Part I
Perspectives and General Methodology in Vitamin Analysis

1
Stable Isotope Dilution Assays in Vitamin Analysis – A Review of Principles and Applications

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1.1
Principle of Stable Isotope Dilution Assays

1.1.1
General Remarks

The past decades have seen an increasing use of compounds labeled with stable isotopes in research. For instance, labeled precursors facilitate metabolism studies as the label can be followed on its way into different metabolites by mass spectrometry. Another application is the labeling of high molecular weight compounds such as proteins to elucidate their three-dimensional structures by modern nuclear resonance spectrometric methods. The third important use of stable isotopes is in trace analysis by stable isotope dilution assays (SIDAs), which is the topic of this review on vitamin quantitation.

The origin of SIDAs can be traced back to the beginning of the twentieth century when Soddy [1] discovered the existence of isotopes and George Hevesy used radioactive isotopes to determine the content of lead in rocks and the solubility of lead salts in water [2]. The conviction that elements are composed of atoms containing identical nuclei was refuted by Aston [3], who detected different atomic species of the noble gas neon by mass spectrometry. This resulted in a new definition of elements, which accordingly comprise mixtures of nuclei showing identical charge but different masses. As the nuclei with identical charges have the same (Greek *isos*) place (*topos*) in the periodic system of the elements, Soddy introduced the term "isotopes" [1]. An element has a natural isotopic distribution and there are two types of isotopes, namely the stable and radioactive ones. For example, carbon shows a natural distribution of C-12 (98%), of C-13 (1.1%), and of C-14, the last of which is radioactive and undergoes β-decay with a half-life of 5370 years.

Radioisotopes have the advantages over their stable analogs of their sensitive detectability and the possible use of low degrees of labeling. However, stable isotopes thereafter found their place as analytical tools when Hevesy and Jacobsen used deuterium oxide to quantify the percentage of extracellular liquid [4]. Interestingly, the percentage of deuterated water was determined by measuring the

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density of the water, as the mass spectrometers at that time showed very low precision.

The term "stable isotope dilution assay" was first introduced in 1940 by Rittenberg and Foster [5], who quantified amino acids in protein hydrolyzates. SIDAs at that time were very tedious, as mass spectrometry required purification of the compounds to be analyzed. Therefore, several chromatographic and recrystallization steps were essential. Finally, coupling of mass spectrometry (MS) to gas chromatography (GC) [6] opened the door to faster and more sensitive methods. In that way, the first modern type of SIDA was performed by Sweeley *et al.* [7], who quantified glucose by GC–MS after trimethylsilylation and used [$^2H_7$]glucose as the internal standard.

Although they are very similar in their properties, isotopes can be enriched or depleted due to their different masses. Mixing an element or compound showing a natural isotopic distribution with such an isotopically different material (Figure 1.1) results in a smaller proportion of the naturally abundant isotopes in the resulting material–which led to the term "dilution" in SIDA.

The principle of SIDA is simply explained in Figure 1.2. After addition of the labeled standard and its equilibration with the analyte, the ratio of the isotopologs remains stable throughout all subsequent analytical steps. This is due to their almost identical chemical and physical properties. A final MS step enables the isotopologs to be differentiated. Consequently, the content of the analyte in the sample can be calculated with the known amount of the internal standard (IS)

