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Evaluation of Flow-Injection Tandem Mass Spectrometry for Rapid and High-Throughput Quantitative Determination of B Vitamins in Nutritional Supplements

Deepak Bhandari and Gary J. Van Berkel*

Organic and Biological Mass Spectrometry Group, Chemical Sciences Division, Oak Ridge National Laboratory (ORNL), Oak Ridge, Tennessee 37831-6131, United States

Supporting Information

ABSTRACT: The use of flow-injection electrospray ionization tandem mass spectrometry for rapid and high-throughput mass spectral analysis of selected B vitamins, viz., B_1, B_2, B_3, B_5, and B_6, in nutritional formulations was demonstrated. A simple and rapid (~5 min) in-tube sample preparation was performed. Automated flow injection introduced 1 μL of the extracts directly into the mass spectrometer ion source without chromatographic separation. Sample-to-sample analysis time was 60 s. Quantitative capabilities of the flow-injection analysis were tested using the method of standard additions and SRM 3280. The quantity determined for each B vitamin in SRM 3280 was within the statistical range provided for the respective certified values. This approach was also applied to two different commercial vitamin supplement tablets and proved to be successful in the quantification of the selected B vitamins, as evidenced by an agreement with the label values and the results obtained using isotope dilution liquid chromatography/mass spectrometry.

KEYWORDS: *Solid−liquid extraction, flow injection, tandem mass spectrometry, electrospray ionization, B vitamins, quantitative analysis, standard addition*

INTRODUCTION

Vitamins are organic micronutrients that have diverse biochemical functions, including protein and mineral metabolism, regulation of cell and tissue growth and differentiation, and prevention of oxidative damage to tissue and organs.[1] On the basis of their chemical and biological activities, vitamins are classified into two categories: fat-soluble vitamins (FSVs) and water-soluble vitamins (WSVs). FSVs include vitamins A, D, E, and K, whereas WSVs include B vitamins (B_1, B_2, B_3, B_5, B_6, B_7, B_9, and B_{12}) and vitamin C. Because not all vitamins are biosynthesized adequately by an organism, they are usually consumed via fortified food or dietary supplements. However, excessive consumption of vitamin formulations is not safe, because it may result in vitamin poisoning or hypervitaminosis.[2] The Food and Nutrition Board of the Institute of Medicine periodically assesses the dietary reference intakes (DRIs) of vitamins and minerals intended for general public and health professionals to ensure that their optimal nutrient needs are satisfied.[3]

Several analytical methodologies have been developed for the quantification of B vitamins in food matrices. Official analytical methods are based on microbiological assays (MBAs) developed by the Association of Analytical Communities (AOAC) and have been established for more than 30 years.[4] These methods are rigorous and time-consuming (up to 72 h for the incubation of a food extract with the growth medium and microbiological culture).[4,5] MBAs for B vitamin analysis were surpassed by relatively faster liquid chromatographic approaches. Initially, chromatography was hyphenated with ultraviolet (UV),[6−11] fluorescence,[12,13] or coulometric detection system.[14] More recently, liquid chromatography/mass spectrometry (LC/MS) has been considered a preferred mode for the quantitative analysis of B vitamins because of high chemical specificity and information-rich content of mass spectral detection. In particular, Chen and Wolf have performed extensive studies on the quantification of B vitamins using LC/MS^n (n = 1 and 2),[11,15−19] as have others.[20−27] Despite LC being the preferred technique of sample introduction for the mass spectral detection of B vitamins in food and nutritional supplements, it is also a sample throughput-limiting step. Including system equilibration time, LC typically requires 25−45 min per injection for the analysis of the selected B vitamins under study. Also, solvent compatibility for LC and MS adds another level of complexity in sample preparation and analysis. Furthermore, the cost of solvents consumed during a typical LC separation and their disposal and the chromatographic columns cannot be neglected. Therefore, development of a faster and more cost-effective analytical technique is of significant interest in the food industry sector for high-throughput monitoring of vitamin contents in raw materials during food processing, packaging, storage, or verifying regulatory compliance prior to market delivery.

