EU, 2013b), and should be considered when determining total pantothenic acid in future reference methods.

## 2.9. Vitamin B<sub>6</sub>

There are six vitamers of vitamin B<sub>6</sub>: pyridoxine (pyridoxol), pyridoxal and pyridoxamine and their corresponding phosphorylated derivatives pyridoxine 5'-phosphate, pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate, with the vitamin Activity of each being identical (Ball, 2006). Vitamin B<sub>6</sub> as pyridoxal 5'-phosphate serves as a cofactor for enzymes in many metabolic reactions.

Vitamin B<sub>6</sub> can be found in many food products either free or as phosphorylated forms, with the highest amounts found in dry yeast and wheat germ. However, for a human diet, animal sources such as meat, and fish as well as fruits, vegetables and cereals are important sources (Ball, 2006). Vitamin B<sub>6</sub> is mainly present as pyridoxine in plants and as pyridoxal and pyridoxamine in animal tissue, often bound to proteins and carbohydrates. In some vegetables vitamin B<sub>6</sub> can also be present as glucosides (Kall, 2003), but the bioavailability of these glucoside forms are regarded as lower (Gregory, 1997; Gregory et al., 1991).

To quantify vitamin B<sub>6</sub> in food products, it is therefore necessary to include an acid hydrolysis and an enzymatic step with a phosphatase as part of the analytical procedure to liberate the three bound forms. This drives the choice for an analytical method to either quantify the three forms separately or chemically transform to a single vitamer form to determine an aggregate total vitamin B<sub>6</sub>. In addition, to quantify the vitamin B<sub>6</sub> glucosides the analytical procedure must include an enzymatic step with glucosidase. There are two European Standards to determine vitamin B<sub>6</sub> in foods. EN 14663:2005 (CEN, 2005) includes an extraction of total vitamin B<sub>6</sub> after an acid hydrolysis and an enzymatic treatment with phosphatase, with pyridoxine, pyridoxamine and pyridoxal subsequently separated by HPLC and quantified by fluorometry. An additional optional enzymatic step with glucosidase has also been included to liberate pyridoxine glucosides. EN 14164:2014 (CEN, 2014d) includes an extraction of vitamin B<sub>6</sub> after an acid hydrolysis and an enzymatic treatment. Pyridoxamine and pyridoxal are both subsequently chemically transformed to pyridoxine, which is separated by HPLC and quantified by fluorometry.

In addition to pyridoxine hydrochloride and pyridoxine 5'-phosphate, European regulations have authorised the use of pyridoxine dipalmitate as a source to fortify foods (EC, 2009) in particular foods for special medical purposes, processed cereal-based food and baby food and total diet replacement for weight control (EU, 2013b). This source of vitamin B<sub>6</sub> is not captured with the current reference methods to quantify vitamin B<sub>6</sub> in foods.

## 2.10. Vitamin B<sub>7</sub>

Biotin (vitamin B<sub>7</sub>), initially named as vitamin H, or in some countries as vitamin B<sub>8</sub>, is an essential micronutrient for all mammals because of its role as a cofactor for carboxylase enzymes. Human cells have four biotin-dependent carboxylases that catalyse key reactions in gluconeogenesis, amino acid catabolism, and fatty acid synthesis. Biotin has three asymmetric carbons, and can therefore exist as eight potential stereoisomers, but only the D-biotin form is both biologically active and found in nature and is the only form of the parent vitamin that requires consideration. Biotin is widely distributed in the human diet, either mostly free in the case of milk, vegetables and fruit, or partly covalently protein-bound as biocytin ( $\epsilon$ -N-biotinyl-L-lysine) in animal tissues and plant seeds (Eitenmiller, Landen Jr., & Ye, 2008; Woollard & Indyk, 2013).

Current global and international reference methods (EU, CEN, Codex, ISO, AOAC, GB, FSANZ) do not adequately define this micronutrient, leaving a gap as to whether the bioavailable biocytin form should be included from a compliance perspective. All current regulatory standards define this vitamin simply as biotin, and most do not reference its stereoisomeric form. Further, the presence of protein bound biocytin in a food will lead to an underestimate of bioavailable biotin if excluded from analysis.