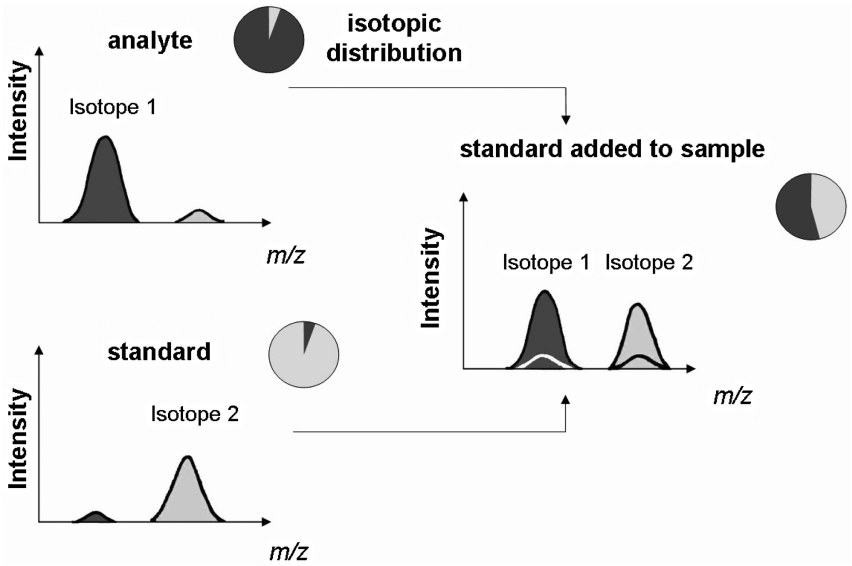

Figure 1.1 Origin of the term "dilution" in stable isotope dilution assays: addition of a standard with a different isotopic distribution – the original isotopic distribution of the analyte has been "diluted."

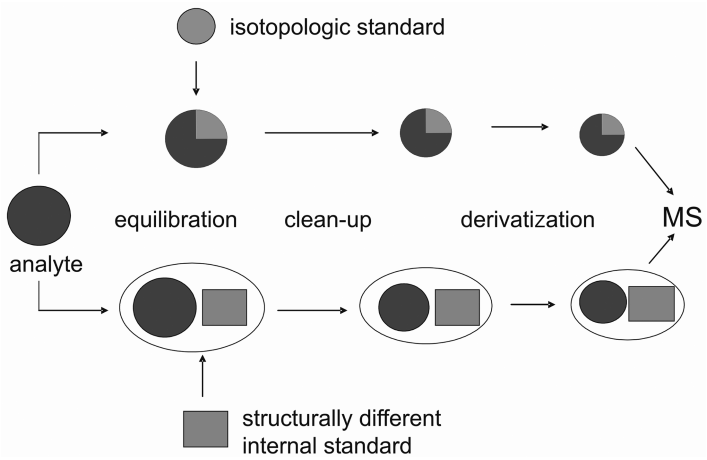

Figure 1.2 The ratio of isotopologic analyte and standard remains stable until final mass spectroscopic analysis. For a structurally different IS, however, the ratio between standard and analyte can alter during sample preparation.

added at the beginning. In contrast, a structurally different internal standard may be discriminated against and, therefore, cause systematic errors and imprecision. Hence losses of the analyte are completely compensated for by identical losses of the isotopolog, whereas the structurally different IS may show different losses.

As SIDAs require more or less elaborate syntheses of labeled compounds, their development was at first restricted to very few applications, in particular to those fields in which highest sensitivity and accuracy were essential. Therefore, toxicology, clinical chemistry, and environmental analysis were the first disciplines to use SIDAs. Subsequently, these methods were transferred to foods and emerged as reference methods for food compounds such as lignans [8] and steroids [9].

However, more recently the direction of research changed and assays developed for foods have opened up new prospects in toxicology and nutrition research. In addition to vitamins such as pantothenic acid [10] and folates [11], further examples have been centered on mycotoxins such as trichothecenes [12, 13] and patulin [14] and on odorants [15].

1.1.2 Benefits and Limitations of Using an Isotopologic Internal Standard

As detailed before, due its ideal compensation for losses, SIDA is a perfect tool for a series of analytical applications, in particular for trace analyses. The latter often demand tedious clean-up procedures due to matrix interferences, which typically evoke losses of the analyte. The use of structurally different ISs requires additional recovery and spiking experiments, which often result in imprecise data. In all these cases, SIDA offers significant benefits.

