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Determination of vitamins in food-matrix Standard Reference Materials

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Abstract

In recent years, the National Institute of Standards and Technology (NIST) has developed several food-matrix Standard Reference Materials (SRMs) characterized for vitamins and other organic nutrients. NIST uses several ''modes'' for assignment of analyte concentrations in SRMs, one of which includes the use of data provided by collaborating laboratories. Certification modes and liquid chromatographic methods that were used by NIST for value assignment of vitamin concentrations in recently introduced food-matrix SRMs are described in this paper. These materials and methods include vitamins D and E in coconut oil (SRM 1563) by gravimetry and multi-dimensional liquid chromatography (LC); vitamins A, E, and several B vitamins by reversed-phase LC and vitamin C by ion-exchange chromatography in infant formula (SRM 1846); and carotenoids and vitamins A and E by reversed-phase liquid chromatography in a baby food composite (SRM 2383). © 2000 Published by Elsevier Science B.V.

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1. Introduction

The National Institute of Standards and Technology (NIST) has recognized the importance of food-matrix reference materials with values assigned for organic nutrients since before 1987, the year in which Standard Reference Material (SRM) 1563 Cholesterol and Fat-Soluble Vitamins in Coconut Oil (Natural and Fortified) was released. This and SRM 1588 Organics in Cod Liver Oil (with an assigned value for α-tocopherol) were the only food-matrix SRMs with values assigned for organic nutrients that were available for almost 10 years. (Additional food-matrix SRMs were available that focused mainly on inorganic analytes of nutritional or toxicological interest). The value-assignment of organic nutrients in food-matrix SRMs at NIST gained momentum through the mid- and late-1990s with the introduction of SRM 1544 Fatty Acids and Cholesterol in a Frozen Diet Composite, SRM 1546 Meat Homogenate, SRM 1548a Typical Diet, SRM 1846 Infant Formula, and SRM 2383 Baby Food Composite.

The Infant Formula Act of 1980 and the Nutrition Labeling and Education Act of 1990 [1,2] have been driving forces behind the introduction of food-matrix SRMs with values assigned for organic nutrients. The Infant Formula Act requires that nutrients contained in infant formula fall within a specified range or above a specified minimum, and the Nutrition Labeling and Education Act requires food processors to provide specific nutrition information on individual sales units of products distributed in the USA. Information about vitamin A and C content is required on the nutrition label, thereby making reference materials with assigned values for these

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vitamins particularly useful; information about additional vitamins can be added to the nutrition label at the manufacturer's discretion.

Well-characterized reference materials are needed by laboratories in the food testing and nutrition communities (including food industry, commercial testing, university research, government regulatory agency, and consumer group laboratories) [3]. Such reference materials will facilitate compliance with nutritional labeling laws, provide traceability for food exports needed for acceptance in many foreign markets, and improve the accuracy of nutrition information that is provided to assist consumers in making sound dietary choices. In 1996, in a study of nutrition labeling accuracy sponsored by the US Food and Drug Administration (FDA), nutrient information on 300 product labels was compared to results obtained by a contract laboratory [4]. Results that were less than 20% different from the labeled information in the direction of no nutritional ''harm'' were considered to be consistent with the labeling information, e.g. the labeled values for cholesterol or sodium could be 20% higher than the measured value and still be ''consistent'' but if the labeled values for these analytes were lower than those measured, the label would not be considered accurate. Vitamin C was accurately labeled in at least 80% of the products and vitamin A in 54%. The lack of labeling accuracy for vitamin A may be attributed to a lack of reliable methods of sample preparation, as well as to a lack of food-matrix reference materials for assessing the reliability and accuracy of such methods during the period in which samples were tested. NIST is working to address both of these issues with the introduction of food-matrix reference materials and the development or adaptation of reliable methods with which to analyze these materials.

NIST uses several ''modes'' for assignment of analyte concentrations in SRMs [5]. Perhaps the most time-honored of these modes is the use of two or more independent methods to generate a certified concentration value. The requirement for independence extends to the methods used for sample preparation as well as to the chromatographic separation and analyte detection, where feasible and appropriate. Another value-assignment mode involves the use of data from outside collaborating laboratories; these laboratories may have been selected by NIST to assist with value assignment of an SRM or they may be participating in a well-defined interlaboratory comparison exercise. Resulting data may be combined with NIST data to provide a certified, reference, or information value; if NIST data are unavailable for a particular analyte, a reference or information value may be assigned. A combination of modes has been used to assign concentrations of fat- and water-soluble vitamins and carotenoids in a variety of food-matrix SRMs. Certification modes and liquid chromatographic (LC) methods that were used by NIST for value assignment of vitamin concentrations in these SRMs are described in this paper.

