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Challenges to Quantify Total Vitamin Activity: How to Combine the Contribution of Diverse Vitamers?

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ABSTRACT

This state-of-the-art review aims to highlight the challenges in quantifying vitamin activity in foods that contain several vitamers of a group, using as examples the fat-soluble vitamins A and D as well as the water-soluble folate. The absorption, metabolism, and physiology of these examples are described along with the current analytical methodology, with an emphasis on approaches to standardization. Moreover, the major food sources for the vitamins are numerated. The article focuses particularly on outlining the so-called SLAMENGHI factors influencing a vitamer's' ability to act as a vitamin, that is, molecular **s**pecies, **l**inkage, **a**mount, **m**atrix, **e**ffectors of absorption, **n**utrition status, **g**enetics, **h**ost-related factors, and the **i**nteraction of these. After summarizing the current approaches to estimating the total content of each vitamin group, the review concludes by outlining the research gaps and future perspectives in vitamin analysis. There are no standardized methods for the quantification of the vitamers of vitamin A, vitamin D, and folate in foods. For folate and β-carotene, a difference in vitamer activity between foods and supplements has been confirmed, whereas no difference has been observed for vitamin D. For differences in vitamer activity between provitamin A carotenoids and retinol, and between 25-hydroxyvitamin D and vitamin D, international consensus is lacking. The challenges facing each of the specific vitamin communities are the gaps in knowledge about bioaccessibility and bioavailability for each of the various vitamers. The differences between the vitamins make it difficult to formulate a common strategy for assessing the quantitative differences between the vitamers. In the future, optimized stationary digestive models and the more advanced dynamic digestive models combined with in vitro models for bioavailability could more closely resemble in vivo results. New knowledge will enable us to transfer nutrient recommendations into improved dietary advice to increase public health throughout the human life cycle. *Curr Dev Nutr* 2019;3:nzz086.

Introduction

Worldwide, the term "vitamin" describes organic compounds that the human body by itself cannot produce in sufficient amounts to prevent the development of deficiency diseases. The Polish biochemist Casimir Funk coined the word "vitamine" when he discovered that thiamine could cure polyneurosis (1). In 1941, Burk and Winzler introduced the word "vitamer," when their investigation of biotin found that several chemical compounds possess biotin activity. At the Vitamin Conference on Gibson Island in July 1942, Burk and Winzler, together with their colleague Hickman, developed the definition of vitamer that embraced compounds that act in a similar way to overcome a given vitamin deficiency (2). The terms vitamine and vitamer have been challenged, and the spelling vitamine was later changed to vitamin, because not all the described compounds were amines like thiamine.

In total 13 vitamin activities have been identified, including the 4 fat-soluble vitamins A, D, E, and K and the 9 water-soluble vitamins B-1 (thiamine), B-2 (riboflavin), B-3 (niacin), B-5

(pantothenic acid), B-6 (pyridoxine), B-7 (biotin), B-9 (folate), B-12 (cobalamin), and C (ascorbic acid). An overview of the vitamers of each vitamin is provided in **Table 1**. Inadequate dietary intake of vitamins can cause lethal vitamin-specific deficiency diseases, which can also be cured by a sufficient intake, though visible signs of the deficiency period can persist. Vitamin intake requirements to prevent deficiency diseases have been established. However, due to challenges in identifying and quantifying the vitamers in foods, and because of the difficulty in understanding the complex bioactivity of vitamers in relation to food sources interacting with the host, it is still difficult to estimate the optimal intake for health through all stages of the lifecycle (3, 4).

Historically, the methods for quantification of each of the vitamins have relied on either a biological assay (i.e., an animal model) for the fat-soluble vitamins or a microbiological assay (i.e., specific bacteria requiring the individual B-vitamins for growth). A chemical method for the quantification of vitamin C was introduced in the 1930s (5), and subsequently chemical methods have gradually been introduced for the quantification of the other vitamins. Information on standardized specific chemical methods is provided in Table 1. The use of specific chemical methods introduced new challenges, including how to convert the specific information on the quantity of each vitamer into vitamin activity, and which compounds are actually vitamers. To estimate total vitamin activity, the respective vitamin activity of each of the vitamers must be understood and estimated. This includes assessments of bioaccessibility and bioavailability. Bioaccessibility describes the amount of an ingested nutrient that is potentially available for absorption, and it depends on digestion and on the release of the vitamer from the food matrix in the gastrointestinal tract (i.e., oral, gastric, and small intestinal). As soon as a food has been digested, bioaccessibility can be measured as either solubility or through a static or dynamic gastrointestinal model. The analytical methods used to estimate the bioaccessible amount of a vitamer can be similar to the methods used for the quantification of vitamers in foods.

By contrast, bioavailability is defined as the amount of an ingested nutrient that is absorbed and available for use in normal physiological functioning and storage (6). In addition to digestion and the release of the vitamer from the food matrix, bioavailability depends on absorption by intestinal cells, transport to body cells, and conversion into the functional form. It may be estimated in vivo through use of, for example, ^{13}C-labeled foods, or in vitro through, for example, the use of Caco-2 cell-line models.

The determinants of bioavailability were originally summarized for carotenoids in the mnemonic acronym "SLAMENGHI." The letters of the acronym represent the molecular **s**pecies of the nutrient (i.e., vitamer); its **l**inkage (i.e., whether it is bound to a protein or present as a glucoside); the **a**mount of vitamer in the meal consumed; the **m**atrix in which the vitamer is present; the **e**ffectors of absorption (i.e., the presence of fat for fat-soluble vitamins); the **n**utrient status (i.e., the difference in uptake when a person is deficient); **g**enetic factors (i.e., whether genetic variants have an effect on the absorption); **h**ost-related factors (i.e., whether sex and age affect absorption), and finally any **i**nteraction between the aforementioned factors (7).

This state-of-the-art review aims to describe current advances in the quantification of total vitamin activity from food, focusing on vitamin A, vitamin D, and folate. A short description will be given of each vitamin group's metabolism and function in the human body, followed by the state-of-the-art analytical methods used for their quantification in food (including efforts at standardization). The review then briefly enumerates the important food sources of the vitamers and current insights into their vitamin activity. Lastly we discuss current research gaps and offer perspectives on quantifying total vitamin activity in our food in the future.

Vitamin A

Retinol refers to a group of closely related fat-soluble compounds present in our diet, that is, all-*trans* retinol, 13-*cis* retinol, retinyl esters, and retinaldehyde. In addition, 3 carotenoids with provitamin A activity have been identified: α-carotene, β-carotene, and β-cryptoxanthin (**Figure 1**). Of these, β-carotene is the predominant carotenoid in the human diet and also believed to have the greatest vitamin A activity, with 12 μg of β-carotene from food being equivalent to 1 μg of retinol, or 1 μg retinol activity equivalent (RAE). For α-carotene and β-cryptoxanthin the amount is double, thus 24 μg of each vitamer is equivalent to 1 μg RAE (8).

Metabolism and function of vitamin A in the human body

Upon entering the small intestine, retinol and carotenoids follow slightly different routes of absorption. Retinol is absorbed together with lipids and other fat-soluble vitamins and packaged in chylomicrons as retinyl esters or retinyl palmitate before being released into the bloodstream or liver (9). Absorption of carotenoids is regulated by scavenger receptor class B type I, which can be repressed by the intestinal transcription factor intestine specific homeobox (ISX) (10, 11). Once absorbed, cytosolic β,β-carotene 15,15′-monooxygenase (BCO1) cleaves β-carotene to retinal, after which it is further converted to retinol and incorporated into chylomicrons and transported into the lymph duct (12, 13). Uncleaved β-carotene remaining in the enterocyte is also incorporated into the chylomicrons to be transported to the liver and peripheral tissues. Retinol is stored in the liver stellate cells as retinyl esters, for the body to call on when needed.