Flow injection is a simple alternative to LC for the mass spectrometric sample introduction. This technique has been used in MS mainly for high-throughput qualitative screening of metabolites, bacteria, and several other targets.[28−30] In this

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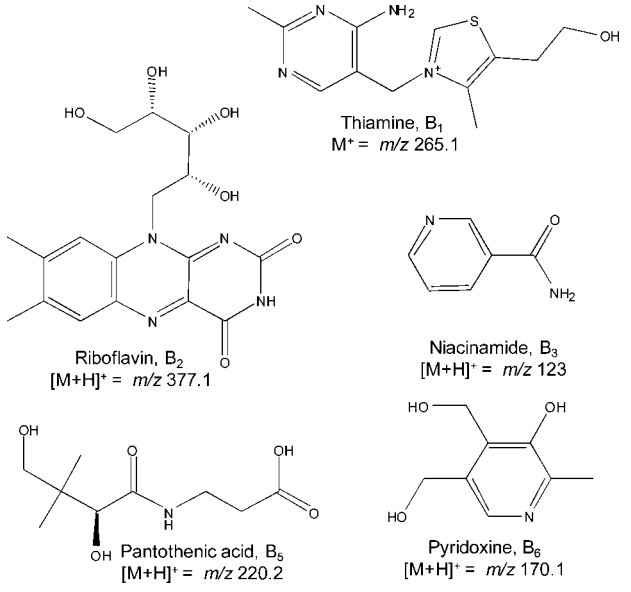
paper, a rapid solid−liquid extraction followed by automated flow injection of samples is investigated for high-throughput electrospray ionization tandem mass spectrometry (ESI−MS/MS) quantification of selected B vitamins in National Institute of Standards and Technology (NIST) standard reference material (SRM 3280) multivitamin/multielement tablets and two over-the-counter vitamin formulations. The quantitative results of this approach are compared to NIST certified values, label values, and that of isotope dilution liquid chromatography/mass spectrometry (ID−LC/MS) analyses of the same materials.

MATERIALS AND METHODS

Materials. SRM 3280 multivitamin/multielement tablets were purchased from NIST (Gaithersburg, MD). Each SRM 3280 tablet contained both WSVs and FSVs and mineral constituents as well as other excipients required for the stability and/or coating of similar commercial formulations.[11] Two other commercial nutritional supplements, (a) a multivitamin/multielement tablet, termed sample "X" and (b) a vitamin B complex tablet, termed sample "Y", were purchased over-the-counter locally. Analytical standard grade thiamine hydrochloride (vitamin B_1), riboflavin (vitamin B_2), niacinamide (vitamin B_3), pantothenic acid hemicalcium salt (vitamin B_5), and pyridoxine hydrochloride (vitamin B_6) were purchased from Sigma-Aldrich (St. Louis, MO). Thiamine chloride-[$^{13}C_3$], niacinamide-[$^{2}H_4$], calcium pantothenate monohydrate-[$^{13}C_3$, ^{15}N], and pyridoxine hydrochloride-[$^{13}C_4$] were purchased from Cambridge Isotope Laboratories (Andover, MA), and riboflavin-[$^{13}C_4$, $^{15}N_2$] was obtained from Sigma-Aldrich (St. Louis, MO). The chemical structures of selected B vitamins and the mass-to-charge ratios of the precursor ions detected are shown in Scheme 1.

Scheme 1. Chemical Structures and Mass-to-Charge Ratios Observed for the B Vitamins Studied Using Flow-Injection ESI−MS/MS



Calibration Solutions. Stock solutions of thiamine hydrochloride, riboflavin, niacinamide, and pyridoxine hydrochloride were prepared in 50:50 (v/v) methanol/5 mM aqueous hydrochloric acid mixtures, while pantothenic acid hemicalcium salt was prepared in 50:50 (v/v) methanol/water. For ID−LC/MS analyses, all stock solutions, including internal standards, were prepared in 100:1 (v/v) water/acetic acid (AA) solutions. All stock solutions were stored at −20 °C prior to their use.