2. Vitamin analyses

Analytical methods in general must be reliable and robust to be useful. The analyte must be stable during the analytical process; both the extraction and chromatographic procedures must be reproducible; the results should be accurate and the uncertainty should be small; the detector should be selective for the analytes of interest; the signal for each analyte of interest should be free from significant interferences; and the method should be free from matrix effects. Additionally, for determination of vitamin concentrations, it is necessary to demonstrate the accuracy of a method by demonstrating that all of the naturally occurring species of a given vitamin are fully extracted and measured. Books and reviews of vitamin determinations have been published (e.g. Refs. [6–10]). Some of the challenges that can be encountered in the determination of vitamin concentrations, for those vitamins that have been measured at NIST, are presented below.

An important aspect of assigning values to analyte concentrations in a certified reference material is analyte stability or the lack thereof. Certain vitamins and carotenoids may function as antioxidants, which makes them unstable by their very nature. Vitamins may be degraded when exposed to air, light, or heat. Such instability is an important consideration, both in the storage of reference materials as well as in the preparation and analysis of test portions. For example, in the case of vitamin C, we have demonstrated

that above 4°C, ascorbic acid is degraded to dehydroascorbic acid and smaller molecules ([11]; see additional discussion below).

The methods used at NIST for the determination of vitamin and carotenoid concentrations in food-matrix reference materials are typically based on LC. The complexity of the analytical methods employed has evolved along with the complexity of the food-matrix SRMs: The fat-soluble vitamins were spiked into SRM 1563 Cholesterol and Fat-Soluble Vitamins in Coconut Oil, and therefore one of the methods used for value-assignment was gravimetry, with confirmation by LC using a straightforward clean-up on a normal-phase chromatographic column prior to the reversed-phase LC analysis. SRM 1846 Infant Formula was fortified by the manufacturer of the material with vitamins at levels in compliance with the Infant Formula Act of 1980, therefore levels of any naturally occurring forms of the vitamins are expected to be overwhelmed by the fortified, known forms, and only the fortified forms need be measured. SRM 2383 Baby Food Composite, as well as forthcoming spinach and baking chocolate SRMs, contain only the naturally occurring forms of the vitamins at their naturally occurring levels, potentially making the analyses and the value assignment more difficult.

NIST has measured niacin and vitamins B_2, B_6, C, A, D, and E, and carotenoids in several of the food-matrix SRMs mentioned above, and these analyses are described here. A summary of the SRMs, analytes for which certified and reference values are provided as a result of NIST analyses, and the methods used for value assignment is provided in Table 1.

2.1. Vitamin C[1]

The extraction process for the measurement of vitamin C must extract both ascorbic acid and dehydroascorbic acid, and prevent the oxidation of ascorbic acid. Dehydroascorbic acid must be completely converted to ascorbic acid, and the ascorbic acid must be stable on the analytical column.

Vitamin C was measured in SRM 1846 Infant Formula, and NIST results were combined with results obtained by a group of collaborating laboratories to provide the certified value [12,13]. A comparison of NIST's value to the certified value is provided in Table 2. NIST's method for vitamin C analysis has been previously published [11]. Briefly, dithiothreitol was added to convert dehydroascorbic acid to ascorbic acid. Proteins were coagulated, and the supernatant solution was analyzed by anion-exchange chromatography using a polymer-coated amino silica column (Capcell Pak NH_2, Shiseido, Tokyo, Japan) with electrochemical detection. This method has the advantage of being done in a single vessel without dilutions or transfers and of measuring the reduced and total content of both L- and D-ascorbic acid isomers by a single method.

Ascorbic acid isomers can be separated on silica-based aminopropyl columns, but ascorbic acid is not stable on these columns [14]. The Capcell column used by NIST separates the ascorbic acid isomers, but does not oxidize the ascorbic acid. The elution position of both dithiothreitol and uric acid (which is also extracted by the sample preparation method) are much more favorable on the Capcell column than on a Fast Acid Analysis column (Bio-Rad, Hercules, CA, USA) used in earlier studies [15]. The selectivity and advantages of using an electrochemical detector for vitamin C analyses have been documented [15,16].