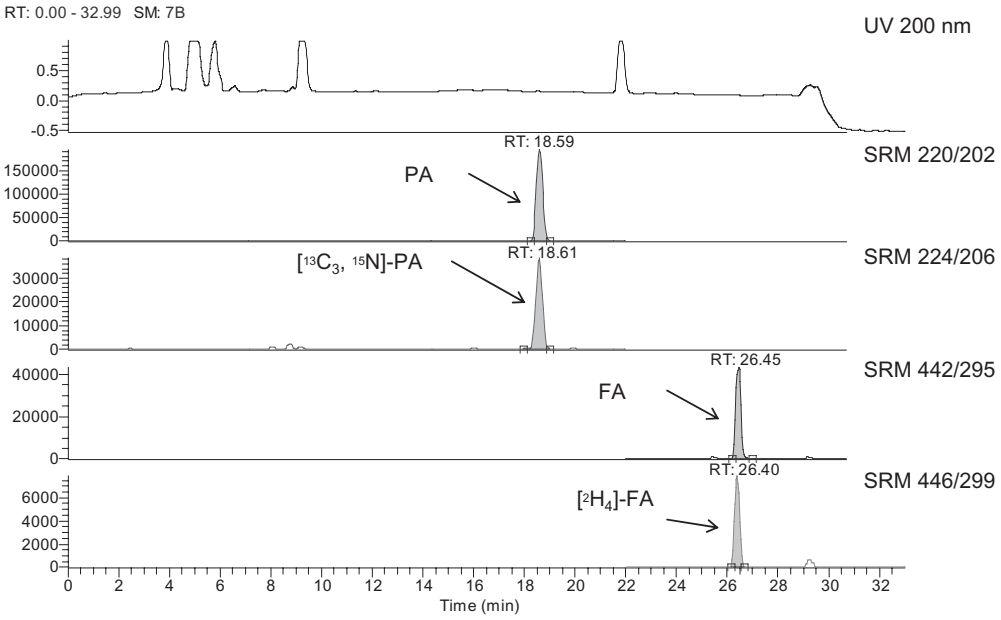

Figure 1.3 MS/MS chromatograms of fortified breakfast cereals containing 7.94 mg per 100 g of pantothenic acid (PA) and 270 µg per 100 g of folic acid (FA). The isotopically labeled internal standards show retention times identical with those their unlabeled isotopologs in the respective traces below.

In addition to compensation for losses, thus resulting in improved accuracy, the use of an isotopologic standard enhances the specificity of the determination. In addition to the specific MS information on the analyte, the IS is eluted at an almost identical retention time and shows a distinct mass shift. Therefore, the analyte can be unequivocally assigned in the chromatogram from a SIDA showing the coeluting peaks in the respective mass traces (Figure 1.3).

A further advantage of adding isotopologic material is to enhance sensitivity by the so-called "carrier effect." Due to adsorption phenomena on glassware or chromatographic columns, a definite amount of the analyte is likely to be lost during sample clean-up. If the total amount of the analyte in an extract is lower than this loss, the compound will no longer be detectable. However, if an isotopologic standard is added in an amount that exceeds this loss, the total sum of standard and analyte is higher than the loss and, therefore, the isotopologs can be detected. Although there are some conflicting opinions on this topic (for a review, see [16]), there are some applications showing a significant enhancement of sensitivity [17]. For vitamins, however, this effect has not yet been demonstrated.

Regarding the major benefits of SIDAs, that is, specificity and ideal compensation for losses, the question arises of whether they have the potential to be "definite methods." According to a definition by Cali and Reed [18], "a definite method is one that, after exhaustive investigation, produces analytical results that are accu-

rate, that is, free of systematic errors, to the extent required for the intended end-use(s)." This definition holds true especially for primary methods, which are "methods having the highest metrological qualities, for which a complete uncertainty statement can be written down in terms of SI units, and whose results are, therefore, accepted without reference to a standard of the quantity being measured" [19]. A SIDA can be traced back to a gravimetric (i.e., primary) measurement and, therefore, is considered a primary method. In the case of being validated intensively for the absence of systematic errors, it has the potential to be accurate, that is, to produce the "true" value.

A SIDA of vitamins is only possible if a combination of chromatography with MS and a labeled IS are available. However, whereas the former has increasingly become basic instrumentation in most laboratories, the latter is a narrow bottleneck for the wider application of SIDAs. Of all the vitamins discovered to date, about 20 have been synthesized as labeled analogs, but only seven of these are commercially available. Hence the compound aimed at often has to be synthesized. There are two aspects that arise as a hindrance before starting with these syntheses. The first is that analysts may hesitate to perform chemical syntheses due to a lack of experience. Although the envisaged syntheses on the microscale require often intense purifications by chromatography, analysts are normally familiar with the necessary methods such as distillation *in vacuo*, purification by high-performance liquid chromatography (HPLC) and checking purity and yield by GC–MS. The procedures are completely detailed in the literature and usually the groups having published these syntheses are willing to give advice in case of practical problems.

The second psychologic hindrance that prevents analysts from synthesizing labeled mycotoxins is the price of labeled educts. However, this is not a convincing argument, as can be explained by the following example: 1 g of a labeled educt may cost around US$1000 and the yield of a multi-step synthesis may be only 1%, both of which are realistic figures. Then, the price for 10 mg of the labeled product is $1000. However, as less than 1 μg of the labeled standard is required for a SIDA, 10 mg of the standard enables at least 10 000 analyses to be performed. Hence the material cost for using a labeled standard is just $0.10 per sample, which is negligible compared with the cost of labor and equipment.