Retinol plays an important role in vision for most animals. In humans, retinal forms part of rhodopsin, which is a photochemical reacting to light exposure. One of the clinical consequences of vitamin A deficiency is therefore impaired vision in dim light, with severe deficiency leading to irreversible blindness (14). Retinol can be irreversibly converted to retinoic acid, which is a major ligand for retinoic acid receptors retinoic acid receptor/retinoid X receptor (RAR/RXR) that bind to promoter regions in DNA to initiate gene transcription (15). This process underlies many metabolic pathways and explains the multiple functionality of vitamin A in, amongst others, embryogenesis, cellular differentiation of mucus-secreting cells, synthesis of glycoproteins, and lipid metabolism.

Intake of retinol in large quantities can lead to hypervitaminosis A and can be toxic because the human body is not equipped with a feedback mechanism that regulates absorption of retinol (16). In contrast, β-carotene absorption is regulated by retinoic acid in a feedback mechanism under control of the homeobox ISX (17). Whereas low concentrations of retinoic acid stimulate absorption, high concentrations inhibit uptake of β-carotene. Therefore, no upper limit

TABLE 1 Summary of the vitamers for each of the 13 vitamins, including information on availability of standardized methods for foods, CRMs for foods, and quantification principle for the state-of-the-art analytical method

Vitamin	Vitamer	Standardized method[1], matrix[2] (organization)	CRM organization[3], matrix[4]	State-of-the-art method and quantification principle[5]
Vitamin A	Total retinol	Foods (CEN)	–	LC-UV
	All-*trans* retinol	Foods (CEN)	–	
	13-*cis*-retinol	Foods (CEN)	–	
	Retinol esters	IF, AN (ISO)	NIST[y]	
	Retinaldehyde	IF, AN (ISO)	–	
	Total β-carotene	–	ERM[d]	LC-VIS
	All-*trans* β-carotene	Foods (CEN)	ERM[d]	
	Total α-carotene	–	ERM[d]	
	All-*trans* α-carotene	–	ERM[d]	
	β-Cryptoxanthin	–	–	
Vitamin D	Vitamin D-3	Foods (CEN)/IF, AN (ISO)	ERM[b]/NIST[y]	LC-MS/MS
	Vitamin D-2	Foods (CEN)/IF, AN (ISO)	NIST[n]	
	25-Hydroxyvitamin D-3	–	NIST[q]	
	25-Hydroxyvitamin D-2	–	–	
Vitamin E	α-Tocopherol, total	Foods (CEN/ISO)	ERM[b]/NIST[y,m]	LC-UV/LC-fluorescence
	α-Tocopherol	IF, AN (ISO)	–	
	α-Tocopheryl acetate	IF, AN (ISO)	NIST[y]	
Vitamin K	Phylloquinone, total	Foods (CEN)	NIST[y]	LC-fluorescence/LC-MS/MS
	Phylloquinone, *trans*	Foods (CEN)	–	
	Phylloquinone, *cis*	Foods (CEN)	–	
	Menaquinone-4	–	–	
	Menaquinone-n (MK-n)	–	–	
Thiamine	Thiamine, total	Foods (CEN)	ERM[a,d,e]/NIST[x,m]	LC-fluorescence/LC-MS/MS
	Thiamine, free	–	NIST[z]	
	Thiamine	–	–	
	Thiamine monophosphate	–	–	
	Thiamine diphosphate	–	–	
	Thiamine triphosphate	–	–	
Riboflavin	Riboflavin, total	Foods (CEN)	ERM[a,e]/NIST[x,n,p]	LC-fluorescence/LC-MS/MS
	Riboflavin	–	–	
	Flavin mononucleotide	–	–	
	Flavin adenine dinucleotide	–	–	
Niacin	Niacin, total	Foods (CEN)	ERM[c]/NIST[x,m,q]	LC-MS/MS
	Niacin, free	–	NIST[z]	
	Nicotinic acid (niacin)	–	NIST[q]	
	Nicotinamide	–	NIST[x,y,m,p,q]	
	Bound forms of niacin	–	–	
Vitamin B-6	Pyridoxine, total	Foods (CEN)	ERM[d,e]/NIST[x,y,p]	LC-fluorescence/LC-MS/MS
	Pyridoxine, free	–	NIST[z]	
	Pyridoxine	–	–	
	Pyridoxal	–	NIST[p]	
	Pyridoxamine	–	NIST[p]	
	Pyridoxine-5′-phosphate	–	–	
	Pyridoxal-5′-phosphate	–	–	
	Pyridoxine-5′-d-glucoside	–	–	
Pantothenic acid	Pantothenic acid, total	Foods (CEN)	NIST[x,y,n,q]	LC-MS/MS
	Pantothenic acid	–	–	
	Coenzyme A	–	–	
Biotin	Biotin, total	Foods (CEN)	NIST[x,y]	LC-MS/MS
	Biotin	–	–	
	Biocytin (*o*-biotinyl lysine)	–	–	

TABLE 1 (*Continued*)

Vitamin	Vitamer	Standardized method[1], matrix[2] (organization)	CRM organization[3], matrix[4]	State-of-the-art method and quantification principle[5]
Folate	Folate, total	FOODs (CEN)	ERM[a,d,e]	LC-MS/MS
	Folic acid	–	NIST[y,m]	
	Dihydrofolate	–	–	
	Tetrahydrofolate (THF)	–	–	
	5-Methyltetrahydrofolate	–	–	
	5-Formyltetrahydrofolate	–	–	
	10-Formyltetrahydrofolate	–	–	
	5,10-Methenyltetrahydrofolate	–	–	
	5,10-Methylenetetrahydrofolate	–	–	
	10-Formyldihydrofolate	–	–	
	10-Formylfolic acid	–	–	
Vitamin B-12	Vitamin B-12, total	Foods (CEN)/IF, AN (ISO)	ERM[e]	LC-MS/MS
	Cyanocobalamin	–	–	
	Methylcobalamin	–	–	
	Adenosylcobalamin	–	–	
	Hydroxycobalamin	–	–	
Vitamin C	Vitamin C, total	IF, AN (ISO)	ERM[c]/NIST[x,y,p]	LC-UV
	Ascorbic acid	–	–	
	Dehydroascorbic acid	–	–	