For an estimation of total biotin in food, a proteolytic step with biotinidase is required to specifically release biotin from any biocytin present in the food, which may then be included with free biotin in the analysis. Prior to end-point analysis, the variation in extraction protocols found in international reference methods related to the analysis of biotin in foods is a challenge, partly due to issues related to availability and specific conditions required for biotinidase (Eitenmiller et al., 2008; Woollard & Indyk, 2013). Variability of vitamin stability and recovery as a result of variations in sample preparation can have a greater significance on the result than the actual end-point detection technique and standardisation of sample preparation across methods is therefore needed to reduce food compositional data variability. Irrespective of whether free or total biotin is estimated, analytical methods report content as biotin. For those analytical methods in which biocytin is included, its bioavailability is accounted for as follows:  $1 \mu\text{g D-biocytin} = 0.656 \mu\text{g D-biotin}$  due to the difference in molecular weight.

Analytical techniques utilised for the determination of biotin content can be categorised as microbiological, biological (no longer used), chromatographic, or biospecific ligand-binding, and have been comprehensively reviewed (Eitenmiller et al., 2008; Woollard & Indyk, 2013). Although the microbiological assay (GB 5009.259–2016) remains the reference method for foods (GB, 2016b), chromatographic methods with MS, UV or fluorescence detection are increasingly utilised (AOAC INTERNATIONAL, 2023k, 2023l; CEN, 2009d; Gill, Saldo, Wood, & Indyk, 2018; Woollard & Indyk, 2013). However, among current global reference methods, whether biocytin-derived biotin is included in the reported measurement is not always apparent, nor consistent. In contrast to most foods, this issue is less significant in the case of infant formulas, where the overwhelming majority of biotin is present in its free supplemental form.

While existing global reference methods target biotin as measurand, they are ambivalent and inconsistent regarding the inclusion of biocytin in the determination of biotin content of a food (AOAC INTERNATIONAL, 2023k, 2023l; CEN, 2009d; GB, 2016b; ISO, 2020c). Codex has adopted the AOAC and its equivalent ISO standard and CEN methods as Type II and III for foods respectively for special dietary uses (Codex, 1999). Depending on the reference method used, inherent differences in analytical results can therefore be expected.

In summary, the gap that currently exists is due to the absence of a more specific definition of biotin, generating confusion, primarily regarding either whether biocytin should be included in an estimate of total biotin, or to measure free biotin alone, the latter resulting in an underestimate of nutritionally available content. For this reason, regulatory standards should be revised and preferably harmonised globally to define the specific forms of biotin to be determined during analysis.

## 2.11. Vitamin B<sub>9</sub>

Folic acid (vitamin B<sub>9</sub>) is the synthetic form of folate used as a reference compound for a group of folate vitamers that have vitamin Activity. 5-Methyltetrahydrofolate and 5-formyltetrahydrofolate are the main vitamers that occur naturally in foods, whether in a free form or as polyglutamates, while folic acid is the primary form used for food fortification. Folic acid is generally not present in nature, or present at very low levels from oxidation of other vitamers. To account for difference in bioavailability of supplemental versus natural occurring folate forms, the unit dietary folate equivalent (DFE) has been introduced. Table 3 gives an overview of units of expression and their definition as per Codex standards (1987; Codex, 1981, 1985) and regional regulations in the US (USFDA, 2016, 2019), EU (EC, 2009, EU, 2011, EU, 2016), China (GB, 2008, 2010, 2011, 2012, 2013, 2021a, 2021b, 2021c) and Australia/New Zealand (FSANZ, 2018a, 2018b, 2018c, 2018d).