Sample Preparation for Flow Injection. The experimental overview of the solid−liquid extraction method employed for flow-injection tandem mass spectrometry is illustrated in Figure 1. The sample preparation procedure was developed using NIST SRM 3280 multivitamin/multielement tablets as a model material. These tablets are produced by NIST for use as controls in laboratories that produce dietary supplements. NIST assigned certified and reference values for more than 13 vitamins, 2 carotenoids, and 24 elements present in SRM 3280.[31] For the sample preparation, 15 tablets (approximate weight of each SRM 3280 tablet was 1.5 g) were ground to a fine powder using a porcelain mortar and pestle. Approximately 0.1 g of the powdered sample was weighed and transferred into a 15 mL centrifuge tube. Extraction solvent A [10 mL of 50:50 (v/v) methanol/5 mM aqueous hydrochloric acid at pH ∼2.85] was added to the centrifuge tube for the extraction of thiamine, riboflavin, niacinamide, and pyridoxine hydrochloride because these vitamins are stable in acidic solutions. Using a separate 0.1 g sample aliquot, solvent B with pH ∼5.5 (10 mL of 50:50 (v/v) methanol/water) was used for the extraction of calcium pantothenate because pantothenic acid hydrolyzes to pantoic acid and β-alanine below pH 4 and above pH 7.[32] The powdered sample aliquot and extraction solvent were first vortexed for 1 min and then centrifuged for 1 min at 5752*g*. The supernatant was transferred to a 500 μL Ultrafree-MC centrifugal filter unit (Millipore, Billerica, MA) and centrifuged for another 1 min at 23008*g*. Alternatively, the supernatant from a 15 mL centrifuge tube was filtered through a 0.45 μm porosity membrane. Finally, the filtrate or eluate was diluted with 50:50:0.1 (v/v/v) methanol/water/formic acid (FA) for standard addition experiments. Overall, sample preparation required 5 min or less and could be multiplexed.

Flow-Injection ESI−MS/MS Analysis. Automated flow injection was performed using an Agilent 1100 series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA). The HPLC system (column compartment was bypassed to allow for direct injection of samples to the mass spectrometer) was coupled to a Thermo Scientific LTQ XL (Thermo Scientific, West Palm Beach, FL) linear ion-trap mass spectrometer equipped with an ESI source operated in positive-ion mode. The 1 μL injection volume was loaded to and dispensed from a syringe at the rate of 20 μL/min. The flow rate of the carrier solvent 50:50:0.1 (v/v/v) methanol/water/FA was 100 μL/min. The mass spectrometer was operated in ESI−MS/MS full-scan product ion mode. Optimized instrumental parameters were as follows: ESI voltage of 4 kV, sheath gas flow of 40 (arbitrary units), auxiliary gas flow of 10 (arbitrary units), capillary temperature of 275 °C, normalized collision energy of 35%, and m/z isolation width of 1.5 Da. The maximum ion injection time for MS/MS analysis was set to 100 ms with automatic gain control on.

ID−LC/MS Analysis. Sample preparation and liquid chromatographic separation for the quantification of B vitamins was adapted from the work of Phinney et al.[27] For multivitamin/multielement tablet SRM 3280 and sample "X", 0.25 g of ground sample, 22.5 mL of 100:1 (v/v) water/AA, and 1.5 mL of each internal standard were added to a 50 mL centrifuge tube (total volume = 30 mL). In the case of sample "Y" (vitamin B complex), a 0.025 g tablet sample aliquot was used because of the low solubility of riboflavin. The contents in the centrifuge tube were vortexed for 0.5 min and sonicated for another 30 min without heat. The tube was then centrifuged at 3000 rpm for 15 min. The supernatant was then filtered through a 0.45 μm porosity filter membrane for ID−LC/MS analysis. The overall time required for the sample preparation was approximately 50 min. The liquid chromatographic separation used a Cadenza CD-C18 stationary phase column (4.6 × 250 mm, 3 μm particles) from Imtakt USA (Philadelphia, PA). Analyses were performed using an Agilent 1100 series HPLC system coupled to a Thermo Scientific LTQ XL linear ion-trap mass spectrometer with an ESI source in positive-ion mode. Mass spectral parameters were the same as those used for flow-injection analysis. Mobile phases A, 20 mM aqueous ammonium formate (pH 4), and B, methanol, were programmed for gradient elution in the following order: isocratic at 100% A (0−6 min), linear gradient from 100 to 50% A (6−20 min), isocratic at 50% A (20−30 min), linear gradient from 50 to 100% A (30−35 min), with

