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Liquid chromatography with isotope-dilution mass spectrometry for determination of water-soluble vitamins in foods

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Abstract Vitamins are essential for improving and maintaining human health, and the main source of vitamins is the diet. Measurement of the quantities of water-soluble vitamins in common food materials is important to understand the impact of vitamin intake on human health, and also to provide necessary information for regulators to determine adequate intakes. Liquid chromatography (LC) and mass spectrometry (MS) based methods for water-soluble vitamin analysis are abundant in the literature, but most focus on only fortified foods or dietary supplements or allow determination of only a single vitamin. In this work, a method based on LC/MS and LC/MS/MS has been developed to allow simultaneous quantitation of eight water-soluble vitamins, including multiple forms of vitamins B_3 and B_6, in a variety of fortified and unfortified food-matrix Standard Reference Materials (SRMs). Optimization of extraction of unbound vitamin forms and confirmation using data from external laboratories ensured accuracy in the assigned values, and addition of stable isotope labeled internal standards for each of the vitamins allowed for increased precision.

Keywords Vitamins · Nutrition · Fortification · Food · Mass spectrometry · Isotope dilution

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Introduction

The intake of vitamins is essential for normal human growth and maintenance of general health through their role in various metabolic processes. Because water-soluble vitamins are not synthesized within the human body, nor are these compounds stored in the body for future use (with the exception of vitamin B_6), these micronutrients must be consumed daily to ensure proper health and function [1]. Ongoing debates surround adequate intake levels; the quality of intake from fortified, enriched, and unfortified sources; the mechanism used for fortification; and the impact of these and other factors on overall health. Underlying each of these debates is the importance of accurate and precise measurement technology that is needed to quantify and understand both vitamin intake and biomarkers for health status.

In the United States, the Food and Drug Administration (US FDA) is responsible for guaranteeing the integrity of the food supply. In 1990, the Nutrition Labeling and Education Act granted US FDA with the authority to require clear and concise labeling of the energy and nutrient content of most foods sold in the US [2]. Currently, labels are required to declare the content of nutrients vitamins A and C, as well as calcium and iron. An update to the nutrition facts label was proposed by US FDA in 2014 to include requirements for vitamin D and potassium as "nutrients of public health significance" [3]. All other vitamins and minerals may be included on a voluntary basis, and often are when a manufacturer intends to demonstrate that a product is nutritious. US FDA requires that information provided about both required and voluntary nutrients be accurate, for which both appropriate analytical methods and a variety of certified reference materials are of utmost importance.

Separate from the legal requirements of nutrient labels, accurate information about the nutrient content of foods is critical for understanding the impact of diet on health status. In

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the US, the major source of information on population health status is the National Health and Nutrition Examination Survey (NHANES), administered by the National Center for Health Statistics of the US Centers for Disease Control and Prevention. The continuous survey that has been ongoing since 1999 includes nutrition and lifestyle survey information as well as clinical tests for biomarkers, including those for a number of vitamins [4]. Understanding the health status of individuals, as well as population trends, depends on the accuracy of information about nutrient content of foods reported on labels and in nutrient databases such as the National Nutrient Database for Standard Reference, maintained by the US Department of Agriculture (USDA) [5]. The accuracy of the information contained in this database relies heavily on the availability of both appropriate analytical methods and a variety of certified reference materials.

Countless analytical approaches for the determination of water-soluble vitamins are described in the literature, from colorimetric and microbiological assays to more specific instrumental techniques [6, 7]. A majority of the publications focus on a single vitamin or suite of vitamers, without attempting to analyze simultaneously thiamine, riboflavin, niacin, niacinamide, pantothenic acid, pyridoxine, pyridoxal, and pyridoxamine. Of the reports that determine multiple water-soluble vitamins simultaneously, a vast majority are applied only to fortified foods and dietary supplements [8–27]. A few papers have described the simultaneous determination of water-soluble vitamins in unfortified foods by liquid chromatography (LC) with ultraviolet (UV) absorbance or fluorescence (FL) detection [28–31], or by LC with mass spectrometry (MS) or tandem MS (MS/MS) [30, 32–34]. Methods based on LC-UV are plagued by matrix interferences, as the wavelengths used for absorption determination (254 to 270, 325, and 360 nm) are common to many organic compounds. In addition, pantothenic acid (vitamin B_5) has no native chromophore and can only be detected with weak absorbance signal at 200 nm [28]. Vitamins B_2 and B_6 have native fluorescence that can be exploited, and with inline detectors can add sensitivity for these compounds in spectrophotometric detection systems [28]. Significant gains can be made in selectivity of detection as well as accuracy and precision of quantitation by utilizing MS or MS/MS with addition of stable isotope labeled internal standards for each vitamin [35]. Only the work of Hälvin et al. describes the use of isotope dilution (ID)MS for determination of water-soluble vitamins in an unfortified food (nutritional yeast) [34]. In the current method, separation and quantitation of eight water-soluble vitamins (thiamine, riboflavin, niacin, niacinamide, pantothenic acid, pyridoxine, pyridoxal, and pyridoxamine) is described using LC/IDMS and LC/IDMS/MS for a wide variety of fortified and unfortified foods.