Most global standards and regional regulations use DFE as the unit to express total folate content of foods, although general rules for nutrition labelling in the EU refer to folic acid in micrograms (EU, 2011). However, specific regulations authorising fortification of food products, such as infant formula, require the use of DFE to express folate content (EU, 2016). European regulation also allows the use of (6S)-5-methyltetrahydrofolic acid, glucosamine salt as a source of folate added for nutritional purposes to food supplements (EU Regulation 2015/414 (EU, 2015)), as well as the use of calcium-L-methylfolate as a source of folate in food for special medical purposes and total diet replacement for weight control (EU, 2013b).

Following a request from the European Commission, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) proposed to use the same factor as for folic acid for conversion of 5-methyltetrahydrofolate into DFE for intakes < 400 µg day<sup>-1</sup>. As such intake levels are unlikely to be exceeded through fortified food consumption, the conversion factor of 1.7 relative to natural folate (NF) could be applied to 5-methyltetrahydrofolate added to foods and to food supplements providing < 400 day<sup>-1</sup>. At 400 µg day<sup>-1</sup>, 5-methyltetrahydrofolate was found to be more bioavailable than folic acid and a conversion factor of 2 is proposed for this intake level and higher (EFSA, 2022).

To be able to verify compliance with the labelled values, an analytical method is needed that can distinguish between supplemental folic acid and/or 5-methyltetrahydrofolate and natural available folates. There is currently no European (CEN) standard available to support this requirement. The current European standard for the determination of folates in foods is based on a microbiological assay (CEN, 2003b) and is only able to assess total folate content. Recently an AOAC Official Method 2011.06 and equivalent ISO Standard 20,631:2023 have been established for determination of folate in infant formula and adult

nutritionals (AOAC INTERNATIONAL, 2023m; ISO, 2023). This LC-MS/MS method quantifies folic acid as well as 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate and has been single-laboratory and multi-laboratory validated for selected foods, infant formula and adult nutritionals (Bhandari, Gao, & Szpylka, 2018; Szpylka, DeVries, Cheney, & House, 2012). A suitable alternative LC-MS/MS method (Loznjak Svarc et al., 2020b; Ložnjak, Striegel, Díaz De la Garza, Rychlik, & Jakobsen, 2020a) for a broad range of food matrices is also standardised by NMKL (NMKL, 2020). The method includes a simplified enzymatic extraction procedure using a plant-based deconjugase and quantifies total folate including folic acid and naturally occurring food folates 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, tetrahydrofolate, 10-formylfolic acid and 5,10-methenyltetrahydrofolate with satisfactory repeatability and reproducibility. To express total folate content in foods, it should be questioned if natural and supplemental 5-methyltetrahydrofolate can be distinguished analytically if the latter is used for fortification.

## 2.12. Vitamin B<sub>12</sub>

Vitamin B<sub>12</sub> is the term used to designate all compounds having the same biological activity as cyanocobalamin. It is present in nature as methylcobalamin, adenosylcobalamin and hydroxycobalamin, the latter originating mainly from the effect of light degradation. Food sources of vitamin B<sub>12</sub> are restricted to those of animal origin including fish, meat, poultry, eggs, and dairy products (Ball, 2006; Watanabe, 2007). Cyanocobalamin, the most stable form, is used in fortification and supplements, although is only present in nature in trace amounts. Vitamin B<sub>12</sub> cobalamin analogues, pseudo-vitamin B<sub>12</sub>, are molecules that, structurally, are very similar to vitamin B<sub>12</sub> and can also be found in nature. However, these are not utilised in the human body and may prevent uptake of physiologically active vitamin B<sub>12</sub> (Carmel, Karnaze, & Weiner, 1988). These vitamin B<sub>12</sub> analogues can be present in food supplements such as Spirulina or seaweeds (e.g., Nori, Wakame).