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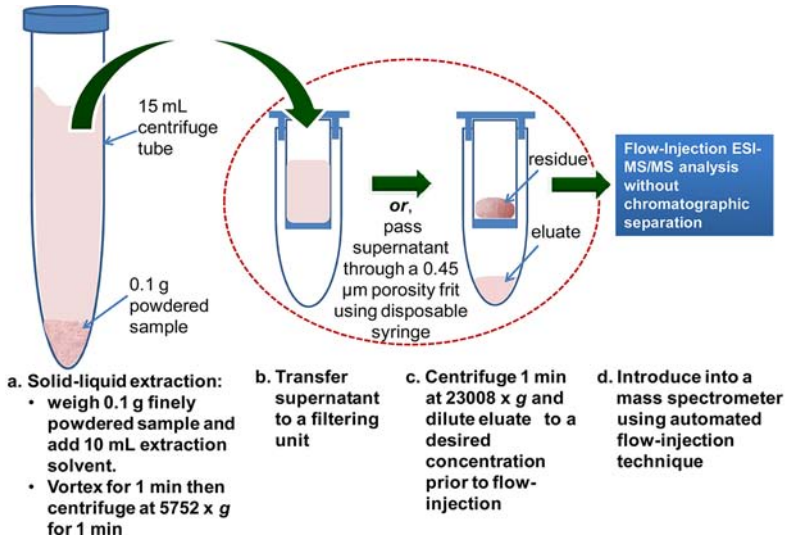

Figure 1. Experimental workflow for the extraction/detection of B vitamins in NIST SRM 3280 multielement/multivitamin tablets.

equilibration at 100% A (35−45 min). The injection volume was 2 μL. The flow rate was 300 μL/min. The LC column was thermostatted at 22 °C.

RESULTS AND DISCUSSION

Optimization of Flow Injection. The flow-injection ESI−MS/MS conditions for the quantification of all selected vitamins were optimized using respective 1 μg/mL standard solutions in 50:50:0.1 (v/v/v) water/methanol/FA and 100:0.1 (v/v) methanol/FA, 100:0.1 (v/v) water/FA, or 50:50:0.1 (v/v/v) water/methanol/FA as the potential carrier solvent systems. Using 100:0.1 (v/v) methanol/FA resulted in the highest signal levels but produced broad product ion peak profiles in some cases. In contrast, employing 100:0.1 (v/v) water/FA resulted in the lowest signal level but generated better peak profiles. As a compromise, 50:50:0.1 (v/v/v) water/methanol/FA was chosen as the carrier solvent. Because the injection volume is directly proportional to the amount of sample matrix introduced into the ion source, the former was kept as low as possible (1 μL) while generating good peak profiles. In addition, matrix interference in flow injection was minimized by the dilution of the sample extract. In this study, sample extracts were diluted at least 100-fold prior to flow injection, depending upon the concentration of analyte in the sample.

To demonstrate the reproducibility of flow injection, the MS/MS product ion chronogram obtained for vitamin B_6 (estimated concentration of 1 μg/mL) during 20 sequential 1 μL injections of a SRM 3280 extract over 22 min is shown in Figure 2a. In addition, Figure 2b shows a representative product ion peak (zoomed in from Figure 2a), exhibiting a signal flow-injection peak profile. For these 20 sequential injections, the relative standard deviation (RSD) of the signal of vitamin B_6 was 4.2% using the peak areas on the ion chronogram.

Method Validation Using NIST SRM 3280 Multivitamin/Multielement Tablets. SRM 3280 multivitamin/multielement tablets were used for the validation of the flow-injection method. A standard addition method, i.e., continuous variation of the standard at a constant total volume, as described by Morris Bader, was employed for the estimation of the unknown concentration of the vitamin samples (c_x) using the following mathematical expression:[33]