To address the needs of food manufacturers and federal partners such as the US FDA, CDC, and USDA, the National Institute of Standards and Technology (NIST) has made available a number of food-matrix Standard Reference Materials (SRMs) with certified and reference values for water-soluble vitamins. These reference materials have been selected and designed to represent common matrix challenges in the analytical measurements of nutrients in foods, and they are distributed throughout the food triangle developed by AOAC INTERNATIONAL [36]. Food-matrix SRMs with both fortified and endogenous levels and forms of water-soluble vitamins are included in the NIST catalog. The analytical approach described herein was utilized in the certification of the mass fraction values of water-soluble vitamins in unfortified food-matrix reference materials such as SRM 1546a Meat Homogenate, SRM 1549a Whole Milk Powder, SRM 1845a Whole Egg Powder, SRM 2383a Baby Food Composite, SRM 2384 Baking Chocolate, SRM 2387 Peanut Butter, SRM 3234 Soy Flour, and SRM 3287 Blueberry (Fruit). The same analytical method was utilized in certification of water-soluble vitamins in fortified materials including SRM 1849a Infant/Adult Nutritional Formula and SRM 3233 Fortified Breakfast Cereal. This collection of reference materials with certified and reference values for water-soluble vitamins in both fortified and unfortified materials is unmatched by any other certified reference material producer.

Materials and methods[1]

Chemicals Thiamine chloride hydrochloride, riboflavin, niacinamide, calcium pantothenate, and pyridoxine hydrochloride were obtained from the U.S. Pharmacopeia (Rockville, MD, USA). Niacin, pyridoxal hydrochloride, and pyridoxamine dihydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Thiamine-[$^{13}C_3$] chloride and pantothenic acid-[$^{13}C_3$,^{15}N] calcium salt monohydrate were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Riboflavin-[$^{13}C_4$,$^{15}N_2$], niacinamide-[$^{2}H_4$], niacin-[$^{2}H_4$], pyridoxal-[$^{2}H_3$] hydrochloride, pyridoxine-[$^{13}C_4$] hydrochloride, and pyridoxamine-[$^{2}H_3$] dihydrochloride were obtained from Isosciences (King of Prussia, PA, USA). Acetic acid, ammonium acetate, and hydrochloric acid used in extraction solvent preparation, as well as formic acid and ammonium formate used in mobile phase preparation, were reagent grade from Sigma. Water used in the extraction and water and methanol used to prepare LC mobile phases were high performance LC (HPLC) grade from J&H Berge (South Plainfield, NJ, USA).

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

Samples Food samples and controls were Standard Reference Materials (SRMs) produced and distributed by the National Institute of Standards and Technology (NIST) in Gaithersburg, MD, USA. Fortified foods included SRM 1849a Infant/Adult Nutritional Formula (milk-based hybrid infant/adult nutritional powders prepared by a manufacturer of infant formula and adult nutritional products) and SRM 3233 Fortified Breakfast Cereal (ground and homogenized fortified commercial cereal). Unfortified foods included SRM 1546a Meat Homogenate (canned mixture of commercially blended pork and chicken products), SRM 1549a Whole Milk Powder (a free-flowing fine powder prepared from whole milk by a dairy distributer), SRM 1845a Whole Egg Powder (a free-flowing, fine powder prepared from USDA-inspected whole eggs by a food distributor), SRM 2383a Baby Food Composite (mixture of fruits, vegetables, macaroni, rice flour, and milk powder, commercially blended and packaged), SRM 2384 Baking Chocolate (commercially prepared from 100 % cocoa beans), SRM 2387 Peanut Butter (creamy peanut butter containing roasted peanuts, sugar, partially hydrogenated vegetable oils, and salt, prepared by a commercial manufacturer), SRM 3234 Soy Flour (defatted soy flour prepared by a food ingredient manufacturer), and SRM 3287 Blueberry (Fruit) (freeze-dried, powdered blueberries). Formulas were stored at – 80 °C; milk powder, egg powder, and baby food were stored at 4 °C; breakfast cereal, meat homogenate, soy flour, and blueberries were stored at room temperature. For determination water-soluble vitamins in SRM 1549a Whole Milk Powder, ERM-BD600 Whole Milk Powder was obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and was used as a control material.

Sample preparation The sample preparation procedure described by Phinney et al. [35] was evaluated and adapted as necessary for each food material. Potential analyte degradation was minimized by conducting all sample preparation under reduced lighting. Two to twelve packets of each food material were selected for analysis, and duplicate subsamples were taken from each packet. The contents of each packet of material were shaken or mixed to distribute the contents. A sample of material (0.5 to 11 g) was weighed into a 50 mL polypropylene centrifuge tube and an appropriate volume of internal standard solution was added to match the expected level of each analyte in each sample. The extraction solvent (1 % acetic acid in water, volume fraction) was added to bring the total solution volume to 30 mL and the contents were mixed well. The tubes were placed in an ultrasonic bath for 30 to 120 min without heating, then centrifuged at 3000 rpm (314 rad/s) for 15 min. Because the baking chocolate required melting, extraction of water-soluble vitamins from this matrix was conducted using a HotBlock (Environmental Express, Charleston, SC, USA) at 90 °C with constant stirring. For some samples (baking chocolate, soy flour, and blueberries), the supernatant was decanted, fresh solvent was added, and the process was repeated for a total of 2 to 4 cycles. Approximately 1.5 mL of the supernatant (or combined supernatant) was transferred to an autosampler vial following filtration through a 0.45 μm regenerated cellulose filter. For each study, a NIST SRM with assigned values for water-soluble vitamins was used as a control and prepared simultaneously in the same manner. Optimized extraction conditions for each matrix are provided in the Electronic Supplementary Material (ESM) Table S3.