1.1.3
Prerequisites for Isotopologic Standards

As outlined before, SIDA is based on an isotopolog ratio that remains stable during all analytical steps. Therefore, a stable labeling step is essential for an IS. As carbon–carbon and carbon–nitrogen bonds are very unlikely to be cleaved, ^{13}C and ^{15}N labels are considered to be very stable. In contrast, losses of ^{18}O or ^{2}H at labile positions can occur. On the one hand, ^{18}O in carboxyl moieties can be exchanged in acidic or basic solutions. On the other, deuterium is susceptible to so-called protium–deuterium exchange if it is activated by adjacent carbonyl groups or aromatic systems.

Moreover, an isotopolog ratio may be altered by isotope effects (IEs), that is, small differences in physical or chemical properties of the isotopologs. IEs are due to different energy contents that are caused by the mass differences of the isotopes. The lowest energy level of a molecule, namely the zero point energy, is given by the ground-state vibration of the bonds at absolute zero, where the population in excited vibrational levels is negligible. As frequencies and energies of vibrations are proportional to the encountered masses, heavier isotopologs possess a lower energy content, resulting in higher energies of bond dissociation. IEs are mainly observable in the case of hydrogen, as the mass difference between ^{1}H and ^{2}H is proportionally much higher than between ^{13}C and ^{12}C or ^{15}N and ^{14}N. In the case of chemical reactions involving C–H bond rupture, IEs result in monodeuterated isotopologs reacting up to seven times more slowly then their light analogues. For labeling with ^{13}C, these effects are several hundred times smaller. However, the kinetic IE of ^{13}C is a valuable diagnostic tool in enzyme studies, as ^{12}C isotopologs show lower reaction rates than their ^{13}C counterparts. This discrimination is commonly measured as the $\delta^{13}C$ value of the products and is dependent on the transitions states of the encountered enzymatic reactions. Therefore, the measurement of $\delta^{13}C$ values allows the characterization of the encountered enzymes and the determination of the products' origin. For instance, the authenticity of alcoholic beverages [20] or valuable spices such as vanilla [21] can be verified.

The primary IE, which affects direct bond cleavage between the label and adjoining atoms, has to be distinguished from the secondary IE, which has an influence on the bonds between unlabeled atoms. A primary IE would cause either loss of labeling or, in MS, would result in different intensities of the fragments bearing the label. Therefore, all derivatizations or MS fragmentations including a primary IE have to be avoided. Although much less pronounced, a secondary IE, however, cannot be excluded in these reactions, but has not yet been reported in the literature.

In contrast to chemical IEs, a physical IE is more often observable. The latter particularly affects chromatographic behavior and results in different retention times of the isotopologs. In particular for multiple labelings with ^{2}H, the IE may evoke even baseline separation, as shown in the case of eightfold deuterated β-carotene [22], which was clearly separated from its unlabeled isotopolog upon HPLC with ultraviolet (UV) detection (Figure 1.4) In most cases, the heavier isotopologs are eluted earlier than their light analogs, which is unexpected, as the heavier isotopologs should have higher boiling temperatures and, therefore, should be eluted later in GC. This behavior therefore is referred to as an inverse IE.

In order to prevent chromatographic separations of the isotopologs during clean-up and thus changes in the isotopolog ratio, isotope effects have to be minimized either by choosing labelings with ^{13}C or ^{15}N or by introducing only the necessary number of ^{2}H atoms.

For unequivocal quantification, the standard has to be distinguishable from the analyte by MS. This requires the presence of the mass increment introduced by the labels either in the molecular ion or in its fragments. Therefore, a loss of the label prior to detection has to be avoided. However, quantitation by liquid chro-

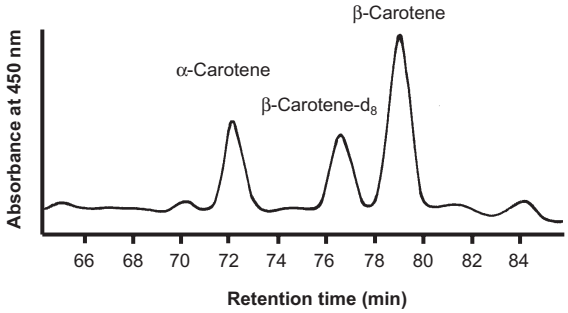

Figure 1.4 Baseline separation of eightfold deuterated β-carotene from its unlabeled isotopolog using HPLC–UV. Reproduced with permission from [22].

matography (LC) coupled with tandem mass spectrometry (MS/MS) is feasible even if the label is lost during the monitored mass transition, as the precursor ions are still differentiated.