The same sample preparation and chromatographic methods were also used for the analysis of vitamin C in SRM 2383 Baby Food Composite. Units of the SRM available for sale have been stored at 4°C. Test samples were stored at this temperature for 5 months to assess the stability of vitamin C [11,17]. The total ascorbic acid concentration had changed very little in those 5 months, but more of the ascorbic acid had been oxidized to dehydroascorbic acid in these samples than in samples stored at −70°C. A small amount (less than 5%) of the total ascorbic acid had degraded beyond dehydroascorbic acid at 4°C after 5 months. Because of the apparent instability, the concentration of vitamin C in SRM 2383 was not assigned.

[1] Certain commercial products are identified to specify adequately the experimental procedure. Such identification does not imply endorsement or recommendation by the National Institute of Standards and Technology, nor does it imply that the materials identified are necessarily the best available for the purpose.

Table 1
Food-matrix SRMs analyzed by NIST for selected fat- and water-soluble vitamins

	Year issued	Certified constituents	Reference constituents	Methods used for value assignment
SRM 1563 Cholesterol and Fat-Soluble Vitamins in Coconut Oil	1987	Ergocalciferol		Gravimetry Multi-dimensional LC
		Tocopheryl acetate		Gravimetry Multi-dimensional LC
			Retinyl acetate	Gravimetry Multi-dimensional LC
SRM 1846 Infant Formula	1996	Retinol		Saponification, reversed-phase LC (RPLC), absorbance detection Interlaboratory comparison exercise (six laboratories)
		α-Tocopherol		Saponification, RPLC, absorbance detection Saponification, RPLC, fluorescence detection Interlaboratory comparison exercise (eight laboratories)
		Vitamin C		Ion-exchange chromatography, electrochemical detection Interlaboratory comparison exercise (eight laboratories)
		Vitamin B_2		Extraction, RPLC, fluorescence detection Interlaboratory comparison exercise (seven laboratories)
		Vitamin B_6		Extraction, RPLC, fluorescence detection Interlaboratory comparison exercise (six laboratories)
		Niacin		Extraction, RPLC, fluorescence detection Interlaboratory comparison exercise (seven laboratories)
SRM 2383 Baby Food Composite	1997	Carotenoids	Carotenoids	Saponification, RPLC, absorbance detection Extraction, RPLC, absorbance detection Extraction, saponification, RPLC, absorbance detection Interlaboratory comparison exercise (22 laboratories)
		Retinol		Saponification, RPLC, absorbance detection Extraction, saponification, RPLC absorbance detection Interlaboratory comparison exercise (30 laboratories)
			Retinyl palmitate	Extraction, RPLC, absorbance detection Interlaboratory comparison exercise (nine laboratories)

Table 1. Continued

	Year issued	Certified constituents	Reference constituents	Methods used for value assignment
		Tocopherols	Tocopherols	Saponification, RPLC, fluorescence detection Extraction, RPLC, fluorescence detection Extraction, saponification, RPLC, fluorescence detection Interlaboratory comparison exercise (36 laboratories)
SRM 2384 Baking Chocolate	In preparation	Tocopherols[a]		
SRM 2385 Spinach	In preparation	Carotenoids[a] Tocopherols[a] Folate[a]		

[a] Proposed constituents to be measured by NIST.

2.2. Vitamins B_2, B_6, and niacin

Because of the existence of a number of vitamers, the B vitamins present a much more complex problem. The B vitamins are frequently measured by the food community using the Association of Official Analytical Chemist's (AOAC's) microbiological methods, which may or may not measure all of the forms, depending on the specifics of the sample preparation procedure involved. Chemical methods can suffer from this limitation, as well.

Niacin is found in foods as nicotinic acid, nicotinamide, nicotinamide adenine dinucleotide (NAD), its reduced form (NADH), nicotinamide adenine dinucleotide phosphate (NADP), and its reduced form (NADPH), and can be covalently bound to carbohydrates and polynucleotides. Upon acid treatment, the NADH and NADPH are converted to the 6-cyclo compounds [18]. Under basic conditions the NAD and NADP are degraded to nicotinaldehyde [19]. Nicotinic acid is released from its covalent carbohydrate link by alkaline hydrolysis. Thus, it is not possible to measure all of the nicotinic acid containing species by a single extraction procedure.