LC/IDMS analysis Samples and standards were analyzed by using an Agilent Series 1200 LC/MSD (Agilent Technologies, Palo Alto, CA, USA) with electrospray ionization in the positive ion mode. A Cadenza CD-C18 column (250× 4.6 mm i.d., 3 μm particles) from Silvertone Sciences (Philadelphia, PA, USA) was used for the analyses without a guard cartridge. Mobile phase A consisted of 20 mol/L ammonium formate in water adjusted to pH 4.0 with formic acid, and mobile phase B was methanol. Gradient elution began after 6 min, from 0 %B to 50 %B over 14 min, followed by a 10 min wash at 100 %B and a 20 min reequilibration at the initial conditions. A slightly modified gradient was used in the analyses of SRM 1849a and SRM 2383a to avoid chromatographic coelutions, which began after 10 min, from 0 %B to 50 %B over 35 min, followed by a 10 min wash at 100 %B and a 10 min reequilibration at the initial conditions. The flow rate for all separations was maintained at 0.8 mL/min and a 5.0 μL injection volume was used for all standards and samples.

Quantitation was performed by IDMS in selected ion monitoring (SIM) mode using the ions for unlabeled and labeled vitamins as described in ESM Table S1. Initially, a labeled internal standard for riboflavin was unavailable and/or the use was cost prohibitive. Therefore labeled pyridoxine and pantothenic acid were used for quantitation of riboflavin in some matrices. The mass spectrometer was operated at a nebulizer pressure of 0.35 MPa (50 psig), a nebulizer gas temperature of 350 °C, a drying gas flow rate of 12 L/min, a capillary voltage of 4000 V, and a fragmentor voltage of 90 V.

Vitamin concentrations in each of the food samples were bracketed with four to five calibration solutions independently prepared in a 1 % solution (volume/volume) of acetic acid. The stock solutions of each vitamin and internal standard were gravimetrically mixed in appropriate ratios to reflect the concentration of each compound in each sample for determination of response factors. All solutions were stored in the refrigerator (4 °C) when not in use. Each calibration solution was injected at least in duplicate, and the response factor was calculated for each injection. An average response factor was used for the calculation of each vitamin concentration in each sample.

LC/IDMS/MS analysis Samples and standards were analyzed by using an Agilent Series 1260 LC (Agilent Technologies)

equipped with an Agilent Series 6410 Triple Quadrupole MS with electrospray ionization in the positive ion mode. The same column and mobile phase compositions were utilized as described above for LC/IDMS analysis. Gradient elution began after 6 min, from 0 %B to 50 %B over 14 min, followed by a 10 min wash at 100 %B and a 20 min reequilibration at the initial conditions. The flow rate for all separations was maintained at 0.8 mL/min and a 10.0 μL injection volume was used for all standards and samples.

Quantitation was performed by IDMS/MS in multiple reaction monitoring (MRM) mode using the timetable, transitions, fragmentor voltages, and collision energies listed in ESM Table S2 for the vitamins and their respective internal standards. Initially, multiple transitions for multiple vitamins were monitored in the same time windows. For baking chocolate and peanut butter analysis, a modified timetable was utilized (ESM Table S2) that allowed each vitamin to be monitored within a unique time window to maximize sensitivity. The mass spectrometer was operated at a nebulizer pressure of 0.10 MPa (15 psig), a drying gas temperature of 300 °C, a drying gas flow rate of 11 L/min, a capillary voltage of 4000 V, and a dwell time of 100 ms.

Vitamin concentrations in each of the food samples were bracketed with five calibration solutions independently prepared in a solution of 0.1 mol/L ammonium acetate, adjusted to pH 2.6 using hydrochloric acid. The stock solutions of each vitamin and internal standard were gravimetrically mixed in appropriate ratios to reflect the concentration of each compound in each sample for determination of response factors. All solutions were stored in the refrigerator (4 °C) when not in use. Each calibration solution was injected at least 5 times, and a response factor was calculated for each transition in each injection. An average response factor was calculated for each transition and each compound, and the average response factor was used for the calculation of each vitamin concentration in each sample. The calculated concentrations for each transition in ESM Table S2 were averaged for each injection, and the average and standard deviation of concentrations determined for all injections (N given in ESM Table S3) were determined. Any outliers were identified using Grubb's Outlier Test and were only removed with 99 % confidence.

Value assignment For the final value assignment of the water-soluble vitamins in each SRM, the mean from the analysis by NIST was used. For some SRMs, the mean of NIST data was combined with the median of the mean results from each collaborating laboratory to determine each certified or reference value. For other SRMs, each certified or reference value was determined solely from the mean of NIST data, with confirmation by data provided by collaborating laboratories. In the case of SRM 1849a, the mean of NIST results was combined with the median of the mean results from each collaborating laboratory, as well as the mean result provided by the material manufacturer. The specific approach to value assignment for a given SRM can be found in the Certificate of Analysis, available on the NIST website [37].