[1]Standardised method in foodstuff for each vitamer or the total for a vitamin provided by CEN, European Committee for Standardisation and ISO, International Organization for Standardization; information on where to obtain (buy) CEN-methods: https://standards.cen.eu/dyn/www/f?p=CENWEB:5 or ISO-methods: https://www.iso.org/home.html
[2]IF, Infant formula; AN, Adult nutritional
[3]NIST, National Institute of Standards and Technology, ERM, European Reference material.
[4]CRMs: [a]BCR-121, wholemeal flour; [b]BCR-122, margarine; [c]BCR-431, brussels sprouts; [d]BCR-485, mixed vegetables; [e]BCR-487, pig liver; [f]ERM-BD600, wholemilk powder (more information at: https://crm.jrc.ec.europa.eu/). NIST: [n]SRM3235, soy milk; [m]SRM3233, fortified breakfast cereal; [p]SRM1549a, whole milk powder; [q]SRM1546a, meat homogenate; [x]SRM1869, infant/adult nutritional formula (milk/whey/soy-based); [y]SRM1849a, infant/adult nutritional formula I (milk-based); [z]SRM3287, blueberry (more information at: https://www-s.nist.gov/srmors/). SRM with reference values is provided in **Supplemental Table 2**.
[5]LC-UV, Liquid chromatography-ultraviolet light detection; LC-VIS, Liquid chromatography-visible light detection; LC-MS/MS, Liquid chromatography-tandem mass spectrometry.
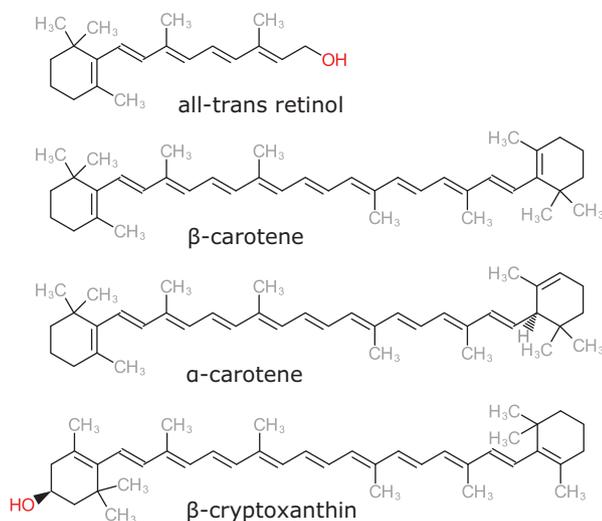

FIGURE 1 Chemical structures of vitamin A and its vitamers present in our diet.

of intake has been defined for β-carotene, whereas there is such a limit for preformed retinol.

In recent years, it has become clear that the bioconversion of provitamin A carotenoids is not restricted to enterocytes, because BCO1 is also expressed in peripheral tissue including the liver, kidney, eyes, skin, uterus, and testes (17, 18). It is still to be elucidated how much the local production of retinol from provitamin A precursors contributes to overall vitamin A requirements.

Analytical methods used for quantification of vitamin A vitamers in food

Methods for quantitative assessment of carotenoids were comprehensively reviewed in 2014 by Amorim-Carrilho et al. (19). There are no standard methods for carotenoid extraction, but it is mostly done with a water-miscible solvent such as acetone, methanol, ethanol, or tetrahydrofuran, followed by partitioning to hexane, petroleum ether, diethyl ether, or dichloromethane. In case chlorophyll or lipids need to be removed from the sample, a saponification step is required. However, this can lead to loss of some of the more polar carotenoids. Reversed-phase HPLC is the preferred method for carotenoid separation coupled with either a diode array (DAD), a visible spectroscopic detector, or an MS detector. The most common mobile-phase solvents used for carotenoids are acetonitrile and methanol, with small amounts of other solvents being added for optimal carotenoid recovery. Because foods are usually composed of a variety of carotenoids, modification of the chromatographic system, e.g. the mobile phase, may be needed for different types of foods. Some standardization in methodology for the analysis of retinol and β-carotene in foods has been undertaken (e.g. Szpylka & De Vries, 2005; Blake, 2007). Some standardization in methodology for the analysis of retinol and β-carotene in foods

has been undertaken (20, 21). Although hampered by the large range of food matrices, official methods have been published for quantifying retinol and β-carotene in foods, which include information on foods included in the validation (22–25). Major critical steps in the procedure are sample collection and sample preparation, due to the vulnerability of vitamers to heat, light, acid, and oxygen exposure. The complexity of assessing retinol and provitamin A carotenoids in food is evidenced by relatively large interlaboratory variability, which appears to be mostly driven by sample preparation and to a lesser extent by the chromatographic system (26, 27). A few of the problems concern the instability of carotenoid internal standards and the lack of a labeled international standard usable in quantification by liquid chromatography–tandem mass spectroscopy (LC-MS/MS). Table 1 provides information on available reference materials. These are limited to retinol and α- and β-carotene, which hampers objective interlaboratory quality control.

Content of vitamin A vitamers in foods

So-called "preformed retinol" is only derived from animal-based or fortified foods, whereas provitamin A carotenoids are mostly derived from brightly colored fruits and vegetables, with dairy products such as margarine and cheese as modest animal-based sources. Abundant β-carotene (values in μg/100 g) is present in sweet potatoes (9444), carrots (8332), and spinach (6288) among the vegetables, and in dried apricots (2163), cantaloupe (2020), and wild plums (1930) among the fruits. Butternut squash (3116) contains an appreciable amount of β-cryptoxanthin, whereas carrots (14,251) are a rich source of α-carotene (28). Over the last decade, biofortified staple crops with increased β-carotene content have been developed through breeding, such as orange-fleshed sweet potato, orange maize, and yellow cassava, which are primarily marketed in low-income countries (29). Although the latter 2 contain relatively low concentrations of β-carotene, the typically large intake in certain populations make them good sources of provitamin A. Transgenic crops with enhanced provitamin A carotenoids, such as rice, wheat, sorghum, soybean, potato, cassava, cauliflower, tomato, and banana, are still undergoing research (30). The final content of provitamin A vitamers in food at the point of consumption largely depends on retention during food processing, storage, and preparation, which is determined by the duration of storage and the intensity of processing and preparation (31, 32).

Methods to estimate activity of vitamin A vitamers

Bioavailability of retinol from food is generally considered to be high, although this has been based on studies using oil as a matrix. The bioefficacy of provitamin A carotenoids from food is determined by the multiplier of bioavailability and bioconversion to vitamin A, usually referred to as the conversion factor. Whereas bioavailability is highly influenced by the food matrix, bioconversion is more driven by factors related to the host. SLAMENGHI factors were first described for carotenoids by West and Castenmiller in 1998 (7). Here we give only a brief summary of some new insights.

Species—It has been suggested that β-cryptoxanthin might be better absorbed compared with β-carotene (33); however, this claim has not yet been substantiated by thorough studies. Recently, vitamin A activity

has also been reported for some ketocarotenoids, namely, sapotexanthin and cryptocapsin (34).

Linkage—Carotenoid esters are frequently found in fruits and vegetables, but to date it is not known how these affect bioavailability.

Amount—The fraction of carotenoids absorbed decreases with increasing dose, as is seen for many nutrients (7); however, the total amount absorbed can still be higher from a larger dose.

Matrix—Liberation of carotenoids from the food matrix is 1 of the main limiting steps in their bioavailability. Green leafy vegetables, such as spinach and kale, are rich in carotenoids, but only around 5–10% of the total carotenoid content is bioavailable. In contrast, carotenoids from fruits show higher bioavailability despite their relatively lower carotenoid content (35–37).

Effectors of absorption—Fat content of the diet is the most important enhancer of carotenoid absorption (38–40), whereas fiber present in the diet can reduce absorption efficiency (41).

Nutrient status—Vitamin A status of the host affects absorption of β-carotene by a negative feedback loop via homeobox ISX (17).

Genetic factors—Common variants in the BCO1 gene have been shown to reduce the conversion of β-carotene by $\leq$69% (42), but several other genes might also influence absorption and conversion (43).

Host-related factors—Iron and zinc deficiency both can affect bioconversion of carotenoids to retinol, and vitamin A metabolism in general. In addition, any disease affecting fat absorption will also affect the absorption of carotenoids.

Interactions—No studies have looked into interactions between the factors stated above, but such interactions might exist.