Microbiological methods such as AOAC 952.20 (AOAC INTERNATIONAL, 2023n), which are still considered as the reference method for foods and food supplements cannot distinguish vitamin B<sub>12</sub> from pseudo-vitamin B<sub>12</sub>. Consequently, certain foods may be claimed to be “rich in vitamin B<sub>12</sub>”, while in fact this may not be the case. Although chromatographic methods may be able to distinguish both forms, they have not included separation of pseudo-vitamin B<sub>12</sub> from active forms as part of method validation. There is currently no European standard for the determination of vitamin B<sub>12</sub> in foods. Recently, an AOAC Official Method 2011.10 and its equivalent ISO standard 20634:2015 have been published for the determination of vitamin B<sub>12</sub> in infant formula and adult nutritionals using HPLC-UV (AOAC INTERNATIONAL, 2023o; ISO, 2015c). In addition, AOAC published another HPLC-UV method (AOAC 2014.02; AOAC INTERNATIONAL, 2023p) for the determination of vitamin B<sub>12</sub>, also for infant formula and adult nutritionals with different sample preparation, but equivalent results as compared with AOAC 2011.10. Future reference methods

applicable to foods that are able to distinguish between vitamin B<sub>12</sub> and its cobalamin analogues are therefore required.

### 2.13. Vitamin C

Vitamin C (ascorbic acid) is widely distributed in fresh fruits and vegetables and is an important antioxidant of reactive oxygen species such as singlet oxygen and plays a role as co-factor for metalloenzymes in the reduction phase (Gropper et al., 2021). The forms L-ascorbic acid and the oxidised form dehydroascorbic acid (DHAA) exhibit similar activity (Frikke-Schmidt, Tveden-Nyborg, & Lykkesfelt, 2016). However, D-isoascorbic acid named erythorbic acid is cheaper to manufacture than L-ascorbic acid and in some countries, it is legal to substitute erythorbate for ascorbate when, for technical reasons, the antioxidant capacity rather than the vitamin C activity is required. In some countries, erythorbate is not permitted as an antioxidant, and is also prohibited for use with raw and unprocessed meats. It should be noted that Codex Guidelines and European regulations (Codex, 1979; EC, 2009; EU, 2013b) allow the use of L-ascorbyl 6-palmitate as a source for vitamin C in addition to L-ascorbic acid and ascorbate salts. Although ascorbyl palmitate does not occur in nature, it does exhibit the full antiscorbutic activity of ascorbic acid.

When analysing vitamin C in food products it is necessary to discriminate between L-ascorbic acid and D-isoascorbic acid and to include their dehydro-forms. Both ascorbic and dehydroascorbic acids should be quantified when estimating the total vitamin C activity of a food product. One of the significant challenges with any analytical method for the determination of vitamin C is the need to stabilise the very labile vitamin, both during extraction and subsequent measurement. Existing reference methods to determine vitamin C in foods can be divided into three categories:

- (1) Titration with 2,6-dichlorophenolindophenol. The advantage of these methods is that they are cheap, rapid and therefore suitable for quality control in a factory environment. The disadvantages are that (i) these methods do not include dehydroascorbic acid, (ii) D-isoascorbic acid will react in the same manner, and (iii) these methods are vulnerable to potential interferences from pigmented and reducing sample components.
- (2) Fluorometric methods. These are based upon oxidation of ascorbic acid to dehydroascorbic acid and reaction with *o*-phenylene diamine (OPDA) to form a quinoxaline. Advantages are that these methods include dehydroascorbic acid and can be applied to coloured extracts and samples containing reducing substances, although suitability to green leafy vegetables has been questioned (Wills, Wimalasiri, & Greenfield, 1983). A disadvantage is that D-isoascorbic acid will react in the same manner.

- (3) HPLC based methods. These methods are based on acid extraction followed by chromatographic separation. For example, Kall and Andersen (1999) described a method for simultaneous determination of ascorbic acid and isoascorbic acid detected by UV, after which dehydroascorbic acid and dehydroisoascorbic acid were quantified following post-column derivatisation with OPDA and fluorescence detection.