Results and discussion

Countless methods for determination of water-soluble vitamins in foods can be found in the scientific literature. A majority of these methods focus on a single vitamin or small group of vitamers, requiring numerous separate analyses for determination of vitamins B_1, B_2, B_3, B_5, and B_6. Some methods have been developed for simultaneous determination of these vitamins in fortified foods, but these methods are only focused on the fortified forms (typically niacinamide, not niacin; and pyridoxine, not pyridoxamine and pyridoxal). Application of these methods to unfortified foods would result in underestimation of the content of vitamins B_3 and B_6, or additional separate analysis for quantification of these endogenous compounds using a different method. Also, in many cases, the extraction conditions for fortified vitamers would not be sufficient to extract the endogenous vitamers. The method described here has been used for simultaneous determination of both fortified and unfortified forms of vitamins B_1, B_2, B_3, B_5, and B_6 in a wide variety of food products.

Sample preparation The sample preparation procedure described by Phinney et al. [35] was modified as necessary to provide exhaustive extraction of the unbound forms of each vitamin in each food material. Extraction conditions of temperature, time, and the number of sequential processes were investigated and evaluated based on overall extraction yield compared with measurement precision. An example of the optimization of the extraction of pyridoxamine from SRM 3234 Soy Flour is demonstrated in Fig. 1, and will be discussed in detail to illustrate the process used for all food materials. First, the effect of sonication temperature was investigated by conducting the extraction of SRM 3234 without added heat, as well as by controlling the bath temperature at 40 °C and 60 °C. The samples extracted at each temperature were analyzed by LC/MS/MS and the resulting mass fractions compared. Optimum conditions were determined based on the balance of high yield and high precision for each of the vitamins. For SRM 3234 Soy Flour, sonication extraction with no added heat was selected for future sample preparation (Fig. 1a). The sonication time was also optimized using the temperature selected for each matrix. Samples were sonicated for 30 min, 60 min, and 120 min with no added heat and the mass fractions resulting from LC/MS/MS analysis were compared. The highest extraction yields for SRM 3234 were obtained following 120 min of sonication, and the increase in uncertainty for pyridoxamine was determined to be

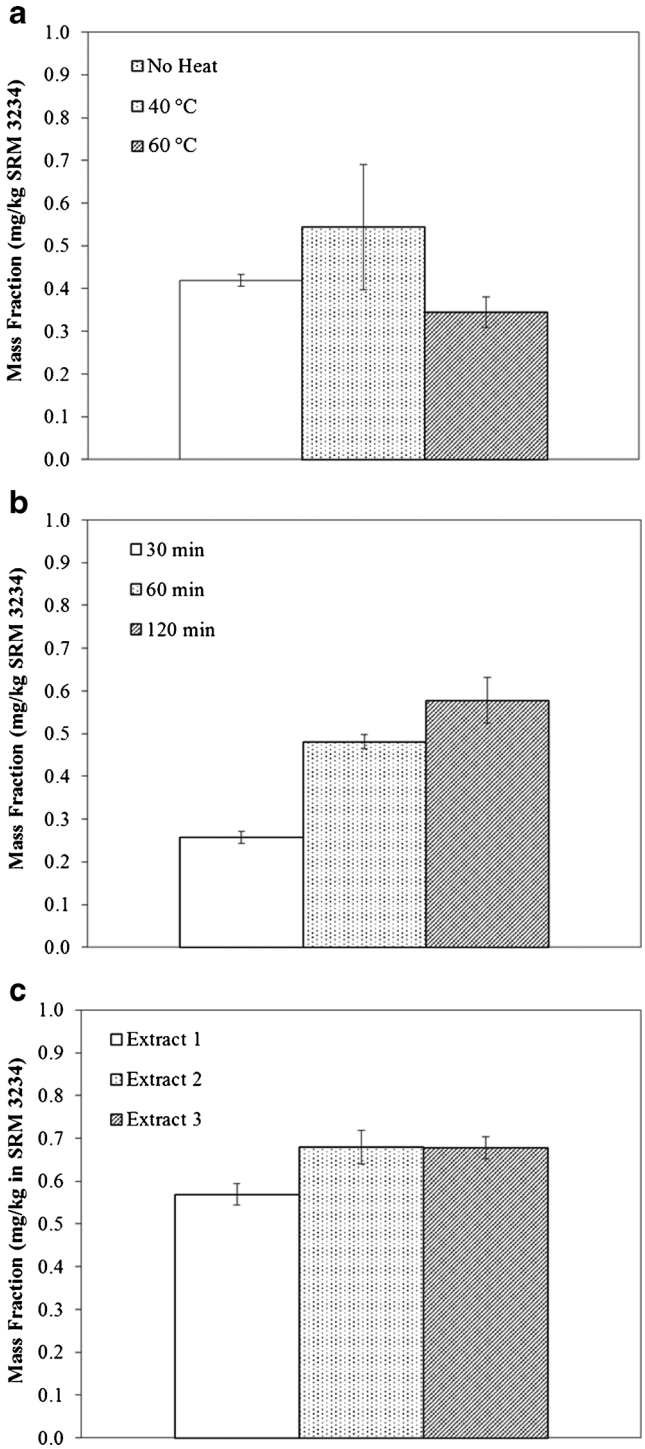

Fig. 1 Optimization of sample preparation conditions for pyridoxamine in SRM 3234 Soy Flour. **a** Sonication temperature; **b** Sonication time; **c** Number of sequential extraction cycles combined prior to LC/IDMS/MS analysis. The error bars represent the standard deviation of duplicate measurements