Vast discrepancy exists between conversion factors reported for different foods and across settings (44, 45). A relatively small number of studies have attempted to assess the conversion factor of β-carotene from an oil-based matrix to retinol, which overall ranged from 2.1 to 3.8. Conversion factors are expressed as the amount (μg) of ingested provitamin A required to form 1 μg of retinol. Until 2001, the average conversion factor of β-carotene from food was considered to be 6, whereas it was 12 for other provitamin A carotenoids. This difference was based on chemical structure, which allows central cleavage of β-carotene to produce 2 retinol molecules, whereas α-carotene and β-cryptoxanthin will only yield 1 retinol molecule. This has resulted in reporting of provitamin A concentrations as retinol equivalents (REs) in food composition tables, taking these conversion factors into account. Based on a range of studies conducted at the end of the previous century, conversion factors for specific fruits and vegetables were shown to vary from 3 to 12 for tubers and fruits, and from 10 to 77 for cooked and raw vegetables (not including tubers) (44, 45). This prompted the Institute of Medicine in the United States to increase the conversion factors to 12 for β-carotene and 24 for other provitamin A carotenoids (RAEs). This was based on food patterns estimated to deliver ~20% of provitamin A carotenoids from fruit and ~80% from vegetables, thereby weighing conversion factors for vegetables more heavily than for fruit. Although many countries have followed suit by using the RAE rather than RE in their national food composition tables and nutrient intake recommendations, the WHO
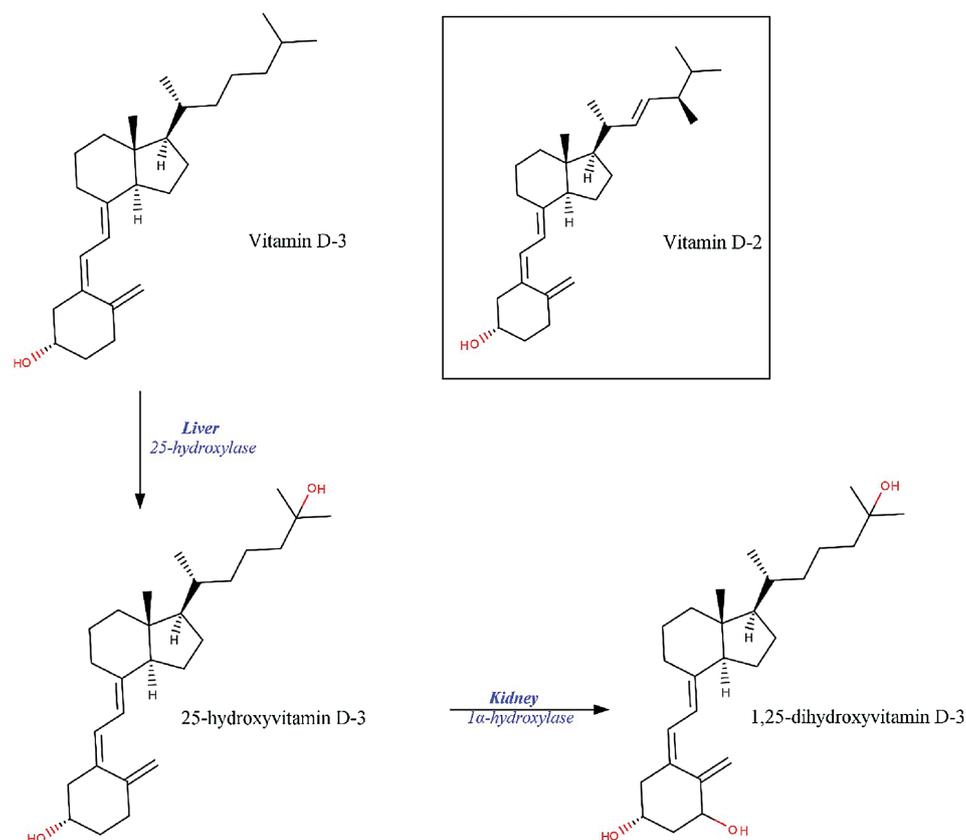
FIGURE 2 Chemical structures of vitamin D-3 and vitamin D-2, and the hydroxylation of vitamin D-3 to 25-hydroxy vitamin D-3 and 1,25-dihydroxyvitamin D-3 in the liver and in the kidney, respectively.

has not yet renewed its intake recommendations published in 2004 and therefore still uses the outdated RE (46). Even so, the revised vitamin A intake recommendations for Europe by the European Food Safety Authority in 2015 retained the old conversion factors (47). This is confusing for anyone trying to assess adequacy of dietary vitamin A intake, and especially relevant for populations with a high dependency on provitamin A intake to fulfill their vitamin A requirements (48).

Estimation of total content of vitamin A

Given the interlaboratory variation, it can be expected that values for food carotenoid concentrations are approximations rather than accurate figures in many cases. One of the pitfalls is the loss of vitamers or conversion of the *trans* to the *cis* configuration during sample preparation. Reliable measurement requires well-trained and experienced laboratory staff, in addition to proper laboratory facilities. Moreover, when the content of provitamin A carotenoids is expressed as vitamin A activity, it should be clear which conversion factors have been used. Lastly, although β-carotene concentration in foods is well characterized, less is known about the concentrations and vitamin activity of α-carotene and β-cryptoxanthin.

Gaps and future perspectives for estimation of vitamin A activity

The large interindividual variation in the bioconversion of β-carotene to vitamin A determines conversion factors—and thereby vitamin A activity—to a large extent. Better insight into the factors related to this variation is required. It might not be appropriate to use food-based conversion factors when bioconversion depends in large part on the host, and it would therefore be more logical to disentangle a bioavailability factor based on the food (food composition tables) and a conversion factor based on the host (nutrient requirements). This implies also that actual concentrations of vitamers should be reported and not be expressed as RE or RAE. Another aspect that needs attention is the lack of knowledge of the vitamin A activity of β-cryptoxanthin, which is suggested to be higher than assumed so far because of better bioavailability in comparison with β-carotene (33). Therefore, studies on conversion factors for β-cryptoxanthin, either synthetic or from food, are required.

Vitamin D

The vitamin D–active compounds are vitamin D-3, 25-hydroxyvitamin D-3 [25(OH)D3], vitamin D-2, 25-hydroxyvitamin D-2 [25(OH)D2], as well as the corresponding metabolites of 1,25-dihydroxyvitamin D [1,25(OH)2D]. Vitamin D-3 is synthesized in the skin of vertebrates from 7-dehydrocholesterol by UVB exposure, and vitamin D-2 is synthesized from ergosterol in fungi and in certain plants contaminated with fungi. Vitamin D compounds are called secosteroids, which means that they are steroids where 1 ring (the B ring between 9 and 10) is open;

the hydroxylated vitamers 25(OH)D3 and 1,25(OH)2D are present due to hydroxylation primarily in the liver and in the kidney, respectively (**Figure 2**). Vitamin D-3 and vitamin D-2 differ only in the side chain, with vitamin D-2 having a double bond (between carbons 22 and 23) and an additional methyl group.

Metabolism and function of vitamin D in the human body

Studies in rats have shown that the parent vitamer, vitamin D, is absorbed by passive diffusion and transported in chylomicrons to be hydroxylated in the liver, but investigation in human cell lines has shown that the absorption of vitamin D is far more complicated and uptake involves proteins in the intestinal cell membrane. Absorption in humans is between 55% to 99% (49). In contrast, 25(OH)D is absorbed via the portal route, but there is no information on amount absorbed (50).