There is no European standard for the determination of vitamin C in foods. Until 2017, AOAC 967.21 (AOAC INTERNATIONAL, 2023q), a titrimetric method applicable to determination of reduced ascorbic acid in citrus and tomato juices, was considered as a reference method (Type II) for the determination of vitamin C in infant formula by Codex. A method for the quantification of ascorbic acid and isoascorbic acid in fortified foods including infant formula and nutritional products (Fontannaz, Kilinc, & Heudi, 2006) was the basis for a recent AOAC official method and ISO standard for vitamin C in infant formula and adult nutritionals (AOAC INTERNATIONAL, 2023r; Campos-Giménez & Martin, 2017; ISO, 2018b). Using this method, applicability to a wider scope of matrices could be demonstrated for a future reference method applicable to foods. However, currently no reference method exists to quantify ascorbyl palmitate when used as a vitamin C source.

### 3. Recommendations

Existing reference methods for the determination of vitamins in foods need to be updated/renewed because of:

- Lack of up-to-date reference methods validated for foods in general
- New units of expression of vitamin Amounts
- Newly authorised molecules for fortification
- Newly identified compounds with or without vitamin Activity
- Advanced analytical technology, e.g. MS/MS as more selective, faster and robust alternative to microbial determination
- Simultaneous multi-vitamin Analysis possible to save time and costs

For each of the vitamins the main recommendations for the development of new reference methods and/or other actions are summarised in Table 4.

As new analytical method development is time consuming and costly, an alternative first step could be to establish method performance requirements for the determination of relevant vitamins in foods. However, trueness is an important criterion typically not included in method performance requirements and the

availability of (certified) reference materials is therefore extremely important. If not available, the creation of these materials could ideally be aligned with developing reference methods.

## Conclusions

Existing reference methods for the determination of vitamins in foods originate from the 1990s, with more recently established reference methods between 2010 and 2021 specifically scoped for infant formula and adult nutritionals. Developments in science, technology and regulatory requirements have identified the need to consider new global reference methods applicable to vitamins and carotenoids to enable the verification of regulatory compliance and determination of food composition.

This area of nutrients may benefit from globally harmonised definitions on what compounds to include or exclude for analysis and their corresponding bioactivity factor where applicable.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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**Table 1. Overview of units of expression and conversion factors according to global and regional regulations for vitamin A including carotenoids<sup>a</sup>**

Source and Authority	Expression / calculation
Pre-formed retinol and retinyl esters	
Codex	$\mu\text{g RE or RAE} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87)$
USFDA	$\mu\text{g RAE} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87)$
EU	$\mu\text{g vitamin A} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87)$
CHINA GB	$\mu\text{g RE} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87)$
FSANZ	$\mu\text{g RE} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87)$
Provitamin A carotenoids (foods)	
Codex	$\mu\text{g RE} = (\mu\text{g } \beta\text{-carotene} / 6) + (\mu\text{g other provitamin A carotenoids} / 12)$ $\mu\text{g RAE} = (\mu\text{g } \beta\text{-carotene} / 12) + (\mu\text{g other provitamin A carotenoids} / 24)$
USFDA	$\mu\text{g RAE} = (\mu\text{g supplemental } \beta\text{-carotene} / 2) + (\mu\text{g supplemental } \alpha\text{-carotene and } \beta\text{-cryptoxanthin} / 4) + (\mu\text{g dietary } \beta\text{-carotene} / 12) + (\mu\text{g dietary } \alpha\text{-carotene and } \beta\text{-cryptoxanthin} / 24)$
EU	No scientific consensus
CHINA GB	$\mu\text{g RE} = (\mu\text{g } \beta\text{-carotene} / 6) + (\mu\text{g other provitamin A carotenoids} / 12)$
FSANZ	$\mu\text{g RE} = (\mu\text{g synthetic all trans } \beta\text{-carotene} / 6) + (\mu\text{g total dietary } \alpha\text{-carotene, } \beta\text{-carotene, } \beta\text{-cryptoxanthin and other provitamin A carotenoids like } \beta\text{-apo-8'-carotenal, } \beta\text{-apo-8'-carotenoic acid ethyl ester} / 12)$
Infant formula and formula for older infants and young children	
Codex	$\text{Codex } \mu\text{g RE} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87)$ (carotenoids excluded)
USFDA	$\mu\text{g RAE} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87) + (\mu\text{g supplemental } \beta\text{-carotene} / 2) + (\mu\text{g supplemental } \alpha\text{-carotene and } \beta\text{-cryptoxanthin} / 4) + (\mu\text{g dietary } \beta\text{-carotene} / 12) + (\mu\text{g dietary } \alpha\text{-carotene and } \beta\text{-cryptoxanthin} / 24)$
EU	$\mu\text{g RE} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87)$ carotenoids excluded
CHINA GB	$\mu\text{g RE} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87)$ carotenoids excluded
FSANZ	$\mu\text{g RE} = \mu\text{g retinol} + (\mu\text{g retinyl palmitate} \times 0.55) + (\mu\text{g retinyl acetate} \times 0.87) + (\mu\text{g synthetic all trans } \beta\text{-carotene} / 6) + (\mu\text{g total dietary } \alpha\text{-carotene, } \beta\text{-carotene, } \beta\text{-cryptoxanthin and other}$