acceptable (Fig. 1b). The increase in uncertainty with extraction time was observed for all low-level analytes in this matrix, and combined with the decreasing trend in the increase in yield with extended extraction time, indicated that the increased yield from further increases in extraction time would be indistinguishable from the measurement uncertainty.

Samples of SRM 3234 were then sonicated for 120 min with no added heat to determine the number of sequential extraction cycles necessary to obtain exhaustive vitamin extraction. After 120 min of sonication, the samples were centrifuged and a small aliquot of the supernatant was removed for LC/MS/MS analysis. The remaining supernatant was decanted into a clean vessel, fresh extraction solvent was added, and the 120 min sonication cycle repeated. Following centrifugation, the supernatant was combined with the supernatant from the first extraction cycle, the solution was mixed well, and an aliquot was removed for LC/MS/MS analysis. This procedure was repeated for a third extraction cycle, and the mass fractions determined by LC/MS/MS were compared. As shown in Fig. 1c for pyridoxamine in SRM 3234, additional pyridoxamine was extracted during the second extraction cycle, but no additional yield was gained by adding a third extraction cycle. For SRM 3234, an extraction protocol involving two sonication cycles of 120 min with no added heat was adopted. This process was repeated for each food material to ensure exhaustive free vitamin extraction, and the optimized conditions for each food material are provided in ESM Table S3.

LC/IDMS analysis The NIST method developed and reported by Phinney et al. [35] was based on liquid chromatography with isotope dilution mass spectrometry (LC/IDMS) for determination of water-soluble vitamins in nutritional formulations such as SRM 3280 Multivitamin/Multielement Tablets and SRM 1849 Infant/Adult Nutritional Formula. Isotope dilution based approaches for quantitation reduce the impact of sample handling and instrument variability, as well as the effect of ion suppression or enhancement from interfering matrix compounds.[35] The chromatographic conditions described by Phinney et al.[35] were used directly for determination of water-soluble vitamins in SRM 1549a Whole Milk Powder, SRM 3233 Fortified Breakfast Cereal, and SRM 3287 Blueberry (Fruit). An example separation including extracted ion chromatograms for seven of the eight water-soluble vitamins in SRM 3233 Fortified Breakfast Cereal with detection by MS in SIM mode is provided in Fig. 2. To resolve some vitamins from interfering matrix components, the gradient was expanded slightly in the certification of SRM 1849a Infant/Adult Nutritional Formula and SRM 2383a Baby Food Composite. In this modified method, the isocratic step at 100 % aqueous was increased from 6 min to 10 min, and the gradient from 0 % to 50 % organic was increased from 14 min to 35 min. These methods provided baseline resolution of all eight water-soluble vitamins of interest, and the mass fractions determined using these methods are listed in Table 1. The ranges given in Table 1 represent the standard deviation of the number of measurements for that matrix, as reported in ESM

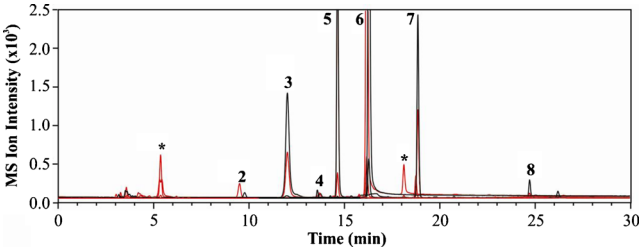

Fig. 2 LC/IDMS separation and detection of water-soluble vitamins in SRM 3233 Fortified Breakfast Cereal. Extracted ion chromatograms for vitamins are shown in the *black* traces, and those for stable isotope labeled internal standards are show in *red* traces. (*asterisk*) Unknown; (*2*) Niacin (*m/z* 124, *m/z* 128); (*3*) Thiamine (*m/z* 265, *m/z* 268); (*4*) Pyridoxal (*m/z* 168, *m/z* 171); (*5*) Pyridoxine (*m/z* 170, *m/z* 174); (*6*) Niacinamide (*m/z* 123, *m/z* 127); (*asterisk*) Unknown; (*7*) Pantothenic Acid (*m/z* 220, *m/z* 224); (*8*) Riboflavin (*m/z* 377, *m/z* 383)

Table S3. The behavior of control samples was determined to be fit for purpose (see ESM Tables S4 through S6).