Pyridoxal, pyridoxamine, pyridoxine, pyridoxal-5′-phosphate, pyridoxamine-5′-phosphate, and pyridoxine-5′-phosphate are active forms of vitamin B_6. The extraction of vitamin B_6 species has been reviewed by Gregory [20]. The completeness of the recovery of the various vitamers from plant species using selective hydrolytic steps has allowed the measurement of glycosylated forms of vitamin B_6 [21]. The binding of several species to proteins and the existence of glycosides in plants also complicates the extraction procedure.

Riboflavin occurs as a free base and the nucleotide. The nucleotide can be converted to riboflavin or measured separately. Additionally, riboflavin occurs as the glycoside or bound to the amino acids histidine, cysteine, and tyrosine [22]. We have also observed that as much as 15% of riboflavin in dilute hydrochloric acid is adsorbed to glass; consequently Teflon vessels must be used for the preparation of standards and extraction procedures.

As with vitamin C, the above three B vitamins were measured in SRM 1846 Infant Formula, and

Table 2
Assigned values (in mg/kg) and NIST results (one standard deviation shown in parentheses) used in value-assignment analyses for selected vitamins in SRM 1846 Infant Formula

	Certified value	NIST result
Vitamin A (*trans*)[a]	5.84±0.68	4.77 (0.49)[b]
Vitamin E[c]	271±25	255 (14)
Vitamin C	1146±66	1230 (39)
Vitamin B_2	17.4±1.0	17.59 (0.49)
Vitamin B_6	8.4±1.0	8.62 (0.36)
Niacin	63.3±7.6	67.3 (1.7)

[a] Vitamin A=*trans*-retinol+*trans*-retinyl palmitate in retinol equivalents.

[b] Result based on the saponification of dry material. Later improvements in sample preparation procedure have resulted in better agreement with the certified value; see discussion in text.

[c] Vitamin E=α-tocopherol+α-tocopheryl acetate. Vitamin E was added to the infant formula as RRR-α-tocopheryl acetate.

NIST results were combined with results obtained by a group of collaborating laboratories to provide the certified value. A comparison of NIST's value to the certified value is provided in Table 2. NIST's sample preparation for the determination of riboflavin (vitamin B_2), pyridoxine (vitamin B_6), and nicotinamide (niacin) concentrations, the vitamin forms added to the material by the manufacturer, involved dissolution of 3- to 5-g test portions in 15 ml water in Teflon beakers, with the addition of 4-deoxypyridoxine as an internal standard. *m*-Phosphoric acid and acetonitrile were added to final volume fractions of 5 and 3.8%, respectively. After mixing, overnight storage at 4°C to coagulate proteins, and centrifugation, a portion of the bottom layer was removed for LC analysis using a monomeric C_{18} column (Vydac 201HS54 ODS, The Separations Group, Hesperia, CA, USA) with fluorescence and absorbance detection. (The method employed is an unpublished method developed by The Separations Group, Hesperia, CA, USA.)

This method worked well for the analysis of SRM 1846, into which known forms of these vitamins were added during material preparation. The method is not, however, applicable to the measurement of the same vitamins in SRM 2383 Baby Food Composite because bound forms of the analytes exist in this material, and those forms are not extracted by a simple dissolution of the sample in water. Vitamins B_2, B_6, and niacin were not measured by NIST in SRM 2383 Baby Food Composite, although reference values for these analytes (as well as vitamins B_1, B_{12}, pantothenic acid, and biotin) were assigned using data reported by collaborating laboratories using both microbiological and chemical methods of analysis [17,23]. These laboratories may have measured free forms of the analyte or total analyte, depending on sample preparation steps involved and the efficiencies of extraction; uncertainties associated with the assigned values may reflect differences in what analytes are being included in a measurement rather than uncertainties resulting from the measurements themselves.

2.3. Vitamins A, D, and E

Vitamin A content can be assessed by measuring retinol and/or retinyl esters. A value for "retinol" can mean that *trans*-retinol was measured, that individual *trans* and *cis* peaks were measured and mathematically summed, or that one total retinol (*trans*-retinol+*cis* isomers of retinol) peak was measured because the analytical column used was incapable of resolving the isomers. *Cis* isomers of retinol are reported to possess 75% of the activity of *trans*-retinol [24]. Several carotenoids can be converted to vitamin A, therefore these should also be measured to truly assess the vitamin A content of foods.

Vitamin D can occur as cholecalciferol (vitamin D_3, derived from the action of sunlight on the 7-dehydrocholesterol found in vertebrates) or ergocalciferol (vitamin D_2, derived from the action of sunlight on the ergosterol found in invertebrates, plants, and fungi). Provitamin D (7-dehydrocholesterol and ergosterol) are converted to pre-vitamin D compounds, which are then thermally converted to the D vitamins. (The D vitamins themselves must be converted to physiologically active forms by the body before they can function in metabolism; these forms are measured in clinical samples, but not typically in foods). While pre-vitamin D is considered to be biologically inactive, it is summed with vitamin D for nutrition labeling purposes.