<sup>a</sup> Abbreviations are: RE, retinol equivalents; RAE, retinol activity equivalents

**Table 2. Overview of units of expression and conversion factors according to global and regional regulations for vitamin E**

Source and Authority	Expression / calculation
<b>Foods</b>	
Codex	1 mg $\alpha$ -tocopherol = 1 mg RRR- $\alpha$ -tocopherol (D- $\alpha$ -tocopherol)
USFDA	1 mg vitamin E (as $\alpha$ -tocopherol) label claim = 1 mg of natural $\alpha$ -tocopherol (D- $\alpha$ -tocopherol) = 2 mg all-rac- $\alpha$ -tocopherol
EU	mg vitamin E
CHINA GB	1 mg $\alpha$ -TE ( $\alpha$ -tocopherol equivalent) = 1 mg D- $\alpha$ -tocopherol $\alpha$ -tocopherol equivalent = $\alpha$ -tocopherol +0.5 $\beta$ -tocopherol +0.1 $\gamma$ -tocopherol +0.3 tocotrienol
FSANZ	Conversion factors for all natural forms not provided. 1 $\mu$ g $\alpha$ -TE = <ul style="list-style-type: none"> <li>• 1.36 DL-<math>\alpha</math>-tocopherol</li> <li>• 1.10 D-<math>\alpha</math>-tocopheryl acetate</li> <li>• 1.49 DL-<math>\alpha</math>-tocopheryl acetate</li> <li>• 1.23 D-<math>\alpha</math>-tocopheryl acid succinate</li> </ul>
<b>Infant formula and formula for older infants and young children</b>	
Codex	1 mg $\alpha$ -TE ( $\alpha$ -tocopherol equivalent) = 1 mg D- $\alpha$ -tocopherol
USFDA	1 mg vitamin E (as $\alpha$ -tocopherol) label claim = 1 mg of natural $\alpha$ -tocopherol (D- $\alpha$ -tocopherol) = 2 mg all-rac- $\alpha$ -tocopherol
EU	mg $\alpha$ -tocopherol, based on vitamin E activity of RRR- $\alpha$ -tocopherol
CHINA GB	1 mg $\alpha$ -TE = <ul style="list-style-type: none"> <li>• 1/0.74 = 1.35 mg DL-<math>\alpha</math>-tocopherol</li> <li>• 1/0.5 = 2 mg <math>\beta</math>-tocopherol</li> <li>• 1/0.1 = 10 mg <math>\gamma</math>-tocopherol</li> <li>• 1/0.01 = 100 mg <math>\delta</math>-tocopherol</li> <li>• 1/0.3 = 3.3 mg tocotrienol</li> </ul>
FSANZ	1 $\mu$ g $\alpha$ -TE = <ul style="list-style-type: none"> <li>• 1.36 DL-<math>\alpha</math>-tocopherol</li> <li>• 1.10 D-<math>\alpha</math>-tocopheryl acetate</li> <li>• 1.49 DL-<math>\alpha</math>-tocopheryl acetate</li> <li>• 1.23 D-<math>\alpha</math>-tocopheryl acid succinate</li> </ul>
<b>Food supplements</b>	
Codex	1 mg $\alpha$ -TE ( $\alpha$ -tocopherol equivalent) = 1 mg D- $\alpha$ -tocopherol
USFDA	1 mg vitamin E (as $\alpha$ -tocopherol) label claim = 1 mg natural $\alpha$ -tocopherol (D- $\alpha$ -tocopherol) = 2 mg all-rac- $\alpha$ -tocopherol
EU	1 mg $\alpha$ -TE = 1 mg of D- $\alpha$ -tocopherol
CHINA GB	<ul style="list-style-type: none"> <li>• 1 mg DL-<math>\alpha</math>-tocopherol = 0.74 mg D-<math>\alpha</math>-tocopherol</li> <li>• 1 mg D-<math>\alpha</math>-tocopherol acetate = 0.91 mg D-<math>\alpha</math>-tocopherol</li> <li>• 1 mg DL-<math>\alpha</math>-tocopherol acetate = 0.67 mg D-<math>\alpha</math>-tocopherol</li> <li>• 1 mg vitamin E calcium succinate (natural type) = 0.78 mg D-<math>\alpha</math>-tocopherol</li> <li>• 1 mg vitamin E calcium succinate (synthetic) = 0.57 mg D-<math>\alpha</math>-tocopherol</li> <li>• 1 mg D-<math>\alpha</math>-tocopherol succinate = 0.81 mg D-<math>\alpha</math>-tocopherol</li> <li>• 1 mg DL-<math>\alpha</math>-tocopherol succinate = 0.60 mg D-<math>\alpha</math>-tocopherol</li> </ul>
FSANZ	1 $\mu$ g $\alpha$ -TE =