A further problem in isotopolog differentiation may arise from spectral overlaps between the standard and analyte. In case of the analyte, the natural abundance of isotopes, in particular of ^{13}C, ^{18}O and ^{34}S, results in isotope clusters of each fragment showing not only the nominal mass m_A, but also to a lesser extent $m_A + 1$, $m_A + 2$, or even higher masses. In particular, ^{13}C in compounds consisting of a higher number of carbons causes a significant abundance of $m + 1$ and $m + 2$ due to the relatively high natural abundance. To avoid overlap of the signals of those natural isotopologs with m_S of the standard, the mass difference therefore has to be sufficient. For vitamins, in which the number of carbon atoms often exceeds 10, generally a mass increment of at least three units is required. However, the number of labels, especially of deuterium, should not be too high, in order to minimize the mentioned chromatographic isotope effect.

In contrast, signals of the labeled material contributing to those of the analyte may also result in spectral overlaps. This may be due to the low isotopic purity of labeled educts or to inadequate labeling during synthetic steps, thus leading to incomplete labels in the standard.

If a spectral overlap cannot be avoided, calculation procedures have been developed that still permit quantification to be achieved. However, these procedures are more complicated the greater the overlap is.

Another important requirement for accurate quantification is complete equilibration between the analyte and standard in the sample to be extracted. As the labeled standard usually is not contained in the sample matrix after its addition, it will likely be recovered to a high extent during the extraction procedure. For the analyte, this might not be true, as it can be trapped in compartments of the matrix and is less extractable by the solvent. Therefore, sufficient time for equilibration of the standard and analyte in all parts of the sample has to be allowed to assure that the analyte and standard show the same concentration ratio in all compartments as far as possible.

1.1.4 Calibration Procedures

From the intensity ratio of suitable ions measured by MS and a calibration function, the isotopolog ratio can be assessed, which directly allows the amount of analyte present in the sample to be calculated. The relation between isotopolog ratio and intensity ratio has to be elucidated by analyzing the intensity ratios of a series of defined standard–analyte mixtures. If there is no spectral overlap, the calibration function is assumed to be linear. However, typically there are still residues of unlabeled analytes in the labeled material and low intensities of natural isotopologs of the analyte contributing to the signals of the standard. Therefore, the calibration function can be expected to be linear only in a restricted region, as illustrated in Figure 1.5. The outlined function appears under the supposition that the standard may contain 2% of unlabeled material und natural isotopologs may contribute 5% intensity to the signal of the standard. In this case, the calibration function can only be assumed to be a straight line for molar ratios of analyte to standard ranging between 0.2 and 5. With excess of the standard or of the analyte, the function is dominated by the unlabeled residues in the standard or by the natural isotopologs of the analyte, respectively.

However, for complicated structures or elaborate syntheses, a spectral overlap often cannot be avoided and, therefore, suitable procedures for calculations are required. In general, there are several ways to cope with this problem: First, hyperbolic or polynomial models have been elaborated [23, 24], which approximate the real calibration relation by a mathematical function. As these procedures are rather complicated, several authors have proposed linearization methods, which convert the nonlinear function into a linear function. In a study on odorants, Fay *et al.* [25] compared four of these methods using [$^2H_1$]benzaldehyde as the IS for quantification of benzaldehyde. This assay shows a spectral overlap of about 12% between

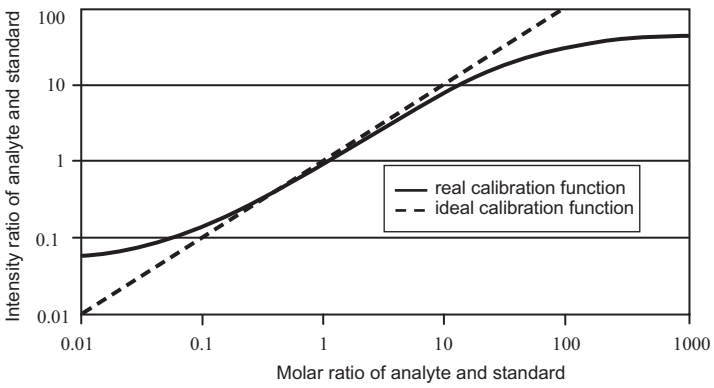

Figure 1.5 "Real" calibration function (dashed line) for a SIDA under the supposition that the standard contains 2% of unlabeled material with natural isotopologs contributing 5% intensity to the standard's signal.