Although the food community only recognizes α-tocopherol and its esters as vitamin E for labeling purposes, δ-, γ-, and β-tocopherol also possess vitamin E activity. Because the other tocopherols have less vitamin E activity than that of α-tocopherol, they must be chromatographically resolved.

2.3.1. SRM 1563 Coconut Oil

SRM 1563 Cholesterol and Fat-Soluble Vitamins in Coconut Oil was the earliest food-matrix SRM available from NIST with values assigned for organic nutrients [25]. SRM 1563 is available in a sales unit containing ampoules of natural and fortified coconut oil; cholesterol, all-*rac*-α-tocopheryl acetate, retinyl acetate, and ergocalciferol were added to the fortified oil, and gravimetry and LC were used to provide assigned values in the classic certification mode – the use of two independent methods. A comparison of gravimetric and LC values to the certified values is provided in Table 3. Recently SRM 1563 was analyzed to reassess the

Table 3
Assigned values (in mg/kg) and NIST results (one standard deviation shown in parentheses) used in value-assignment analyses for selected vitamins in SRM 1563-2 Cholesterol and Fat-Soluble Vitamins in Coconut Oil (Fortified)

Vitamin added	Assigned value	Gravimetry	LC result
Retinyl acetate[a]	8.2	12.6	11.9 (0.3)
Ergocalciferol	10.9±0.8	10.5	11.3 (0.2)
All-*rac*-α-Tocopheryl acetate	158±6	158.2	158.0 (4.5)

[a] Concentration changed following original certification analyses; see text for stability details.

concentrations of the fat-soluble vitamins. The method used in the original value assignment, which has been published previously, employed on-line gel permeation chromatography (GPC) to isolate the vitamins from the bulk of the lipid matrix [26]. For the reassessment performed recently, the GPC was performed off-line.

Normal-phase liquid chromatography was used to separate the three vitamins following the GPC clean-up. An unknown compound co-eluted with α-tocopheryl acetate using the normal-phase column, making it necessary to also analyze the samples using reversed-phase liquid chromatography. For the normal-phase separation, a semipreparative amino-cyano column (Partisil M9, Whatman, Clifton, NJ, USA) was used. An analytical C_{18} column (Vydac C_{18} 201TP54, The Separations Group, Hesperia, CA, USA) was used for the determination of the concentration of α-tocopheryl acetate.

NIST generally assesses SRM stability periodically throughout an SRM's lifetime. Analyte concentrations in SRM 1563 were originally value assigned in 1987, and vitamin concentrations in the material were measured again in 1990 and found to be within the uncertainties specified in the original Certificate of Analysis. As a result of analyses of SRM 1563 in 1996, the value for retinyl acetate was changed from ''certified'' to ''reference'' because it had decreased by about 30%. In 1987, the certified value for retinyl acetate was 12.2±0.8 mg/kg. The new reference value assigned in 1996 is 8.2 mg/kg.

2.3.2. SRM 1846 Infant Formula

Vitamins A and E were measured in SRM 1846 Infant Formula, and NIST results were combined with results obtained by a group of collaborating laboratories to provide the certified value [12,13]. A comparison of NIST's values to the certified values for vitamins A and E is provided in Table 2. The NIST method used for sample preparation in the certification analyses for these vitamins in SRM 1846 has been previously published [12]. The method used for saponification of SRM 2383, discussed below, has been found to be effective for the infant formula, as well, and is now used for both sample types [27].

In the certification analyses for vitamins A and E in SRM 1846, extracts were injected onto a monomeric [28] non-endcapped C_{18} column (YMC-Pack ODS-AL, YMC, Wilmington, NC, USA). Along with α-tocopherol (and α-tocopheryl acetate), recognized as vitamin E by the food industry, δ- and γ-tocopherol were also measured by NIST; because a single method was used for analysis (without confirmation by an outside laboratory using another method or by an interlaboratory comparison exercise), reference values are provided for these analytes in SRM 1846.