- 
- 1.36 DL- $\alpha$ -tocopherol
  - 1.10 D- $\alpha$ -tocopheryl acetate
  - 1.49 DL- $\alpha$ -tocopheryl acetate
  - 1.23 D- $\alpha$ -tocopheryl acid succinate
- 



**Table 3. Overview of units of expression and conversion factors according to global and regional regulations for folate**

Source and Authority	Expression / calculation
Foods	
Codex	1 µg dietary folate equivalents (DFE) • 1 µg food folate • 0.6 µg folic acid added to food or as supplement consumed with food • 0.5 µg folic acid as supplement taken on an empty stomach
USFDA	1 µg dietary folate equivalents (DFE) = µg naturally occurring folate + (1.7 × µg folic acid)
EU	µg folic acid
CHINA GB	1 µg dietary folate equivalents (µg DFE) = naturally occurring folic acid in food (µg) + 1.7 × folic acid as supplement (µg)
FSANZ	µg folic acid
Infant formula and formula for older infants and young children	
Codex	µg folic acid/100 kcal/100 kJ
USFDA	DFE = dietary folate equivalents; 1 DFE = 1 µg naturally occurring folate = 0.6 µg folic acid.
EU	1 µg dietary folate equivalents (DFE) = 1 µg food folate = 0.6 µg folic acid from formula
CHINA GB	µg folic acid
FSANZ	µg folic acid

