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Rapid Method for the Determination of Thiamine and Pantothenic Acid in Infant Formula and Milk-Based Nutritional Products by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

Background: Thiamine and pantothenic acid play a critical role in numerous metabolic reactions and are typically supplemented in infant and adult nutritional formulas as thiamine chloride hydrochloride and calcium pantothenate salts. **Objective:** A rapid compliance method for the analysis of thiamine and pantothenic acid applicable to infant formula and milk-based nutritional products is described. **Method:** Proteins are removed by centrifugal ultrafiltration, followed by analysis by reversed-phase liquid chromatography–tandem mass spectrometry (LC-MS/MS), with quantitation accomplished by internal standard technique. **Results:** The method was shown to be accurate, with acceptable recovery (thiamine, 99.3–101.1%; pantothenic acid, 99.2–108.6%). A certified reference material (NIST 1849a), showed no statistical bias ($\alpha = 0.05$) for thiamine ($P = 0.64$); although a statistically significant bias ($P = 0.01$) for pantothenic acid was found, the nominal bias was only 4.7% (mean = 7.1 mg/hg; certified value = 6.8 mg/hg). A comparison of results by LC-MS/MS and current methods showed negligible bias (mean bias: thiamine, 0.01 mg/hg; pantothenic acid, 0.17 mg/hg) and no statistical significance ($\alpha = 0.05$; thiamine, $P = 0.399$; pantothenic acid, $P = 0.058$). Acceptable precision was demonstrated with a repeatability of 7.2% repeatability relative standard deviation (RSD_r) (HorRat: 0.6) and an intermediate precision of 7.0% RSD for thiamine, and a repeatability of 5.7% RSD_r (HorRat: 0.5) and an intermediate precision of 6.1% RSD for pantothenic acid. **Conclusions:** This rapid method is intended

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for use in high-throughput laboratories as part of routine product compliance release testing of thiamine and pantothenic acid in manufactured infant and milk-based nutritional products.

Introduction

Thiamine (vitamin B₁) is a water-soluble vitamin and is present in foods and tissues as free thiamine or as mono-, pyro-, and tri-phosphorylated forms. In its biologically active form as thiamine pyrophosphate, thiamine is a cofactor in several important metabolic pathways in the formation of carbohydrates and amino acids. As it is a water-soluble vitamin, thiamine is not stored in the body and the excess is excreted in urine. Thiamine is found in various foods, including wholegrain bread and cereals, peas, beans, nuts, brown rice, and meat, and is absent in highly purified cereal products (1). In bovine milk, endogenous vitamin B₁ is produced by rumen microorganisms and is primarily found as free thiamine, with lower levels of phosphorylated and protein-bound vitamin, with typical concentrations of 15–40 µg/dL (2, 3). International regulatory limits for thiamine in infant formula are set at a 60 µg/100kcal minimum (325 µg/hg) and a guidance upper limit of 300 µg/100kcal (1625 µg/hg) (4).

Pantothenic acid (vitamin B₅) is a water-soluble vitamin and is an integral component of coenzyme A and acyl-carrier protein, which perform multiple roles within intermediate cellular metabolism. Pantothenic acid is found in foods, including bovine milk, predominantly in its free form, although it is also present at lower levels in its bound forms (1). International regulatory limits for pantothenic acid in infant formula are set at a 400 µg/100kcal minimum (2167 µg/hg) and a guidance upper limit of 2000 µg/100kcal (10833 µg/hg) (4).

As the UV absorbance of thiamine is low, fluorescence detection after oxidation of thiamine to thiochrome by alkaline hexacyanoferrate provides improved sensitivity. This technique is used in AOAC Official Method 986.27 (5) for the analysis of thiamine in infant formulas, and this oxidation step has been incorporated in many HPLC methods, in both pre-column and post-column modes, for the analysis of thiamine in foods (6–9). LC-MS methods for thiamine with other B-group vitamins have been developed more recently (10, 11), including a method that has become an AOAC Final Action method (AOAC 2015.14; 12).