the analyte and the standard. The authors demonstrated that the method of Colby and McCaman [26] did not yield a linear function. By contrast, both the average mass approach [27] and linearization using isotopic enrichment factors [28] gave straight lines, whose calibration points, however, were spread very inhomogeneously. The only procedure giving a calibration line with homogeneously distributed calibration points was the method of Bush and Trager [29], which notably includes only rather simple calculations.

The third way to cope with a nonlinear calibration function is the "bracketing" approach [30], which requires the measurement of further calibration points lying in the proximity of the intensity ratio measured in the sample. Although this method is widely accepted as the most accurate one, it is very elaborate and, therefore, is only seldom applied.

1.2 Application of Stable Isotope Dilution Assays to Vitamins

1.2.1 Fat-Soluble Vitamins

This group of vitamins is naturally embedded in food lipids. For their analysis, these compounds have to be separated from fat prior to detection. Due to their low volatility, GC applications are scarce. Moreover, the hydrophobicity of these vitamins is responsible for a high retention on common reversed phases in HPLC, which limits their separation on these phases. As a consequence thereof and as LC–MS combinations need aqueous mobile phases, reports on LC–MS detection are also rare. However, with the development of new reversed-phase materials, LC–MS applications have become increasingly popular.

For fat-soluble vitamins, the use of stable isotopologs is restricted to β-carotene, vitamin A, α-tocopherol, vitamin K, and vitamin D. To date, nearly all applications have been directed towards quantitation of these vitamins in blood or studies of their bioavailability. SIDAs in foods have not been reported so far.

1.2.1.1 Vitamin A

For vitamin A, most applications of labeled compounds have been dedicated to the administration of labeled β-carotene and quantification of the generated retinol in blood serum. For this purpose, β-carotene and retinol have been labeled with deuterium [22] or carbon-13 [31]. These compounds have been applied in several studies, and the bioefficacy of β-carotene was found to range between 30 and 40% [32].

1.2.1.2 Vitamin E

Because it functions as an antioxidant in tissues, an important part of tocopherol research is dedicated to studying its reaction with oxygen and to elucidating the respective reaction products. An extensive SIDA for the quantification of

α-tocopherol and its oxidation products such as α-tocopherolquinone, α-tocopherolhydroquinone, and several epoxy-α-tocopherolquinones by using their deuterated analogs was presented by Liebler *et al.* [33]. They oxidized endogenous α-tocopherol in rat liver microsomes with the radical generator azobis(amidinopropane) and quantified the isotopologs by GC–MS after trimethylsilylation.

1.2.1.3 Vitamin D

Cholecalciferol (vitamin D_3) and its major metabolites calcidiol and calcitriol were quantified in human blood by two research groups in the 1980s. Whereas Zagalak and Borschberg [34] used [$^2H_8$]cholecalciferol along with [$^2H_3$]calcitriol as IS, Coldwell *et al.* [35] applied $^2H_6$-labeled isotopologs of cholecalciferol, calcidiol, calcitriol, and some other metabolites for SIDAs. As a further isotopolog of vitamin D_3, [$^2H_7$] cholecalciferol was synthesized by Kamao *et al.* [36] and used in a SIDA for several fat-soluble vitamins in human breast milk.

An actual LC–MS/MS method for vitamin D in mineral tablets and baby food is presented in Chapter 17.

1.2.1.4 Vitamin K

One of the seldom used SIDAs for fat-soluble vitamins has been reported for vitamin $K_{1(20)}$ in plasma using GC–MS [37]. The IS was [$^2H_3$]phylloquinone, which was prepared by deuteromethylation of 1,4-naphthoquinone and subsequent coupling with phytol. The suitability of this IS, however, has been called into question for LC–MS, as the deuterium label is at the acidic 2-methyl position and, therefore, is highly susceptible to H–D exchange in aqueous eluents. In contrast, Suhara *et al.* synthesized $^{18}O_2$-labeled phylloquinone homologs starting from 2-methyl-1,4-naphthoquinone diacetate in a four-step procedure including an oxidation in the presence of $H_2{}^{18}O$ [38]. These compounds were used as ISs in a multiple SIDA for the quantitation of vitamin K along with vitamin K_1, K_4, and K_7 compounds in human breast milk [36]. Further applications of these compounds to foods have not yet been reported.