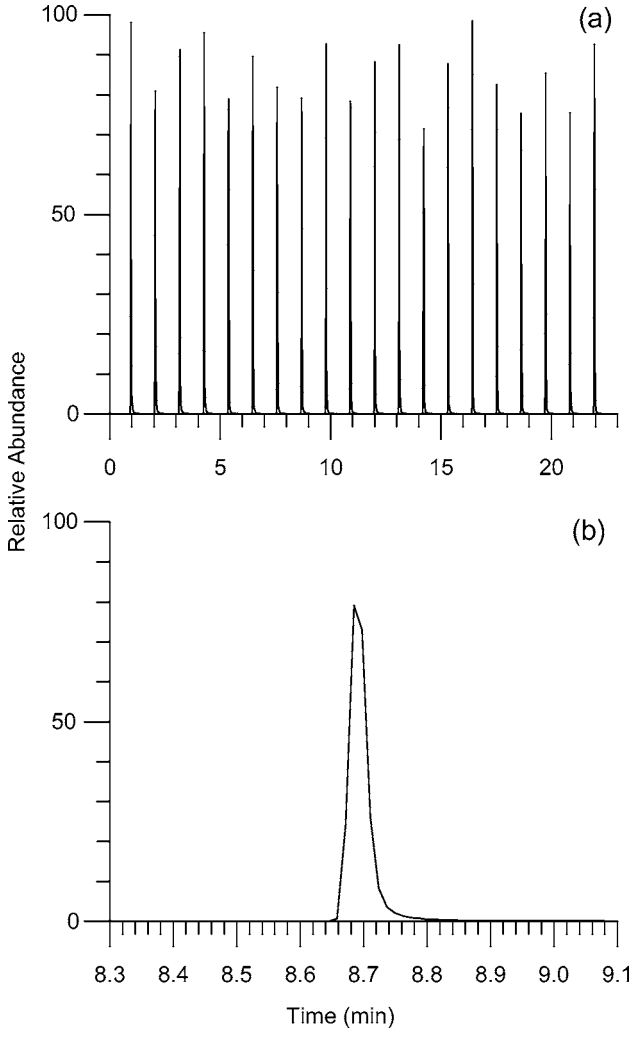

Figure 2. (a) Flow-injection MS/MS ion chronogram of m/z 170.1 → 152 for 20 sequential 1 μL injections of NIST SRM 3280 extract containing approximately 1 μg/mL pyridoxine hydrochloride. The estimated RSD from the peak area is 4.2%. (b) Representative peak profile, zoomed in from panel a. The sample-to-sample analysis time was approximately 1 min.

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$$c_x = \frac{ac_sV_s}{mV_x} \quad (1)$$

where a is the y intercept of the calibration curve obtained from the standard addition method (see the Supporting Information for details), V_s is the fixed unit volume of the standard solution, which was added in its integer multiple to a sample, c_s is the concentration of the standard solution, m is the slope of the calibration curve, and V_x is the volume of the unknown sample. Peak areas of respective product ions were integrated and plotted against the number of standard additions (N), running from 0 to 5.

Table 1 shows respective m/z values used for the quantification of the selected B vitamins. Precursor and product ions were selected on the basis of observed peaks in direct infusion of respective standard solutions. The product ion spectra of all selected B vitamins generated via collision-induced dissociation (CID) are shown in Figure 3. Full-scan mass spectra of vitamin B_1 exhibited its quaternary ammonium ion at m/z 265.1, while the base peak in the product ion spectrum was the pyrimidinic ring at m/z 122.1. Vitamins B_2 and B_3 produced protonated molecular ions $[M + H]^+$ at m/z 377.1 and 123.0, respectively. The product ion of vitamin B_2 at m/z 243.1 was due to the loss of ribitol. Vitamin B_3 generated a product ion peak corresponding to the pyridinic ring at m/z 80. Because vitamin B_5 was fortified as calcium pantothenate in nutritional formulation, it forms pantothenic acid in aqueous solution and appeared in protonated form at m/z 220.2. Similarly, vitamin B_6 appeared as protonated pyridoxine ion at m/z 170.1. Vitamins B_5 and B_6 produced product ion peaks at m/z 202.1 and 152.0, respectively, corresponding to the loss of water from protonated pantothenic acid and protonated pyridoxine, respectively.