**Table 4. Method precision from AOAC Method 2016.05 MLT for infant formula**

Vitamin	Recommendations/needs	Limitation
Vitamin A (and carotenoids)	<p>Reference methods for the determination of provitamin A carotenoids including <math>\alpha</math>-carotene, <math>\beta</math>-carotene, and <math>\beta</math>-cryptoxanthin in foods.</p> <p>Reference methods carotenoids in food supplements.</p> <p>Propose globally harmonized definition of vitamin A.</p>	<p>New expression of vitamin A in label requirements: REA (Retinol Activity Equivalent).</p> <p>Need for chiral column chromatographic methods able to confirm economic motivated adulteration e.g. botanical versus synthetic zeaxanthin, or claims on “wild salmon”.</p> <p>Regulatory definitions of vitamin A are focused on “all-trans” retinol only, whereas 13-cis retinol has 0.75 vitamin A activity as compared to “all-trans”.</p>
Vitamin E	<p>Reference methods using chiral column chromatography that can distinguish between natural and synthetic sources of <math>\alpha</math>-tocopherol.</p> <p>Validate future reference methods for all sources allowed for food fortification.</p>	<p>To express vitamin E correctly on a product label requires the use of different conversion factors for natural and synthetic sources of <math>\alpha</math>-tocopherol.</p> <p>Current reference methods are not validated for tocopheryl succinate and tocopheryl polyethylene glycol-1000 succinate.</p>
Vitamin D	<p>Future reference method including the 25-hydroxyvitamin D metabolites in addition to parent vitamin D2 and vitamin D3.</p> <p>Fit for purpose method applicable to demonstrate compliance with narrow European regulatory range for vitamin D in infant formula.</p>	<p>25-Hydroxy-vitamin D3 has 2.5 times more bioactivity as vitamin D3 and contributes significantly to vitamin D intake. Reference methods are lacking.</p> <p>Precision of current reference methods too high.</p>
Vitamin K	<p>Propose globally harmonized definition for vitamin K1 excluding cis-vitamin K1.</p> <p>Reference methods for the determination of phyloquinone and the menaquinones as well as dihydro-K1.</p>	<p>cis-Vitamin K1 has no vitamin K activity and currently considered as part of this vitamin.</p> <p>Lack of reference methods to determine these compounds in foods.</p>
Vitamin B1	<p>Consider thiamine metabolite 2-(1-hydroxyethyl)thiamine (HET) as vitamin B1.</p> <p>Propose globally harmonized convention how to express vitamin B1 on product label.</p>	<p>HET has equal bioactivity as compared to vitamin B1, currently not included and validated in reference methods, while present in foods.</p> <p>Vitamin B1 can be expressed as thiamine, thiamine chloride or thiamine chloride hydrochloride. This is confusing for enforcers of label claims.</p>

Vitamin B2	Reference methods for simultaneous quantitative determination of multiple B vitamins including B1, B2, B3, B6 in foods by LC-MS/MS.	This would increase specificity, sensitivity, efficiency and reduce costs as compared to individual LCFLD and/or microbial assays.
Vitamin B3	Include newly authorized molecules to fortify foods and food supplements in Europe: inositol hexanicotinate and nicotinamide riboside chloride in future validated reference methods.	New molecules not included/ validated with current available reference methods.
Vitamin B5	Replace current microbial assays for foods with LC-MS/MS and include authorized molecule pantothenol in validation of fortified foods and food supplements.	LC-MS/MS technology makes method more selective, faster and robust as compared to microbial assays. Current reference methods not validated for pantothenol as vitamin B5 source in foods and food supplements.
Vitamin B6	European regulation authorizes the use of pyridoxine dipalmitate in foods and food supplements.	Current reference methods do not include this vitamin B6 molecule.
Vitamin B7	Propose globally harmonized definition which includes biocytin.	Current reference methods ambivalent and inconsistent regarding inclusion of biocytin.
Vitamin B9	Replace current microbial reference methods with LC-MS/MS enabling the individual quantification of folate vitamers.	Total folate in foods is generally expressed as Dietary Folate Equivalents which require the use of different conversion factors for natural and synthetic sources of folate. Microbial methods are not able to distinguish between natural and synthetic sources of folate.
Vitamin B12	Reference methods applicable to foods and able to distinguish between vitamin B12 and analogues.	Microbial methods still considered as reference for foods. They cannot distinguish vitamin B12 from pseudo-B12.
Vitamin C	Reference methods for vitamin C in foods.	No fit for purpose reference methods validated for foods in general.