The conventional method to determine pantothenic acid is a microbiological assay (MBA) using *Lactobacillus plantarum*, and this technique is an Official Method (AOAC 992.07) for the determination of pantothenic acid in infant formula (13). However, MBA methods are time consuming, suffer from poor precision, and are prone to interference in complex food matrixes.

Enzyme-linked immunosorbent assays (14, 15) and a radioimmunoassay method (16) have been developed as sensitive and less labor-intensive alternatives to MBA. A surface plasmon resonance

biosensor immunoassay has also been developed for the quantification of free pantothenic acid in foods (17). The lack of a strong chromophore results in chromatography with low specificity and sensitivity, with such methods suitable for fortified foods only (18). An LC method with post-column derivatization and fluorescence detection that overcomes the limitations in LC-UV methods has been reported (19). More recently, LC-MS methods for the determination of pantothenic acid in foods (20, 21) have been developed, including an AOAC Method (AOAC 2012.16; 22).

LC-MS is rapidly becoming the technique of choice for the analysis of B-group vitamins in foods, with many methods being applied to the simultaneous analysis of two or more of these vitamins (11, 23–25). The purpose of this study was to develop a rapid method with minimal sample preparation for the simultaneous analysis of thiamine and pantothenic acid in infant formulas and milk-based nutritional products. LC-MS/MS offers a number of advantages over other methods, including accuracy afforded from the use of stable isotope-labelled internal standards and specificity accruing from multiple reaction monitoring (MRM). Method performance was evaluated using a wide range of infant formulas, and was validated in accordance with single-laboratory validation procedures endorsed by the AOAC Expert Review Panel (26).

Method

Apparatus

- (a) HPLC system.—Nexera X2 UHPLC system consisting of two LC-30AD pumps, an SIL-30AC autosampler, a CTO-20AC column oven, a CBM-20A control module, and a DGU-20A5R degasser unit (Shimadzu, Kyoto, Japan) or equivalent.
- (b) Mass spectrometer.—6500 QTrap triple quadrupole detector with Analyst software version 1.6 (Sciex, Foster City, CA) or equivalent.
- (c) HPLC column.—Luna PFP 100 × 2.0 mm, 3 μm (Phenomenex, Torrance, CA) or equivalent.
- (d) Centrifuge.—Heraeus Multifuge X3 centrifuge (ThermoFisher, Waltham, MA) or equivalent.
- (e) Analytical balance.—Mettler-Toledo (Columbus, OH) AE 260 analytical delta range (± 0.1 mg) or equivalent, calibrated with National Institute of Standards and Technology (NIST; Gaithersburg, MD) traceable calibration weights.
- (f) Vortex mixer.—Genius 3 (IKA, Wilmington, NC) or equivalent.
- (g) UV spectrophotometer.—UV-1800 (Shimadzu, Kyoto, Japan) or equivalent.
- (h) Micropipettes.—Eppendorf Research plus, 20, 200, and 1000 μL (Hauppauge, NY) or equivalent.
- (i) Centrifugal ultrafiltration tubes.—4 mL, 3 kDa (Merck Millipore, Carrigtwohill, Cork, Ireland).
- (j) Syringes.—3 mL Luer-lock (Hapool, Shandong, China) or equivalent.

- (k) Syringe filters.—Nylon, 0.2 µm pore size 13 mm id (Merck Millipore, Carrigtwohill, Cork, Ireland) or equivalent.
- (l) Centrifuge tubes.—Polypropylene, 50 mL (ThermoFisher, Waltham, MA) or equivalent.
- (m) pH meter.—Orion SA520 (ThermoFisher, Waltham, MA) or equivalent.
- (n) Graduated cylinders.—1000 mL.
- (o) Volumetric flasks.—10, 25, 50, and 100 mL.
- (p) Reagent bottles.—500 and 1000 mL.
- (q) HPLC injection vials.—Amber, 1 mL with Teflon-coated caps.