Table 1. Mass-to-Charge Ratios of Precursor and Product Ions Used for the Quantification of B Vitamins

vitamin	precursor ion (m/z)	product ion (m/z)
thiamine hydrochloride, B_1	265.1	122.1
riboflavin, B_2	377.1	243.1
niacinamide, B_3	123.0	80.0
calcium pantothenate, B_5	220.2	202.2
pyridoxine hydrochloride, B_6	170.1	152.0

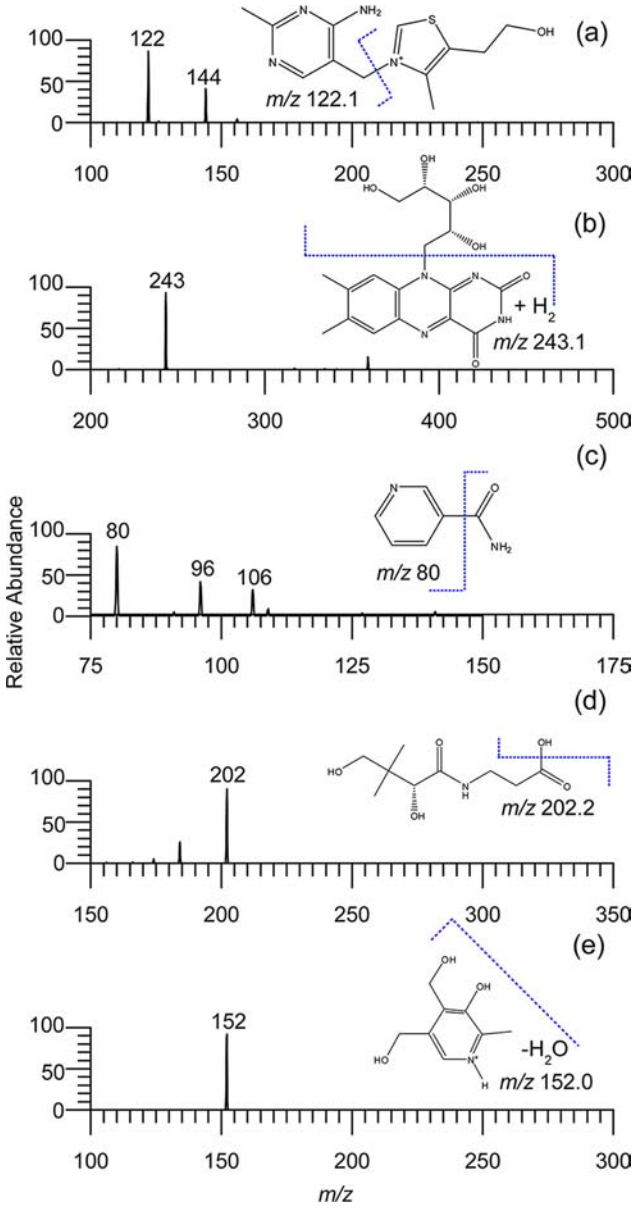

Figure 3. Product ion scan mass spectra and corresponding MS/MS fragmentation pathway for (a) thiamine (vitamin B_1), (b) riboflavin (vitamin B_2), (c) niacinamide (vitamin B_3), (d) pantothenic acid (vitamin B_5), and (e) pyridoxine (vitamin B_6).

When eq 1 was employed for results obtained for three weighed sample aliquots with three replicate injections per sample, the concentration of the B vitamins in SRM 3280 was calculated (see the Supporting Information for a representative mathematical calculation). These concentrations along with NIST certified values[11] and results of ID−LC/MS analyses accomplished by our group, Chen et al.,[15] and Phinney et al.[27] are graphically summarized in Figure 4a (also see Table S2 of the Supporting Information for numeric values). There was good agreement between values obtained with flow-injection ESI−MS/MS and those obtained with the other methods for all selected B vitamins (note that riboflavin was not measured by Chen et al.,[15] and therefore, no comparison could be made).

Quantification of B Vitamins in Commercial Dietary Supplement Tablets. The same flow-injection ESI−MS/MS method validated for the quantification of selected B vitamins in NIST SRM 3280 was implemented for commercial vitamin tablets. Samples from two different commercial brands were selected for the study. Sample X was a multivitamin/multielement tablet, with B vitamin content comparable to SRM 3280, while sample Y was a vitamin B complex with a higher concentration of B vitamins than SRM 3280. The solid−liquid extraction procedure and other instrumental parameters employed for both commercial samples were similar to those used in the analysis of SRM 3280. However, only a 0.01 g aliquot of sample Y was used because of the anticipated higher concentration of less soluble riboflavin (vitamin B_2). Also, note that vitamin B_1 in both samples X and Y was fortified as thiamine mononitrate rather than the hydrochloride salt, as was the case in SRM 3280. The mononitrate salt of vitamin B_1 was quantified using thiamine hydrochloride standard, and the results were converted to a mass equivalent for the mononitrate salt. Our results indicated up to 40% higher amount of B vitamins in these commercial vitamin samples than their label value (see panels b and c of Figure 4 and also Table S3 of the