In combination with the optimized sample extraction conditions detailed above, 27 certified values and 2 reference values were assigned for water-soluble vitamins in 5 food-matrix SRMs. Two of these materials, SRM 1849a Infant/Adult Nutritional Formula and SRM 3233 Fortified Breakfast Cereal, were fortified with these vitamins, while the other three materials contained only endogenous vitamins. Natural forms of vitamins B_3 and B_6 (niacin and pyridoxal, respectively), present at only 2 to 3 % of the fortified levels, were also detected in SRM 3233 and reference values for these components were assigned using this approach. For nearly all vitamins in all matrices, the precision of the method was good, with relative standard deviations (RSDs) of less than 10 %. Some exceptions included riboflavin in SRM 1849a, where the RSD was 11.2 %, and pyridoxal in SRM 3233 with an RSD of 15.0 %. The large uncertainty on the mass fraction for riboflavin in this matrix can be explained by the use of a non-matched stable isotope labeled internal standard. As described in ESM Table S1, other labeled vitamins were used as the internal standard for quantitation of riboflavin due to limited availability of riboflavin-[$^{13}C_4$,$^{15}N_2$] in the early stages of the work. The large uncertainty on the pyridoxal mass fraction in SRM 3233 is attributed to the lower level of this component in this matrix relative to the concentrations of other vitamins, as the IDMS signal for pyridoxal was very low. Using a larger sample size for extraction would have overloaded the signal for other components such as niacinamide and pantothenic acid, and in the interest of quantification of all vitamins in a single chromatographic run, the precision for pyridoxal was sacrificed slightly.

LC/IDMS/MS analysis To increase selectivity for endogenous vitamins in food matrix SRMs, the LC/IDMS approach described above was transferred to a triple quadrupole (MS/MS) system. The ability to operate in the multiple reaction monitoring (MRM) mode and to select multiple transitions for each vitamin provides increased confidence in the identification and quantitation of each analyte. The chromatographic method described above was modified slightly for the LC/IDMS/MS determination of vitamins in food matrix SRMs by replacing the isocratic hold at 50 % organic in favor of a step to 100 % organic from 20 to 20.1 min and a subsequent hold at 100 % organic for 10 min. This adjustment improved the peak shape for and reproducibility of the riboflavin determination, and simultaneously improved the overall reproducibility by washing the chromatographic system to remove matrix components. An example separation for the eight water-soluble vitamins in SRM 2387 Peanut Butter with MS/MS detection in the MRM mode is provided in Fig. 3. This method provided baseline resolution of all eight water-soluble vitamins of interest, and the mass fractions determined using LC/IDMS/MS are listed in Table 2. The ranges given in Table 2 represent the

Table 1 Mass fraction values for water-soluble vitamins in food Standard Reference Materials determined by LC/IDMS. The number of samples analyzed is as given in Table S-3 (Online Resource); ranges represent the standard deviation on those replicate measurements

	Mass Fraction (mg/kg SRM)				
Sample	SRM 1549a Whole Milk Powder	SRM 1849a Infant/Adult Nutritional Formula	SRM 2383a Baby Food Composite	SRM 3233 Fortified Breakfast Cereal	SRM 3287 Blueberry (Fruit)
Thiamine	1.555±0.043	12.09±0.12	0.768±0.023	51.9±3.0	1.679±0.046
Riboflavin	9.66±0.45	20.4±2.3	0.597±0.051	76.5±2.8	nd[a]
Niacinamide	5.76±0.14	103.9±1.3	3.58±0.12	776±11	2.86±0.14
Niacin	nd[a]	nd[a]	1.789±0.091	16.53±0.52	nd[a]
Pantothenic Acid	34.82±0.88	67.3±1.3	1.586±0.047	500±22	3.36±0.30
Pyridoxamine	0.255±0.012	nd[a]	0.1587±0.0047	nd[a]	nd[a]
Pyridoxal	1.786±0.050	nd[a]	nd[a]	2.16±0.32	0.498±0.025
Pyridoxine	nd[a]	13.91±0.13	0.0517±0.0031	73.7±2.1	0.760±0.019

[a] Not determined

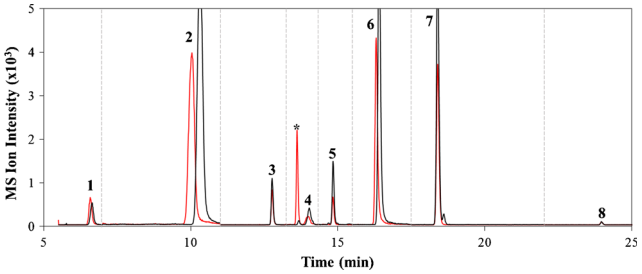

Fig. 3 LC/IDMS/MS separation and detection of water-soluble vitamins in SRM 2387 Peanut Butter. The dashed lines mark the MS time window for monitoring a given transition. Vitamin transitions are shown in the *black* traces, and stable isotope labeled internal standard transitions are show in *red* traces. (*1*) Pyridoxamine (*m/z* 169 → 152, *m/z* 172 → 155); (*2*) Niacin (*m/z* 124 → 80, *m/z* 128 → 84); (*3*) Thiamine (*m/z* 266 → 123, *m/z* 269 → 123); (*asterisk*) Unknown; (*4*) Pyridoxal (*m/z* 168 → 150, *m/z* 171 → 153); (*5*) Pyridoxine (*m/z* 170 → 152, *m/z* 174 → 156); (*6*) Niacinamide (*m/z* 123 → 80, *m/z* 127 → 80); (*7*) Pantothenic Acid (*m/z* 220 → 90, *m/z* 224 → 94); (*8*) Riboflavin (*m/z* 377 → 243, *m/z* 383 → 249)

standard deviation of the number of measurements for that matrix, as reported in ESM Table S3. The behavior of control samples was determined to be fit for purpose (see ESM Tables S4 through S6).