Vitamin D plays an essential role for the homeostasis of calcium and phosphorus, and the diseases rickets in children and osteomalacia in the elderly are the outcomes of vitamin D deficiency. The accepted biomarker for vitamin D status is the concentration of 25(OH)D in serum, which represents the amount of vitamin D-3 generated by UVB exposure of the skin and vitamin D and 25(OH)D from the diet. The concentrations for insufficiency and deficiency are disputed, but 50 nmol/L and 30 nmol/L, respectively, of 25(OH)D are generally accepted (51, 52). The prevalence for deficiency depends on age and country, and is reported to be between 4% and 18% in Germany, the United Kingdom, the United States, and Canada (52), but ≤80% in Middle Eastern countries (53).

Besides bone health, vitamin D status has been associated with nonskeletal diseases, but the recent results from 3 intervention studies, which investigated the effect of supplementation of vitamin D-3 at levels of 2000 IU daily (54), 100,000 IU monthly (55), and 4000 IU daily (56), did not show decreased risk for development of cancer (54, 55), cardiovascular incident (54, 55), falls (55), fractures (55), or type 2 diabetes (56). However, it is argued that these studies lacked a criterion for vitamin D status, which at the start was between 64 and 77 nmol/L. Thus, a majority of the participants had a vitamin D status that is regarded as sufficient. A recent meta-analysis identified association between low vitamin D status and increased risk of all-cause mortality (57).

Analytical methods used for quantification of vitamin D vitamers in food

Content of vitamin D in food was originally assessed by biological assay until the introduction of chemical methods in the 1980s. The biological assay measures the ability to cure rickets in deficient rats, whereas chickens were used to differentiate between vitamin D-3 vitamers and vitamin D-2 vitamers (58, 59). The chemical methods introduced in the 1980 s focused on the parent forms vitamin D-3 and vitamin D-2, and comparing the 2 methods showed similar results for fortified food with vitamin D-3 (60). Since the mid-1990s the contents of 25(OH)D vitamers in meat, offal, eggs, and dairy products through the use of LC-UV/DAD methods have been published (61–63), and more recently LC-MS/MS methods for milk, pork, and liver have been reported (64–66). The principle of all the chemical methods is an alkaline saponification during which an antioxidant is added to protect vitamin D. The nonsaponifiable

extracts include the vitamin D vitamers and is subsequently cleaned up using liquid-liquid extraction with an organic solvent, for example, a combination of *n*-heptane and ethylacetate or iso-octane. Further clean-up steps can include solid-phase extraction (SPE) and/or preparative LC before detection and quantification by use of reversed-phase LC-UV/DAD. If reversed-phase LC-MS/MS is used for quantification, the matrix and the concentration determine if a solid-phase extraction is necessary. Additionally, a derivatization step utilizing a Diels–Alder reaction with, for example, 4-phenyl-1,2,4-triazoline-3,5-dionediene, is generally applied to enhance ionization, thereby increasing specificity and lowering the limit of quantification. However, there are no reports comparing the biological and chemical methods. Standardized methods for quantification of vitamin D-3 and vitamin D-2 in foods are available (67, 68), and recently, an LC-MS/MS method for infant formula and adult nutritionals was published (69, 70). Thus, no standardized method is available for the content of 25(OH)D3 and 25(OH)D2, but an interlaboratory trial, where the participating laboratories (5 in total) used their own in-house, single-laboratory validated methods, reported reproducibility between 8% and 24% in a variety of food matrices (71).

A few certified reference materials like meat homogenate, whole milk powder, and eggs are available (72, 73); see Table 1 for more information.

Content of vitamin D vitamers in foods

Only a limited number of foods naturally contain vitamin D. Vitamin D-3 is primarily found in food of animal origin, with the highest content in fish, and decreasing content in eggs, offal, meat, and dairy products. Except for fish and eggs, the concentration of vitamin D-3 is generally <1 µg/100 g, and decreasing amounts are found in liver, chicken, beef, pork, and dairy products. It should be noted that in Atlantic salmon (*Salmo salar*), the vitamin D-3 concentration in wild salmon is reported as 6.7–26.6 µg/100 g whereas it is lower in farmed salmon (2.3–9.1 µg/100 g), although data are limited (74). In general vitamin D-3 is accompanied by the vitamer 25(OH)D3, though in salmon to a lesser degree than in eggs, pork, beef, and dairy products, being ~5%, ~25%, 20–150%, ~250%, and 50–90% of the concentration of vitamin D-3, respectively (63, 74–77). In general, lean cuts of meat have a lower concentration of vitamin D and 25(OH)D than fatty parts (63, 66, 76). However, for wild fish, for example, mackerel and herring, in which fat content varies through the season, no association between fat and vitamin D-3 occurs (78). The vitamers, vitamin D-2 and 25(OH)D2, are also present in dairy products and beef (63, 77). In wild mushrooms and UVB-treated mushrooms vitamin D-2 is the major vitamer (79).

Increased concentrations of vitamin D-3 in feed for hens, pigs, and cattle yield products with higher concentrations of vitamin D-3, as in eggs, pork, and beef (62, 80, 81). Exposure to sun or artificial UVB will similarly increase the content of vitamin D-3 in dairy products and pork (66, 82, 83).

Feed regulations in the European Union have approved the use of 25(OH)D3 in feed for poultry, pigs, and cattle (84, 85). If 25(OH)D3 is used in the feeding of hens and pigs the content of 25(OH)D3 will increase while content of vitamin D-3 will decrease in eggs and pork, respectively (62, 80). If the animal is fed 25(OH)D3 from birth to slaughter, the content of vitamin D-3 in meat, for example, is negligible (86). Apart from vitamin D and 25(OH)D, the dihydroxyvitamin D vitamer 1,25(OH)2D has been reported in milk from humans and cows (65).

In many countries certain foods, especially milk and margarine, are either voluntarily or mandatorily fortified (51), either with vitamin D-3 or vitamin D-2.

Methods to estimate activity of vitamin D vitamers

The implementation of feeding systems that increase the content of 25(OH)D3 in food products or use of UVB exposure in mushrooms to generate products containing vitamin D-2 highlights the need to know the contribution to vitamin D activity from metabolites other than vitamin D-3. Based on recent reviews the SLAMENGHI factors affecting the absorption can be summarized (49, 50, 87, 88) as follows:

Species—The difference between the vitamers, 25(OH)D3 and vitamin D-2, compared with vitamin D-3 is thoroughly covered due to use of different estimates in food databases. The difference between the vitamers has been investigated in animal, human, and recently in vitro models.

25(OH)D3 versus vitamin D-3

In a review of data estimating the biological activity of 25(OH)D3 compared with vitamin D-3 using animal models it was concluded, "Based on calcification score testing in rachitic rats the biological activity of 25OHD is about 1.5 times greater than that of vitamin D. Based on the ability to enhance calcium absorption studies have demonstrated 25OHD3 to be 5 times as active as vitamin D3. These kinds of studies measure the acute effect of vitamin and can differentiate more precisely between effects of native vitamin D and metabolites. However, intestinal absorption of calcium is not a clinical end point parameter" (89). Testing of 25(OH)D3 in the animal assay estimated 25(OH)D3 to have 1.7 times greater vitamin activity than vitamin D-3 (90). Quesada-Gomez and Bouillon (2018) (87) reviewed the results from 4 human intervention studies (91–94), which included administration of 5–50 μg/d of 25(OH)D3 and vitamin D-3 in a parallel design. Administration of 1 μg of vitamin D-3 or 25(OH)D3 daily increased vitamin D status by either 1.5 nmol/L or 4.8 nmol/L, respectively. However, based on human intervention studies that administered 5–50 μg of vitamin D-3 daily, it is estimated that 1 μg/d of vitamin D-3 will increase vitamin D status by 2 nmol/L (95). Similarly, studies were identified that administered 25(OH)D3 (91–93, 96, 97). Combining the results from these studies revealed that administration of 1 μg/d of 25(OH)D3 increased vitamin D status by 3.9 nmol/L (range 1.8–5.0 nmol/L). Detailed information about the human intervention studies and the calculations are provided in **Supplemental Table 1** and **Supplemental Figure 1**.