Reagents

- (a) Formic acid (CHOOH).—LC-MS grade or equivalent.
- (b) Acetic acid (CH₃COOH).—Reagent grade or equivalent.
- (c) Ammonium formate (NH₄COO).—LC-MS grade or equivalent.
- (d) Ammonium acetate (NH₄CH₃COO).—Reagent grade or equivalent.
- (e) Methanol (CH₃OH).—LC-MS grade or equivalent.
- (f) Hydrochloric acid (HCl).—0.1 M pre-standardized Titri-Packs.
- (g) Water (H₂O).—HPLC grade or equivalent.
- (h) Thiamine hydrochloride.—Purity ≥ 99.0% (Sigma-Aldrich, St. Louis, MO) or equivalent.
- (i) Calcium pantothenate.—Purity ≥ 99.0% (Sigma-Aldrich, St. Louis, MO) or equivalent.
- (j) ¹³C₄-thiamine hydrochloride.—Purity ≥ 95.0%, isotope ≥ 95.0% (IsoSciences, King of Prussia, PA) or equivalent.
- (k) ¹³C₃¹⁵N₁-calcium pantothenate.—Purity ≥ 95.0%, isotope ≥ 95.0% (IsoSciences, King of Prussia, PA) or equivalent.

Solutions

- (a) Acetate buffer solution (50 mM, pH = 4.0).—To 2.32 g of ammonium acetate, add 500 mL of water, adjust to pH = 4.0 with acetic acid, and make to 600 mL.
- (b) Mobile phase A (ammonium formate 20 mM, pH = 3.0).—To 1.26 g of ammonium formate, add 950 mL of water, adjust to pH = 3.0 with formic acid, and make to 1 L.
- (c) Mobile phase B (methanol, 100% v/v).

Standards

Stable isotope-labeled stock standards of ¹³C₄-thiamine (100 mg/mL) and ¹³C₃¹⁵N₁-pantothenic acid (400 mg/mL) were separately prepared by dissolving 5 and 20 mg, respectively, in 50 mL of water.

Aliquots (~1.3 mL) of these standards were transferred to cryogenic vials and stored at –80 °C. A mixed stable isotope labelled internal standard solution was prepared by diluting 1 mL of each stable isotope-labelled stock standard in 25 mL of water.

A thiamine non-labelled stock standard (~60 mg/mL) was made by dissolving 15 mg in 250 mL of water. The accurate concentration of this solution was determined by UV absorbance measurement of a purity solution made by diluting 1 mL of stock standard solution in 25 mL of 0.1 M HCl ($E^{1\%}_{1\text{ cm}}$, 421 dL/g.cm at 247 nm; 27). Pantothenic acid lacks a specific chromophore and hence accurate measurement of its concentration cannot be determined spectrophotometrically. Calcium pantothenate was dried at 102 °C for 2 h, cooled over silica gel, and then used immediately. A pantothenic acid non-labelled stock standard (~1010 mg/mL) was made by accurately weighing ~55 mg of dried calcium pantothenate and dissolving in 50 mL of water.

A mixed thiamine and pantothenic acid working standard was prepared by diluting 6 mL of thiamine and 1 mL of pantothenic acid non-labelled stock standards in 10 mL of water. Calibration standards were made by pipetting 1.0 mL of stable isotope-labelled internal standard and 10, 50, 200, 500, or 1000 mL of mixed thiamine and pantothenic acid working standard into separate 50 mL volumetric flasks and diluting to volume with water.

Samples

Method performance was evaluated using a range of infant formulas that were fortified with thiamine and pantothenic acid, including the NIST 1849a certified reference material (CRM). An unfortified whole milk powder was used for the recovery study.