In addition to the values assigned using the LC/IDMS approach, 22 certified and 18 reference values have been assigned in 5 unfortified food-matrix SRMs using data obtained by the LC/IDMS/MS method. The precision of the values determined by LC/IDMS/MS is comparable to that of values determined by LC/IDMS, with RSDs of less than 10 % for most compounds. Exceptions include slightly higher RSDs for thiamine (12.4 %), riboflavin (13.8 %), niacin (11.7 %), and pyridoxine (12.6 %) in SRM 1546a Meat Homogenate, for pyridoxamine in SRM 1845a Whole Egg Powder (11.4 %), for riboflavin in SRM 2387 Peanut Butter (13.3 %), and for pyridoxine in SRM 3234 Soy Flour (26.7 %). The higher RSDs in the meat homogenate were likely a result of the low signal to noise for many of the compounds and may have been alleviated by using a larger sample size for extraction. Unique matrix components in the meat homogenate extract may have also been more abundant than in other matrices, resulting in reduced precision. The higher RSD for pyridoxamine in the egg powder was also related to low signal to noise, with the mass fraction for pyridoxamine one to three orders of magnitude lower than all other water-soluble vitamins in the egg powder. Low signal to noise was also the case for riboflavin in the peanut butter, with measured peak areas an order of magnitude below those of the other vitamins in that matrix. For pyridoxine in the soy flour, increased variability was observed with increasing extraction time and temperature throughout the method development and extraction optimization. A longer extraction time was necessary to fully extract some of the other vitamins from SRM 3234 Soy Flour, at the cost of slightly higher uncertainty in the value for pyridoxine.

A direct comparison of the LC/IDMS and LC/IDMS/MS methods is possible using data collected for water-soluble vitamins in SRM 1549a Whole Milk Powder. The entire raw data sets for thiamine, riboflavin, niacinamide, pantothenic acid, pyridoxamine, and pyridoxal are depicted in Tukey boxplots in Fig. 4a-f, respectively. These plots illustrate the distribution of the data within the method data set, where the top and bottom of the box represent the first and third quartiles, respectively, and the inner horizontal band represents the mean value for the data set. The top and bottom whiskers represent the limits of 1.5IQR (interquartile range) of the upper and lower quartiles, respectively. The LC/IDMS data was collected in a single batch, as duplicates from ten packets. The LC/IDMS/MS data was collected in three independent batches, each as duplicates from two packets prepared and analyzed on three separate occasions over a period of six months. For thiamine, niacinamide, and pyridoxamine, the overlap in the data sets is visually obvious indicating that the

Table 2 Mass fraction values for water-soluble vitamins in food Standard Reference Materials determined by LC/IDMS/MS. The number of samples analyzed is as given in ESM Table S3; ranges represent the standard deviation on those replicate measurements

	Mass fraction (mg/kg SRM)					
Sample	SRM 1549a Whole Milk Powder	SRM 1546a Meat Homogenate	SRM 1845a Whole Egg Powder	SRM 2384 Baking Chocolate	SRM 2387 Peanut Butter	SRM 3234 Soy Flour
Thiamine	1.357±0.074	0.685±0.085	1.532±0.092	1.74±0.14	0.614±0.012	8.08±0.43
Riboflavin	11.56±0.31	0.352±0.049	16.43±0.17	2.61±0.17	0.181±0.024	3.157±0.081
Niacinamide	6.06±0.44	38.2±1.6	1.421±0.030	1.357±0.075	3.023±0.072	10.78±0.26
Niacin	nd[a]	0.401±0.047	nd[a]	10.90±0.83	38.6±1.3	4.31±0.36
Pantothenic Acid	32.59±0.57	4.42±0.23	59.97±0.51	3.55±0.27	9.48±0.10	10.75±0.23
Pyridoxamine	0.263±0.016	0.272±0.026	0.0472±0.0054	0.1191±0.093	0.0864±0.0072	0.836±0.035
Pyridoxal	1.643±0.081	nd[a]	0.133±0.011	nd[a]	0.1135±0.0043	1.32±0.12
Pyridoxine	nd[a]	0.0446±0.0056	nd[a]	0.1289±0.0091	0.1175±0.0094	0.57±0.15