From a human intervention study using a crossover design, it was estimated that administration of 25(OH)D3 increased vitamin D status 1.5 times more than compared to the increase obtained by administration of vitamin D-3 (98). Furthermore, another study estimated the differences in the AUC after a bolus of the 2 metabolites; it showed that 25(OH)D3 increased vitamin D status 2–3 times more than vitamin D-3 (96). Recently, in a human cell line (Caco-2), 25(OH)D3 was taken up more efficiently than vitamin D-3, and more solubilized into mixed micelles by a factor of ∼1.8 (99).

Vitamin D-2 versus vitamin D-3

As mentioned in the Introduction, vitamin D-3 and vitamin D-2 are regarded as equal, even though this consensus has been challenged by findings that administering vitamin D-2 has less effect on vitamin D status than administration of vitamin D-3 (98, 100).

Linkage—Vitamin D and 25(OH)D in food are regarded as nonbound, which is the form absorbed, but esterified vitamin D can be present. This has not been properly investigated (49).

Amount—Although not investigated for vitamin D and 25OHD in foods, a study in human cell line model (Caco-2) indicates that absorption is depending on concentration of vitamin D, due to the involvement of proteins in the absorption process (50).

Matrix—Food matrix seems not to have an effect, except for bread baked with vitamin D-2–enriched yeast, which showed no bioavailability in humans (50, 101). However, this has not been investigated for 25(OH)D.

Effectors of absorption—These could include fat, dietary fiber, and other vitamin D vitamers. The length of fatty acids might influence the absorption, because MUFAs enhanced the increase in vitamin D status, whereas PUFA decreased it (49). However, no information on 25(OH)D is available, and more research is needed to investigate the how fat and other compounds possibly affect the absorption of D vitamers.

Nutrient status—The expectation would be a lower absorption with higher vitamin D status; however, only 1 specific study in rats has shown this effect (50). During the last 20 y vitamin D-3 has been included in a large number of human intervention studies, although these have not directly focused on absorption. Based on studies administering <30 μg/d vitamin D-3, a curvilinear association between daily intake and vitamin D status was estimated (102). Administration of 25(OH)D3 revealed no effect on vitamin D status (87). However, a steady state for vitamin D status occurs independent of the vitamer administered.

Genetic factors—These have been shown to have a significant impact on vitamin D status e.g. the polymorphisms of the vitamin D binding protein GC, which is essential for vitamin D transport and of the 25-hydroxylase CYP2R1, which is essential for the hydroxylation of vitamin D to 25(OH)D are associated with increased vitamin D status obtained by supplementation and UVB exposure (103). However, a genome-wide association study of 80,000 persons provided evidence against large interactions between common single-nucleotide polymorphisms and dietary vitamin D intake in the population studied (104).

Host-related factors—Few studies have investigated the effect of age, but apparently no differences were identified (50). No information exists for differences between sex, but in studies where women and men have been included no differences were observed (92). Vitamin D status is inversely related to BMI, but this has not been related to differences in absorption (50).

Interaction of the above factors—Not investigated.

Estimation of total content of vitamin D

Estimations of the content of vitamin D in food by the biological methods that quantify total vitamin D activity in deficient rats are limited (59). An IU of vitamin D is defined as 0.025 μg vitamin D-3, and vitamin D-2 is regarded as equivalent to vitamin D-3. For 25(OH)D, it could be valuable to transform the content to IU, but this is currently not possible because no international consensus is available; however, for this purpose it could be an advantage to establish a new unit, the
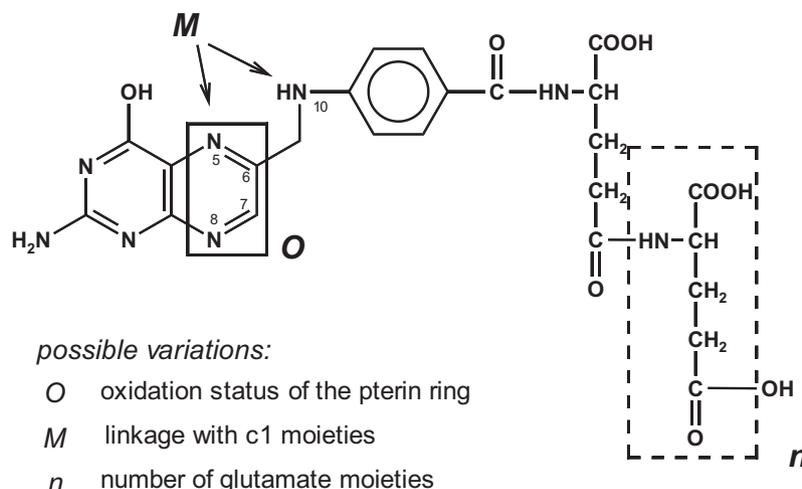
FIGURE 3 Structural variety of folates with natural bioactive forms occurring as reduced tetrahydro forms at O and glutamate distribution ranging from $n = 0$ to 7.

vitamin D equivalent (VDE), where 1 VDE equals 1 μg vitamin D-3. As reviewed above, no method to estimate the difference between the 2 metabolites has been established, and in humans, 1 μg 25(OH)D3 has been estimated to be in the range 1–5 VDE. A factor of 5 (∼5 VDE) is currently used in some food databases (e.g., in the United Kingdom and Denmark), whereas a factor of 1 (∼1 VDE) for 25(OH)D3 is included in other food databases (e.g., in the Netherlands and Canada). No database differentiates between vitamin D-2 and vitamin D-3. In the Codex Alimentarius International Food Standards (CODEX), the issue regarding a difference between vitamin D-2 and vitamin D-3 has been on the agenda, but not the challenges for estimating the vitamin D activity of 25(OH)D3 (105).

Gaps and future perspectives for estimation of vitamin D activity

Data for contents of 25(OH)D metabolites in foods are steadily growing and being incorporated into food databases. In contrast, limited data are available for contents of 1,25-dihydroxy metabolites; the metabolites should be included in future analytical methods in order to investigate if the content of the metabolite contributes significantly to vitamin D activity.

However, the main gap is how to estimate the total vitamin D activity based on the content of mainly vitamin D-3, vitamin D-2, 25(OH)D3, and 25(OH)D2. The food matrix seems so far not to be important, but limited data are available, therefore different food matrices should be investigated. If the method of choice is a human intervention study, it should take into account that an increase in vitamin D status is host dependent, as well as dependent on the amount administered, therefore a crossover design should be prioritized. An alternative would be to investigate the use of human cell lines in an effort to estimate the difference between the parent vitamer and the 25-hydroxylated vitamers. Another technique would be to use ^{13}C-labeled vitamin D vitamers, which have successfully been used for investigation of the metabolism and storage of vitamin D-3 in an animal model (106).