Sample preparation

Approximately 2 g of powder was weighed accurately into a 50 mL disposable centrifuge tube, 30 mL of buffer solution and 1.0 mL of stable isotope-labelled internal standard were added, and the solution was vortex mixed for 30 s and placed on horizontal shaker for 10 min. A 4 mL aliquot of the sample extract was transferred to an ultracentrifuge tube and centrifuged for 40 min at 2700 × g. An aliquot of the filtrate was then syringe filtered into an HPLC vial ready for analysis.

LC–MS analysis

- (a) HPLC system.—Nexera X2 UHPLC system.
- (b) HPLC column.—Luna PFP 100 × 2.0 mm, 3 μm.
- (c) Column temperature.—35 °C.
- (d) Chiller temperature.—15 °C.

- (e) Injection volume.—2 μL .
- (f) Binary gradient.—Settings in Table 1.
- (g) Detection.—Electrospray ionization in positive mode with MRM; settings in Tables 2 and 3.

Calculations

For each vitamin, a linear calibration plot was constructed, plotting the ratio of peak areas against the ratio of concentrations. The concentration (in mg/hg) of thiamine hydrochloride or pantothenic acid in powder samples, respectively, is given by the following equation:

$$\text{Result} = \frac{A_{\text{NV}}}{A_{\text{IS}}} \times \frac{1}{L} \times \frac{(C_{\text{IS}} \times V_{\text{IS}})}{M_{\text{S}}} \times \frac{100}{1000}$$

where

Result = concentration of thiamine hydrochloride or pantothenic acid in the sample (mg/hg);

A_{NV} = peak area of thiamine or pantothenic acid in the sample;

A_{IS} = peak area of $^{13}\text{C}_4$ -thiamine or $^{13}\text{C}_3^{15}\text{N}_1$ -pantothenic acid in the sample;

L = slope of the calibration curve;

C_{IS} = concentration of $^{13}\text{C}_4$ -thiamine or $^{13}\text{C}_3^{15}\text{N}_1$ -pantothenic acid in the internal standard (mg/mL);

V_{IS} = volume of internal standard spiked to the sample (mL);

M_{S} = mass of the sample (g);

1000 = concentration conversion factor (mg/g to mg/g);

100 = mass conversion factor (mg/g to mg/hg).

Results and Discussion

Method optimization

Simplified extraction techniques can be used for the rapid, routine product compliance release analysis of thiamine and pantothenic acid in infant formulas, because the free vitamers, both endogenous and supplemented, are the predominant forms present. Ultracentrifugation is a convenient technique for removing protein from milk products and minimizes the potential degradation of labile vitamins under alternative conditions that are typically employed and that incorporate heat or acids.

Optimization of the ion source parameters was performed by direct infusion of thiamine and pantothenic acid standards. Product ion scans were used to identify major fragment ions and the most intense MRM transitions were selected for quantitation, with the second most intense ion being

selected as the qualifier ion. Unambiguous identification of thiamine and pantothenic acid was assured by selecting a specific elution time window for monitoring characteristic quantifier and qualifier ions, with performance criteria for the ion ratios set at $\pm 25\%$. The chromatography was optimized for the elution of thiamine and pantothenic acid with a run time of 10 min (Figure 1).

Method validation

Detector linearity was performed by the analysis of combined thiamine and pantothenic acid standard solutions at seven concentration levels (thiamine, 0.05–17 mg/hg; pantothenic acid, 0.2–82 mg/hg). Linearity was evaluated by least-squares regression analysis of peak area ratio versus concentration ratio, with acceptable r^2 values of 0.9999 being obtained for both thiamine and pantothenic acid; residual plots were assessed as a further test of linearity, with the deviation from the line of best fit being $< 2.5\%$.

A milk-based infant formula powder was spiked with a known concentration of mixed vitamin standard solution and recovery was calculated. Powder samples were spiked with mixed standard solutions to 0, 50, and 100% of nominal 1.3 mg/hg (thiamine) and 1.4 mg/hg (pantothenic acid). The recovery was estimated to be between 98.4 and 101.1% for thiamine and between 99.2 and 110.0% for pantothenic acid. The average recovery obtained was within the expected range of 90–110% for each sample.