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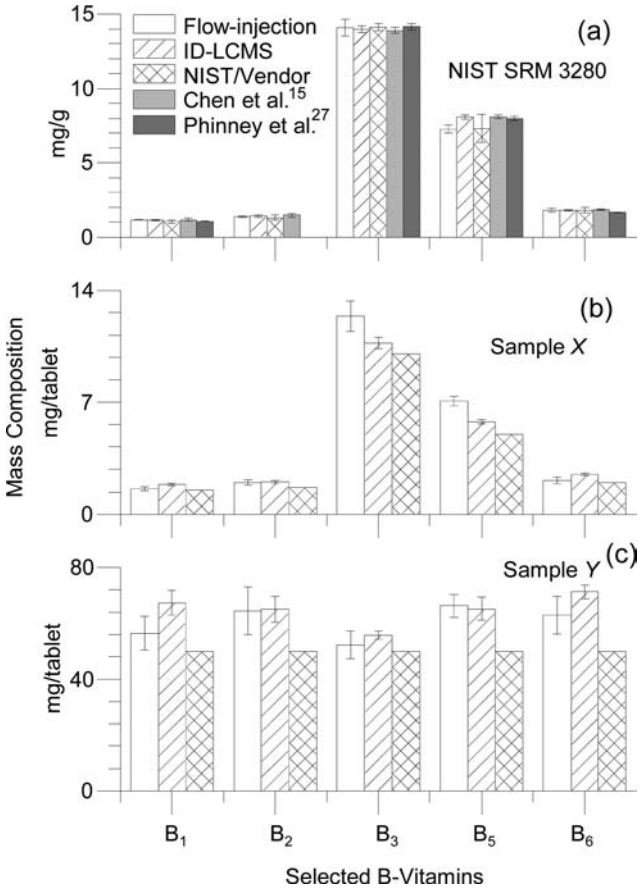

Figure 4. Composition of (a) NIST SRM 3280, (b) commercial multivitamin/multielement (sample "X"), and (c) vitamin B complex (sample "Y") tablets using flow-injection (white bars), ID−LC/MS (hatched bars), and NIST certified values or vendors' label (cross-hatched bars). Gray and dark bars represent work by Chen et al.[15] and Phinney et al.,[27] respectively. The average weight of each SRM 3280, sample X, and sample Y tablet was 1.5, 1.96, and 0.48 g, respectively. Error values in our study represent the standard deviation of three different sample extracts with triplicate injections for each sample.

Supporting Information). For this reason, an additional ID−LC/MS analysis of these tablets was performed to confirm this result. Similar to the flow-injection results, B vitamin contents of both samples obtained by ID−LC/MS were higher than the label values (panels b and c of Figure 4). In addition, taking into account the statistical errors, there was a significant overlap in the results obtained via flow injection and ID−LC/MS for both samples "X" and "Y", except for a slight discrepancy in vitamin B_3 and B_5 content in sample "X". A similar deviation was observed in SRM 3280 for vitamin B_5 (see Figure 4a). However, in SRM 3280, the variation in results obtained via flow injection and ID−LC/MS was within the wide statistical error in the NIST certified value. In contrast, statistical errors of B vitamin content were not provided for either commercial sample. Nonetheless, our results suggest that the B vitamins in both over-the-counter nutritional supplements were (truly) higher than the provided label values. Overfortification of B vitamins in food and nutritional supplements, a common practice in food industries to ensure proper ingredient levels throughout the shelf life of the product, may account for this observation.[34]