Until such results have been published, the vitamin D vitamers should be regarded as equal, that is, 1 μg 25(OH)D = 1 VDE, and the content of vitamin D is the sum of each of the vitamin D vitamers.

Folate

The folate group consists of a variety of compounds. Along with various pteroyl monoglutamic forms, folates in food primarily occur as pteroyl polyglutamates, which can contain 2 to 8 glutamyl residues in the molecule (**Figure 3**).

Metabolism and function of folate in the human body

Folates are essential in metabolism primarily for the transfer of methyl and formyl groups, and they perform key functions in the metabolism of amino acids and nucleic acids. Prior to absorption, polyglutamate forms have to be converted into monoglutamates. This cleavage is performed by gamma-glutamyl hydrolase (GGH), which is either secreted from the pancreas or present in the intestinal brush border. After deconjugation, the monoglutamates are absorbed via transmembrane folate carrier proteins into intestinal epithelium cells, where they are reduced to tetrahydrofolate and transformed to 5-methyltetrahydrofolate (107). The latter is then transported in the blood circulation after passing through the liver via the portal vein. Surveys of folate status in several countries have been based on plasma or erythrocyte folate concentrations and revealed that often a significant part of the population had inadequate folate status. In Europe, for example, this applied to ∼10% of the French population, to 4% of pregnant women in Switzerland, and to >40% of a Swedish cohort of pregnant women (108). This form of dietary deficiency is particularly significant for pregnant women or women of childbearing age because it is strongly associated with an increased frequency of neural tube defects in the developing fetus. There is also increasing evidence that inadequate folate intake increases the susceptibility to cardiovascular disease, colorectal cancer, and Alzheimer disease (109).

Analytical methods used for quantification of folate vitamers in food

Various methods are now used to quantify total folate, of which microbiological and chromatography-based determinations constitute the most important ones. Microbiological assays (MAs) quantify folates by the growth of microorganisms, such as *Lactobacillus casei*, on food

extracts containing folates. Due to its sensitivity and the need for only folic acid as calibrator, the MA is the gold standard in food folate analysis (110). Based on MAs, several reference materials such as mixed vegetables (73) or single food items ranging from meat to wheat flour (72) are commercially available and can assist in evaluating the assay's performance. More information is provided in Table 1. Despite the sensitivity of MAs for determining the overall folate content, they are not suited to the differential detection of individual vitamers. In addition, specific growth factors or inhibitors can provoke stimulation or inhibition of the microorganisms. Therefore, along with the variable response of the microorganisms to the single vitamers, the quantitative result can be compromised; the significant variations revealed by comparative studies can be attributed to these issues (111, 112).

Compared with the MA described above, the chemical analytical methods have the advantage of being able to distinguish between the individual folate vitamers. The most selective detection of folates in the field of LC methods is offered by MS. To compensate for losses of unstable folates during extraction and due to ionization interferences during MS, stable isotope dilution assays (SIDAs) have proved their strength in determining folate concentrations in food (113–116).

With SIDAs, stable isotopically labeled vitamers are used as internal standards (117) and LC-MS/MS allows accurate quantification of the single vitamers.

Several comparisons of these methods have been reported in the literature, often revealing higher total folate content from the MA than the chromatographic methods (118). A recent comparison between LC-MS/MS and MA supported these findings (119). For the latter comparative study, deconjugation of polyglutamates into monoglutamates was performed using pteroyl polyglutamate hydrolase (PPH) from chicken pancreas in combination with PPH from rat serum, at a pH of 5.0. After the experimental confirmation of enzyme activity with the test substrate pteroyl triglutamate, complete polyglutamate deconjugation in the sample matrix was to be expected. No significant differences between the methods were observed for broccoli, pea soup, or a reference material consisting of a mixture of vegetables. Substantial differences were seen with a wheat germ sample: the values from quantification by LC-MS/MS were ~35% lower than those from the MA. Further analysis showed that deconjugation was not complete, and that diglutamates as well as residues of higher polyglutamates were detectable in their respective LC-MS/MS traces. These results were used to develop the first reported method for quantifying the diglutamates (119). Although this method returned higher values for quantification using LC-MS/MS, an optimized enzyme treatment made it possible to determine a concentration of 392 µg/100 g for the wheat germ, which offered a good approximation of the result from the MA. In addition, the dependency of the MA calibration on the choice of folate must be taken into account, because this can result in differences of ≤20% in the total folate content (119). Screening for diglutamates was therefore included into the method in order to verify the completeness of deconjugation. Novel developments target improving deconjugation efficiency by using genetically engineered plant GGH (115, 120).

Content of folate vitamers in foods

Staple foods such as wheat or corn products, potato, or rice contain only minuscule amounts of folates (121) unless they are fortified with synthetic folic acid, as mandated in >50 countries, mainly on the South

and North American continents. A folate-rich diet requires careful selection of the food; in general, green vegetables, particularly legumes (122), and some fruits contain high concentrations of folates. Among the foods with highest folate content, cow peas are a very good source with >400 µg/100 g dry mass (123). This content is exceeded by the "king of fruit," the durian, which has the highest concentration of any fruit, with >400 µg/100 g fresh mass (124); this is consumed almost exclusively in Southeast Asia and China. Tropical fruits (125) have high potential as folate sources, for example, the giant granadilla with 270 µg/100 g fresh mass (N Weber, Technical University of Munich, personal communication, 2019), along with fruits from regions of high biodiversity, for example, the Australian green plum (126). Green vegetables generally are good folate sources, with the prominent ones being spinach with >227 µg/100 g (113) or broccoli with 183 µg/100 g (119). Among fruits, strawberries as highly appreciated by consumers worldwide and can contain >150 µg/100 g (127). Further promising sources include microalgae or yeasts (128).

Methods to estimate activity of folate vitamers

A first estimation of vitamin activity can be performed by MA, and it has already been shown that the MA reveals different responses to the vitamers (119). Interestingly, commonly used MAs are able to detect polyglutamates containing up to 3 glutamate residues with almost equivalent responses, which is a further asset of the MA (129). As conflicting results between the MA and chromatographic methods have also been attributed to additional compounds showing folate activity, the MA has also been used to assess folate derivatives modified during storage or food processing. In this regard, the oxidation product of 5-methyltetrahydrofolate, the pyrazino-s-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate (abbreviated as MeFox), revealed no response in an MA (119), and during testing the bioactivity of folate glycation products, *L. casei* revealed neither a positive response nor an inhibition of growth when exposed to N^2-[1-(carboxy)ethyl]folic acid or N^2-[1-(carboxy)methyl]folic acid (130).

As indicated by this short overview on the MA response to several vitamers, the SLAMENGHI factors mentioned before are also applicable to folate:

Species and Linkage—The vitamers differ in their biokinetics behavior; for example, 5-methyltetrahydrofolate applied in short-term human studies has an earlier t_{max}, i.e. its peak in plasma folate appears earlier than that of folic acid (131). The differences in overall bioavailability, in particular for the polyglutamylated forms, are still under discussion, with several studies giving inconclusive results (132).

Amount—Previous human studies indicate a linear relation between dose and plasma response after saturation of folate stores and below doses that will saturate the folate carriers. However, due to the uncertainties in response quantitation, this is an assumption.