1.2.2 Water-Soluble Vitamins

Because of their low hydrophobicity and low retention on reversed phases, this group of vitamins is very suited to LC–MS in aqueous solvents. Numerous applications of LC–MS have been published, so it is not surprising that some SIDAs have also been developed. In the following sections, such assays for pyridoxin, niacin, folic acid, and pantothenic acid are described.

1.2.2.1 Vitamin B_6

Due to the wide variety of pyridoxine vitamers, SIDA applications for this group of vitamins are rare. The only assay reported in the literature dates back to 1985, when Hachey *et al.* [39] used deuterated analogs of pyridoxine, pyridoxal, pyridoxamine, and pyridoxic acid to quantify these compounds and their phosphates in

guinea pig liver, human urine, feces, and goat milk. In the last case, pyridoxal phosphate was found to be the main vitamer contributing almost 70% to total pyridoxine.

1.2.2.2 Niacin

As [$^2H_4$]nicotinic acid is commercially available, it has been used as the basis of SIDAs for the niacin group. Following the first application to determine nicotinic acid and six of its metabolites in urine by Li *et al.* [40], the first SIDA for foods was presented by Goldschmidt and Wolf [41]. However, the latter assay did not measure nicotinamide, which might also contribute to niacin activity in food samples. Nevertheless, the assay showed excellent accuracy for the analysis of certified reference materials such as wheat flour, milk powder, and multivitamin tablets.

1.2.2.3 Ascorbic Acid

Although its importance for the diet is well documented, LC–MS quantitations of ascorbic acid are surprisingly scarce. Examples of the rare reports are a study of degradation products of dehydroascorbic acid and ascorbic acid [42] and the most recent quantitation of vitamin C along with nine other vitamins in multivitamin products by Chen *et al.* [43].

Moreover, labeled ascorbic acid for LC–MS has not yet been used. The only SIDA for vitamin C was reported in 1988, when Ellerbe *et al.* applied ^{13}C-labeled ascorbic acid to milk powder analysis by GC–MS of the *tert*-butyldimethyl derivatives [44].

1.2.2.4 Folic Acid

The first SIDA for folic acid in fortified foods was published by Pawlosky and Flanagan [45], who used commercially available [$^{13}C_5$]folic acid ([$^{13}C_5$]PteGlu) as the IS. One year later, we synthesized fourfold deuterated folic acid along with the most abundant folate monoglutamates for quantifying endogenous food folates and folic acid in fortified foods [46]. Further developments included the use of chicken pancreas in addition to rat plasma for improved deconjugation [47] and the use of 4-morpholinoethanesulfonic acid (MES) for enhancing folate stability [48]. In particular, the need for labeled folate tracers in bioavailability research spurred the generation of new stable isotope-labeled folates. Starting with the first dual label study using [$^2H_2$]PteGlu and [$^2H_4$]PteGlu by Gregory and Quinlivan [49], early investigations were restricted to the sole measurement of total urinary folate isotopologs by GC–MS [50, 51] and often were hampered by spectral overlap due to insufficient mass increments interfering with naturally occurring isotopologs. For this reason, syntheses of differently labeled folates have been developed, such as [$^2H_4$]PteGlu and [$^{13}C_6$]PteGlu labeled in the glutamate moiety and the benzene moiety, respectively [52]. For tracer studies, these folates were suitable for so-called extrinsic labeling, that is, by simple addition to foods. However, as added substances may not behave like the endogenously occurring folates, so-called intrinsically labeled foods were produced by growing spinach in a ^{15}N-labeled environment

[53], which generated $^{15}N_{1-7}$-labeled 5-methyltetrahydrofolate (5-CH_3-H_4folate) in the latter vegetable.