[a] Not determined

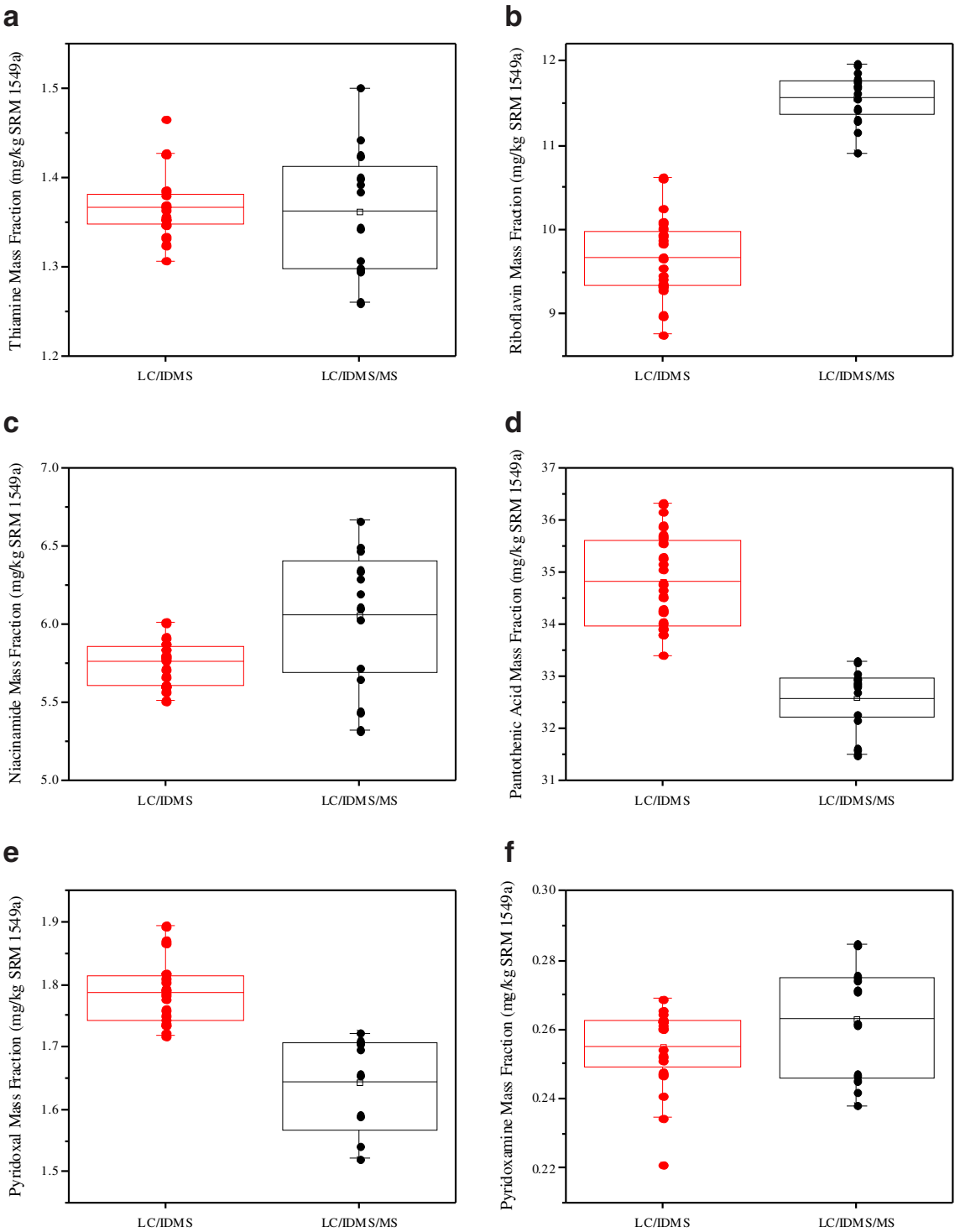

Fig. 4 Tukey boxplots depicting the data for water-soluble vitamins in SRM 1549a Whole Milk Powder as determined by LC/IDMS (*red*, N=10, analyzed in the same batch) and LC/IDMS/MS (*black*, N=6, analyzed in 3 batches). The top and bottom of the box represent the first and third quartiles, respectively. The band inside the box represents the mean value for the data set. The top and bottom whiskers represent the limits of 1.5IQR (interquartile range) of the upper and lower quartiles, respectively. **a** Thiamine; **b** Riboflavin; **c** Niacinamide; **d** Pantothenic acid; **e** Pyridoxamine; **f** Pyridoxal

two methods are providing equivalent data. For riboflavin, pantothenic acid, and pyridoxal, the data sets appear less consistent. In the case of riboflavin, the inclusion of an isotopically labeled internal standard for the LC/IDMS/MS analysis, which was not available when the LC/IDMS results were obtained, resulted in more accurate and precise results.

The cause of the slight differences in results for pantothenic acid and pyridoxal is not obvious, but inclusion of both data sets provides greater confidence in the trueness of the certified value, despite the resulting wider uncertainty. For each of these analytes, the certified value was calculated as the mean of the mean value for each method.

Conclusions

Improvements in methods for determination of water-soluble vitamins in foods and dietary supplements have addressed needs for increased throughput, increased selectivity, and sufficient accuracy and precision, but no method has addressed all of these needs simultaneously. The method described here allows increased throughput by allowing simultaneous determination of eight water-soluble vitamins, allows increased selectivity by identifying multiple forms of vitamins B_3 and B_6 that are commonly found in unfortified foods, and provides for improvements in accuracy and precision of methods through incorporation of IDMS as well as the generation of quality control materials available for use in any laboratory. Results obtained using this method were consistent with data obtained from collaborating laboratories using a variety of methods, and an extraction protocol has been identified for a variety of foods that can be translated to an experimental starting point for extraction of water-soluble vitamins from nearly any sample matrix. This method will be used in the future for assigning values for water-soluble vitamins to other food and dietary supplement SRMs prepared by NIST.

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