Flow Injection versus ID−LC/MS for High-Throughput Sample Analysis. As stated earlier, all three different vitamin tablets under study were analyzed via both flow injection and ID−LC/MS and proved to be successful in the quantitative determination of selected B vitamins. With ID−LC/MS, all five B vitamins were analyzed simultaneously. On the other hand, in a flow-injection ESI−MS/MS analysis, the number of analytes that could be measured in one injection was determined by physical and chemical properties of the targeted analytes (e.g., individual solubilities, sensitivities for degradation, concentrations, etc.) and by instrumental parameters, such as ion injection time, scan speed, and acquisition time. Under our flow-injection experimental conditions, one determination of the five selected B vitamins required two injections at each standard addition. Four B vitamins, viz., B_1, B_2, B_3, and B_6, extracted in 50:50 (v/v) methanol/5 mM aqueous hydrochloric acid were quantified during one injection, while B_5 extracted in 50:50 (v/v) methanol/water was measured in a separate injection. Injection-to-injection time was approximately 1 min. For the quantitative analysis using the method of standard additions, 36 injections were made, i.e., 6 samples with 3 injections for the 2 different extracts per sample, over a 40 min time course. In contrast, the NIST certified ID−LC/MS method employing stable isotope internal standards first required an external calibration curve to be obtained, which took several hours to acquire. After that, an extract was injected every 45 min, resulting in a sample analysis time of 135 min, considering 3 replicate analyses per extract. That means that an analysis of the samples using the flow-injection technique was approximately 3−3.5 times faster than using ID−LC/MS, not even considering the time necessary to create a calibration curve used for the latter. In addition, eliminating the need of stable isotopes of B vitamins and a chromatographic column made the flow-injection method more economical than ID−LC/MS.

Limit of Detection (LOD) and Limit of Quantification (LOQ) using Flow Injection. Because blank samples were not available for the three investigated solid tablets, the LOD and LOQ values of selected B vitamins were estimated using the standard addition method. Table 2 summarizes the predicted values for all selected vitamins in the different sample matrices. In this study, the LODs and LOQs of B vitamins in sample Y (vitamin B complex) were lower than those determined using data from analyses of SRM 3280 and sample X (commercial multivitamin/multielement supplement). This was most likely due to less matrix effect, resulting from the 100 times greater dilution of the sample Y extract than that of the other two samples. Note that the total content of B vitamins in sample Y was much higher than the other two samples, and hence, for the

Table 2. Detection and Quantification Limits of B Vitamins

	NIST SRM 3280		sample X		sample Y	
vitamin	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)
thiamine hydrochloride, B_1	5.2	17.3	1.4	4.6	0.04	0.13
riboflavin, B_2	25.5	85.0	22.5	75.0	0.24	0.80
niacinamide, B_3	48.2	160.6	13.0	43.3	9.9	33.0
calcium pantothenate, B_5	16.9	56.3	13.9	46.4	3.01	10.05
pyridoxine hydrochloride, B_6	17.6	58.7	4.2	14.0	0.04	0.13

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best estimation of LOD and LOQ, the extract of sample Y was diluted more than those of SRM 3280 and sample X.

In conclusion, the utility of flow-injection ESI−MS/MS has been systematically evaluated for rapid and high-throughput surveillance of B vitamins in nutritional formulations. The results presented showed that solid−liquid extraction followed by flow-injection ESI−MS/MS can quantitatively detect B vitamins with milligram per gram concentrations in nutritional supplements without any chromatographic separation. It was also observed that LODs of B vitamins down to the sub-nanogram per gram level could be achieved, depending upon the chemical nature of vitamins and sample matrices. The quantitative values obtained with this analytical approach were in good agreement with published data and our own ID−LC/MS measurements. Moreover, the analyses were 3−3.5 times faster than the chromatographic approach and more economical by eliminating the need of costly isotope-labeled standards and a chromatographic column. Therefore, flow-injection ESI−MS/MS is a viable technique for quick assessment of B vitamins in dietary formulations and should be evaluated for the determination of other vitamins in similar matrices for food safety and quality purposes. In general, the results of this study show that flow-injection tandem mass spectrometry has tremendous potential for the high-throughput quantitative determination of target analytes in complex matrices and, therefore, warrants further study in broader analytical applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Representative mathematical calculation for the standard addition method and numerical values obtained for B vitamins in our study compared to the results of others in a tabular format. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: 865-574-1922. Fax: 865-576-8559. E-mail: vanberkelgj@ornl.gov.

Notes

The authors declare no competing financial interest.

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