A further methodological improvement arose from the use of LC–MS in folate isotopolog analysis, when Wright *et al.* [54] measured $^{13}C_6$-labeled, $^{15}N_{1-7}$-labeled, and unlabeled 5-CH_3-H_4folate using [$^2H_2$]folic acid as the IS in the single ion monitoring mode. However, the use of a structurally different IS such as [$^2H_2$] PteGlu may decrease the accuracy by ion suppression and, moreover, quantitation was hampered by spectral overlap of [$^{15}N_{1-7}$]-5-CH_3-H_4folate with, on the one hand, [$^{13}C_6$]-5-CH_3-H_4folate, and, on the other, unlabeled 5-CH_3-H_4folate in single-stage LC–MS. This handicap was overcome by Melse-Boonstra *et al.* [55], who measured $^{13}C_6$-labeled along with $^{13}C_{11}$-labeled 5-CH_3-H_4folate as tracer isotopologs and simultaneously quantified unlabeled 5-CH_3-H_4folate by using [$^{13}C_5$]-5-CH_3-H_4folate as the IS. In the latter study, spectral overlaps of 5-CH_3-H_4folate isotopologs were avoided by labeling different moieties of the target molecule and their differentiation by LC–MS/MS. However, this investigation was restricted to plasma 5-CH_3-H_4folate without an application to food samples.

With more ^{13}C-labeled folates now being offered commercially, SIDA for folates in foods is currently being used by various groups throughout the world. Of all folate vitamers, H_4folate, 5-CH_3-H_4folate, 5-formyl-H_4folate, 5,10-methenyl-H_4folate and folic acid are currently available as $^{13}C_5$-labeled isotopologs. Whereas SIDA for monoglutamates can be considered a fairly well-established technology, the analysis of endogenous folates is still challenging. In particular, the polyglutamates and their complete conversion to the monoglutamates are still not fully understood and are the target of further research. The most recent approach is the use of [$^{13}C_5$]pteroylheptaglutamate for confirmation and quantitation of the degree of deconjugation. The latter compound has been produced by a Merrifield-like solid phase synthesis [48].

1.2.2.5 **Pantothenic Acid**

Along with folic acid, pantothenic acid was one of the first vitamins to be quantified by SIDA based on LC–MS. An extensive description of [$^{13}C_3$,^{15}N]pantothenic acid used as internal standard and recent results are presented in Chapter 9.

1.3 Outlook

Although numerous labeled vitamins are used in clinical chemistry and bioavailability research, they have been only marginally applied in the SIDA of foods (Table 1.1). However, with the advances in LC–MS/MS in vitamin analysis, labeled ISs will gain more importance. In particular for the analysis of multivitamin products, multiparametric SIDAs will be developed, when the labeled standards become available, as can be seen from the past for folic acid and pantothenic acid [56]. In particular for the analysis of reference materials, SIDAs are essential to establish the certified reference contents. In this context, a SIDA for the analysis

Table 1.1 Applications of SIDAs to vitamins in foods.

Vitamin	Internal standard	Food sample	Reference
Retinyl acetate	[$^2H_6$]Retinyl acetate	Human milk	Kamao *et al.* [36]
β-Carotene	[$^2H_6$]-β-Carotene	Human milk	Kamao *et al.* [36]
Cholecalciferol	[$^2H_7$]Cholecalciferol	Human milk	Kamao *et al.* [36]
α-Tocopherol	[$^2H_6$]-α-Tocopherol	Human milk	Kamao *et al.* [36]
Phylloquinone, menaquinone-4, menaquinone-7	$^{18}O_2$-labeled analogs	Human milk	Kamao *et al.* [36]
Pyridoxine, pyridoxamine, pyridoxal, pyridoxic acid	$^2H_2$-labeledanalogs	Liver, goat milk	Hachey *et al.* [39]
Ascorbic acid	[$^{13}C_6$]Ascorbic acid	Bovine milk	Ellerbe *et al.* [44]
Pantothenic acid	[$^{13}C_3$,^{15}N] Pantothenic acid	Various foods	Rychlik [10, 56]
Folic acid	[$^{13}C_5$]Folic acid	Various foods	Pawlosky and Flanagan [45]
5-Methyltetrahydrofolate, folic acid	$^{13}C_5$-labeled analogs	Various foods	Pawlosky *et al.* [58]
Tetrahydrofolate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 10-formylfolate, folic acid	$^2H_4$-labeled analogs	Various foods	Freisleben *et al.* [11]
Niacin	[$^2H_4$]Nicotinic acid	Various foods	Goldschmidt and Wolf [41]

of seven water-soluble vitamins in an infant/adult nutritional formula has been reported very recently [57].

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