Two separate saponification procedures were employed for the determination of retinol/retinyl palmitate and the tocopherols in the original certification analyses. The longer time required to saponify retinyl palmitate destroyed some of the α-tocopherol, therefore both vitamins A and E could not be measured simultaneously. (Later experiments, discussed below, showed that it was possible to obtain accurate results for both vitamins A and E following a single sample preparation if the encapsulation surrounding the retinyl palmitate was ruptured by reconstituting the infant formula in hot water and homogenizing it for a short time).

Fat-soluble vitamin concentrations in SRM 1846 have been monitored over time. Concentrations for analytes in SRM 1846 were originally assigned in 1996; the certified value for vitamin A (*trans*-retinol) is 5.84±0.68 mg/kg. Concentrations of retinol and the tocopherols have not changed in the intervening years although, curiously, the retinol seems to have

become more difficult to extract. Previously, it was possible for analysts at NIST to extract retinol from samples that were reconstituted in water at room temperature prior to saponification; the analysis of two samples in such a manner gave a mean result of 5.39 mg/kg approximately 1 year after certification analyses were completed. In another analysis a year later, it was necessary to reconstitute the infant formula in water at 90°C (to break the beadlets in which the individual fat-soluble vitamins are encapsulated, apparently) to obtain a result in agreement with the certified value; the *trans*-retinol concentration found in these analyses was 5.86 mg/kg ($n=2$). And most recently, in 1999, it was necessary to homogenize the sample for 30 s following reconstitution in water at 90°C; the mean result for two analyses was 5.51 mg/kg. It has always been more difficult to extract retinol and retinyl palmitate than the tocopherols from SRM 1846, but the reason why it would become increasingly difficult is not clear. The vitamin A concentration does, however, appear to have remained stable.

2.3.3. SRM 2383 Baby Food Composite

Retinol/retinyl palmitate, tocopherols, and carotenoids were measured in SRM 2383 Baby Food Composite, and NIST results were combined with results obtained in two sets of interlaboratory comparison exercises to provide the certified and reference values [17,23]. NIST analyses employed a combination of three sample preparation techniques and three LC methods, as discussed below. A comparison of NIST's values to selected certified and reference values using several of the combinations of sample preparation and LC methods is provided in Table 4.

SRM 2383 was prepared from a mixture of foods that were combined in proportions to provide comparable levels of xanthophylls (hydroxy carotenoids) and carotenes (hydrocarbon carotenoids); powdered infant formula was added to provide fat-soluble vitamins. The ingredients contained xanthophyll esters as well as esters of vitamins A and E (retinyl palmitate and α-tocopheryl acetate). Test portions of SRM 2383 were extracted or saponified in order to

Table 4
Certified and reference values (in mg/kg) and NIST results (one standard deviation provided in parentheses) using three different analytical columns for selected vitamins and carotenoids in SRM 2383 Baby Food Composite

	Assigned value (mg/kg)	Saponified food		Extracted/saponified		Extracted food,
		LC method 1[a]	LC method 3[a]	LC method 1[a]	LC method 2[a]	LC method 3[a]
trans-Retinol[b]	0.80±0.16	0.74 (0.01)		0.73 (0.04)	0.85 (0.03)	
α-Tocopherol[b]	25.0±3.3	23.5 (0.3)		24.3 (1.2)		
Lutein (includes esters)[b]	1.16±0.33	1.01 (0.02)	3.13 (0.01)[c]	1.20 (0.05)	1.07 (0.11)	
Lutein (free)[d]	0.75±0.35			0.60 (0.01)	0.67 (0.09)	
Zeaxanthin (includes esters)[b]	0.86 ±0.14	0.79 (0.01)	0.96 (0.03)	0.88 (0.02)	0.85 (0.02)	
Zeaxanthin (free)[d]	0.46 ±0.10			0.46 (0.02)	0.50 (0.05)	0.40 (0.01)
trans-Lycopene[e]	6.3±1.2	6.57 (0.05)		6.77 (0.26)		
Total lycopene[e,f]	7.0±1.5	7.19 (0.05)	6.58 (0.33)[g]	7.73 (0.23)		7.32 (0.38)
trans-β-Carotene[e]	2.40±0.80	2.12 (0.03)	2.56 (0.08)	2.43 (0.05)	1.75 (0.22)	2.73 (0.15)
Total β-carotene[e,f]	3.12±0.63	2.92 (0.03)	3.56 (0.11)	3.12 (0.08)	2.61 (0.24)	3.62 (0.15)

[a] LC method 1=analysis using the intermediate C_{18} column; LC method 2=analysis using the polymeric C_{18} column; LC method 3=analysis using the polymeric C_{30} column. Details of chromatographic conditions are provided in Refs. [29–31].