Matrix—This factor has been assessed in more detail using bioaccessibility models investigating the influence of the food matrix on deconjugation or stability of folates during digestion. As a result of such in vitro studies a measure of bioaccessibility is obtained, which in the case of folates can be defined as the amount of monoglutamates obtained after simulation relative to the total amount of folates including polyglutamates initially present in the food. Several in vitro

simulations of digestion have been applied, with probably the most prominent being the TNO (the Netherlands Organisation for Applied Scientific Research) - intestinal model, a dynamic computer-controlled digestion model. It simulates the transit time, peristaltic mixing, and diffusion-based absorption of in vivo studies (133). Other approaches have used a simulated digestion model with respect to folate stability and efficiency of deconjugation by applying porcine brush border membrane to realistically simulate digestion in the small intestine (134). When comparing the latter bioaccessibility results with those from a human bioavailability study, the impact of both stability and deconjugation on bioaccessibility was confirmed. The most labile vitamer, tetrahydrofolate, can be degraded and significant loss affects all vitamers unless stabilized by antioxidants like ascorbic acid.

When studying bioavailability in vivo, short-term studies often apply an AUC approach. Using AUC a defined amount of folates is administered followed by frequent blood sampling within a defined period of time. The bioavailability is then calculated relative to the AUC resulting after the ingestion of a reference substance. Generally, folic acid has been used as the reference, but recent studies favor 5-methyltetrahydrofolate as the reference due to its more similar kinetics to that of native food folates.

Effectors of absorption—Deconjugase inhibitors have to be named, and organic acids such as citric acid have been identified to impair deconjugation and absorption.

Nutrient status—There are no reliable data on this factor, which is a severe gap in bioavailability studies, where folate saturation of the subject is the usual protocol. However, it is not known how folate transport and absorption are affected.

Genetic factors—Several polymorphisms of genes involved in folate transport and metabolism can affect folate status (135, 136). These gene variants likely explain the large interindividual variations in bioavailability (sometimes with a CV >100%) reported in human studies (137). This finding highlights further the need to aim for individual dietary recommendations in the future.

Host-related factors—No data are available.

Interaction of the above factors—Not investigated.

The current dietary recommendations are based on the studies of Sauberlich et al. (138), who determined in a long-term study a 50% bioavailability of food folates relative to pteroylmonoglutamic acid. However, this general figure has to be questioned because recent human studies have revealed new bioavailability data for several foodstuffs, such as 73% for spinach and 33% for wheat germ. Moreover, even for the same type of food, highly divergent bioavailabilities have been reported, for example, 9% versus >64% for 2 different types of Camembert cheeses (137, 139).

The results clearly underline the importance of the food matrix, even within the same type of food product, in terms of folate bioavailability. Moreover, the human studies revealed a huge interindividual variability of folate bioavailability, which indicates the need for more individual than general recommendations. Nevertheless, the generally assumed 50% bioavailability of food folates led to the definition of dietary folate equivalents, which are equivalent to the amount of natural folates in a given food or, in case of food fortified with folic acid, multiplied by a factor of 1.7 (140).

Estimation of total content of folate

The result of the MA is considered to give a value for the total content of folate because the MA responds to all monoglutamates and partly to the polyglutamates. As discussed above, the somewhat different response of the microorganisms to the different vitamers has to be considered, and the addition of further PPH activity if polyglutamates consisting of more than 3 glutamate residues are expected. If the vitamer distribution is known approximately, the result of the MA can be more accurate if the major vitamer is used as calibrant (119). However, in laboratory comparisons, the MA revealed CVs >24%, which points to significant uncertainty (112).

For chromatographic methods, which usually measure the content of the vitamers after deconjugation to monoglutamates, total folate content is calculated as the sum of all resulting monoglutamates, and given as folic acid. A comprehensive chromatographic method to detect and accurately quantify all vitamers as they appear natively in foods is currently not available.

Gaps and future perspectives for estimation of folate activity

Notwithstanding the recent improvements in folate analysis, the sensitive and accurate quantitation of folates in foods is still challenging.

Although the MA is still the reference method for foods, the LC-MS/MS–based methods are increasingly being used for blood analysis and generally accepted (141). In infant foods and adult nutritionals a standardized LC-MS/MS method is available (142), and standardization of the recently published LC-MS/MS method in food (115) is currently in progress (J Jakobsen, Technical University of Denmark, personal communication, 2019).

A bottleneck for implementation of an LC-MS/MS method has been the availability and cost of the isotopically labeled internal standards, but this is becoming less relevant as several suppliers now provide almost all relevant vitamer isotopologues at reasonable prices. A series of labeled standards for starting with the LC-MS/MS method would currently cost around 2000€ (143) and would be sufficient for 2000 samples.

Nevertheless, LC-MS/MS requires thorough validation including accurate addition of isotopologic standards as well as ensuring complete deconjugation and consideration of endogenous vitamer contents in the application of enzymes during sample preparation.

To avoid the deconjugation issue, the development of methods to quantify native polyglutamates is even more challenging for the following reasons: *1)* the polyglutamates are present in relatively low concentrations, which requires even more sensitive methods; *2)* the lack of polyglutamic reference compounds; and *3)* the lack of isotopically labeled polyglutamic vitamers—the polyglutamates show different binding behaviors than the monoglutamates on commonly used SPE materials for extract clean-up.

To unravel further the relations between native folate vitamers, their precursors, or metabolites, nontargeted metabolomics might offer some inroads, but lack of sensitivity of the applied high-resolution methodologies is still the roadblock to folate metabolomics. This is obvious from current physiological studies using metabolomics, where folates generally are not detected although they must be present (144).

Discussion and Conclusion

Of the 13 vitamins, 3 have been highlighted. These represent diverse aspects of the challenges in quantifying total vitamin activity. There are no standardized methods for quantification of the vitamers of vitamin A, vitamin D, and folate in foods, and it is troublesome and costly to perform a multilaboratory test, which is required for standardization. Through the AOAC Stakeholder Panel on Infant Formula and Adult Nutritional an effort has been underway since 2010 to establish standardized methods for the vitamins in the matrices of infant formula and adult nutritionals. These methods will provide a good starting point for the future standardization of methods for measuring the natural content of vitamins in foods. Because internationally standardized methods are lacking, the current data in food databases are established using single-laboratory validated methods, proficiency testing systems, certified reference materials, and/or the accreditation of a laboratory according to ISO17025.

For folate and β-carotene, a difference in vitamer activity between foods and supplements has been confirmed, whereas no difference has been observed for vitamin D. For differences in vitamer activity between provitamin A carotenoids and retinol, and between 25(OH)D and vitamin D, international consensus is lacking. To obtain this in the future, essential information on vitamers' bioaccessibility and bioavailability from diverse foods and supplements is necessary. In addition, a new vitamin D unit is justified where the proposed unit is the VDE, which would be consistent in principle with the units for vitamin A and folate. The challenges facing each of the specific vitamin communities are the gaps in knowledge about bioaccessibility and bioavailability for each of the various vitamers. The differences between the vitamins make it difficult to formulate a common strategy for assessing the quantitative differences between the vitamers. In vivo studies in humans using foods labeled with stable isotopes are the best available option, but in the future, optimized stationary digestive models and the more advanced dynamic digestive models combined with in vitro models for bioavailability might more closely resemble in vivo results.

Lastly, knowledge of differences in absorption and bioconversion between individuals will contribute to the future estimation of nutrient recommendations as part of risk-benefit analyses. It is difficult to estimate the total vitamin activity in a food. However, we must take on this task and establish new models and methods that can reliably assess the vitamin activity of today's foods and of new foods that will be brought to consumers in the future. This knowledge will enable us to transfer nutrient recommendations into improved dietary advice to increase public health throughout the human life cycle.

Acknowledgments

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