[b] Concentration in saponified test portions.

[c] Unknown component(s) co-eluted with lutein using the polymeric C_{30} column; data were not included in value assignment. (Analysis of a quality control sample [SRM 968b Fat-Soluble Vitamins and Cholesterol in Human Serum] indicated that the system was properly calibrated for the analysis of lutein).

[d] Concentration in extracted (unsaponified) test portions; measurement of ''free'' (unesterified) analyte.

[e] Data for the analysis of extracted test portions and saponified extracts combined.

[f] Concentration is the sum of *cis* and *trans* isomers.

[g] Measurements on saponified food using LC method 3 were performed 1 month after jars of SRM 2383 were originally opened. There were no changes in concentrations of the other carotenoids, but lycopene had apparently degraded and results were not used for value assignment.

measure both free and esterified forms of these analytes; the food was saponified directly and extracts were saponified, as well, to provide a third variation on the sample preparation method [27]. (Saponification of the food itself (rather than an extract) appears capable of successfully extracting vitamins A and E (and carotenoids) from a variety of food matrices. SRM 1846 Infant Formula and SRM 2383 Baby Food Composite contain mass fractions of about 27 and 4.5% fat, respectively, yet the saponification method works equally well with either matrix). Because of the presence of esters, concentrations are expected to vary based on the type of sample preparation employed; results for SRM 2383 were divided as follows: data for saponified test portions were used for value assignment of retinol, α-tocopherol, and xanthophyll (lutein, zeaxanthin, and β-cryptoxanthin) concentrations; data for unsaponified test portions were used for value assignment of retinyl palmitate and ''free'' (unesterified) α-tocopherol and xanthophyll concentrations; and data for saponified and unsaponified test portions were combined for δ-tocopherol, γ-tocopherol, and carotene (*trans*- and total lycopene, α-carotene, and β-carotene) concentrations.

Details of the three LC methods used for analysis have been previously published [29–31]. Retinol, retinyl palmitate, tocopherols, and carotenoids were measured using a C_{18} analytical column (Bakerbond C_{18}, J.T. Baker, Phillipsburg, NJ, USA) with selectivity characteristics intermediate to those of monomeric and polymeric stationary phases (as reflected in the separation of polycyclic aromatic hydrocarbons; see Ref. [28]). Retinol and carotenoids were measured using a polymeric [28] C_{18} column (Vydac 201TP, The Separations Group). The carotenoids were also measured using a NIST-engineered polymeric C_{30} column (prepared in-house but commercially available from YMC–Waters, Milford, MA, USA) [32].

These three columns exhibit different selectivity; a comparison of chromatograms of saponified test portions is provided in Fig. 1. The main differences among the three sets of chromatograms are related to the columns' abilities to separate geometric isomers. The polymeric C_{30} column is able to resolve the 9-, 13-, and 15-*cis* isomers of β-carotene, while the 13- and 15-*cis* isomers co-elute on the intermediate and polymeric C_{18} columns. The lycopene isomers were not resolved using the polymeric C_{30} column and conditions employed in this value-assignment exercise, but many were resolved by the intermediate C_{18} column; lycopene was not measured using the polymeric C_{18} column, but lycopene would elute after β-carotene using this column, as it does with the C_{30} column.

Co-eluting compounds on one column may become apparent by comparing results among the three columns (Table 4), and this is one of the advantages in using multiple methods for analysis. Data for lutein in saponified test portions using the C_{30} column were not included in value assignment because of the presence of an unknown co-eluting peak. Relative standard deviations (RSDs) for the ''free'' (unesterified) xanthophylls are higher than the results for saponified samples, in which the xanthophyll esters are included; presumably some de-esterification may occur during the extraction process. Differences among results for the xanthophylls may also be due to differences in chromatographic resolution of isomers and unidentified polar carotenoid peaks. Results obtained by NIST for retinol (vitamin A) and α-tocopherol (vitamin E) in saponified samples were in good agreement with results obtained by laboratories participating in interlaboratory comparison exercises, and agree well with the certified values. Results obtained by NIST for total and *trans*-lycopene are in good agreement with each other, but the reference values are somewhat lower than the NIST values because lower results were obtained in an interlaboratory comparison exercise. Results for *trans*-β-carotene do not agree as well as results for total β-carotene, probably because of isomerization during sample preparation.

3. Conclusions

NIST currently has methods for the measurement of naturally occurring levels of vitamins C, A and E, and carotenoids, as well as for fortified levels of some of the B vitamins. These methods have been applied to several food-matrix SRMs now available from NIST. Unfortunately, LC methods are not yet in place at NIST for the measurement of naturally occurring forms and levels of the B vitamins.

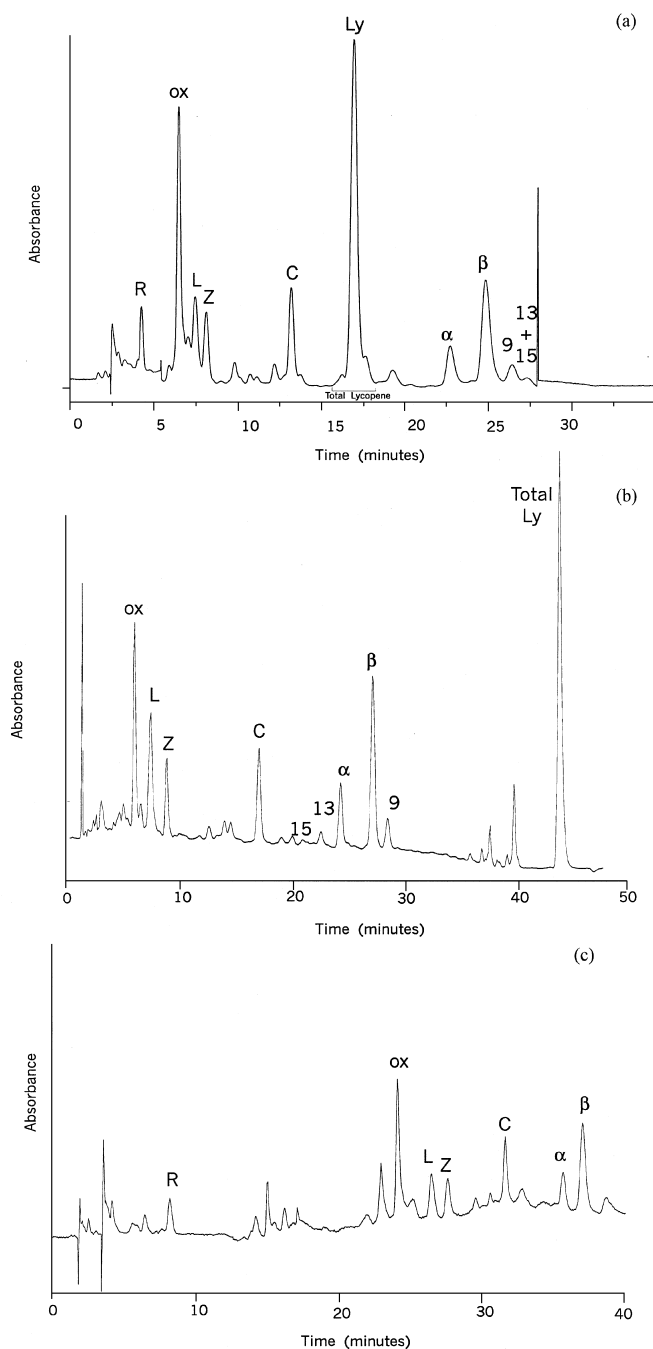

Fig. 1. Chromatograms showing the separation of retinol and carotenoids on the intermediate C_{18} column (a), the polymeric C_{30} column (b), and the polymeric C_{18} column (c) in saponified test portions. Peak identities are: R=retinol, ox=*trans*-β-apo-10′-carotenal oxime (internal standard), L=lutein, Z=zeaxanthin, C=β-cryptoxanthin, Ly=*trans*-lycopene, α=*trans*-α-carotene, β=*trans*-β-carotene, 9=9-*cis*-β-carotene, 13=13-*cis*-β-carotene, and 15=15-*cis*-β-carotene. Chromatographic conditions are described in Refs. [29–31] for (a), (b), and (c), respectively. The absorbance of retinol was measured at 325 nm and the absorbances of the internal standard and the carotenoids were measured at 450 nm using a programmable UV–Vis absorbance detector.

Methods are currently under development for the measurement of folates in food, with the intent of providing folate values in the candidate spinach SRM. As defined by the recent modes of certification document [5], a NIST certified value must include some analytical measurements performed at NIST; therefore, development of chromatographic methods for additional vitamins is necessary to provide certified values for food-matrix SRMs with naturally occurring levels and forms of these vitamins.

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