Bias against a CRM was evaluated by replicate analyses ($n = 20$) of NIST 1849a, with differences between the measured value and the certified value being determined with the mean and standard deviation of the differences, and the t -test statistic and the P -values were calculated. No statistical bias ($\alpha = 0.05$) between the measured results and the certified values for thiamine was found ($P = 0.64$); however, a statically significant bias ($P < 0.01$) for pantothenic acid was found, although the nominal bias was only 4.7% (mean = 7.1 mg/hg; certified value = 6.8 mg/hg).

The method was compared with an HPLC-fluorescence method for thiamine (6) and a surface-plasmon-resonance based biosensor immunoassay method for pantothenic acid (17); both are currently used for routine product release analysis. A total of 20 samples, including a range of infant and growing-up formulas, was tested over five different days and a paired t -test was used to evaluate bias. The bias between the measured results obtained by LC-MS/MS and the current analytical methods was negligible (mean bias: thiamine, 0.01 mg/hg; pantothenic acid, 0.17 mg/hg) and not statistically significant ($\alpha = 0.05$; thiamine, $P = 0.399$; pantothenic acid, $P = 0.058$). Bland-Altman plots illustrate the level of agreement between the analytical methods for the determination of thiamine and pantothenic acid in infant formulas (Figures 2 and 3).

Method precision was evaluated by the analysis of duplicate pairs of an in-house quality control sample infant formula powder ($n = 10$) and a NIST 1849a CRM infant formula ($n = 10$). Both samples selected for precision studies were analyzed as 20 replicate pairs by two different analysts on several different days. Acceptable precision was demonstrated, with a repeatability of 7.2% RSD_r (HorRat: 0.6) and an intermediate precision of 6.8–7.2% RSD for thiamine, and a repeatability of 5.7% RSD_r (HorRat: 0.5) and an intermediate precision of 3.7–8.6% RSD for pantothenic acid.

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The method detection limit (MDL) is the smallest amount of an analyte that can be quantitated with reliability, estimated from the precision of the complete method applied to a sample containing low levels of analyte (28). The MDLs for thiamine and pantothenic acid were estimated by replicate analysis ($n = 8$) of a buttermilk powder sample containing endogenous levels. Buttermilk powder was chosen because of its relatively lower concentration of endogenous thiamine and pantothenic acid compared with whole milk powder. The measured values for the MDL were 4 and 30 $\mu\text{g}/\text{hg}$ for thiamine and pantothenic acid, respectively, signifying that the described method is suitable for application to fortified infant formulas, as these typically contain thiamine and pantothenic acid at concentrations that are at least an order of magnitude higher.

The robustness of the method was assessed by conducting a Plackett-Burman trial (29, 30) in the manner described previously (31). The seven factors assessed were: sample weight (1.8 and 2.2 g); buffer volume (28 and 32 mL); shaker time (8 and 12 min); centrifuge speed (2500 and 2700 \times g); centrifuge time (35 and 45 min); filter type (PTFE and nylon); and a dummy factor. The method was found to be robust for the method parameters evaluated, and the results obtained were normally distributed, with variances conforming to that expected by chance (Figures 4 and 5). As with similar methods exploiting stable isotope-labelled internal standard quantitation, critical method parameters included accurate measurement of sample weight and the ratio of internal standard in samples and calibration standards. Both parameters were tightly controlled by the use of appropriately calibrated pipettes and balances.

The method described is capable of yielding a single result in approximately 2 h, with the procedure being capable of significant sample throughput, with more than 50 samples per day completed by a single analyst.

Conclusions

A rapid LC-MS/MS method intended for use in high-throughput laboratories as part of routine product compliance release testing of thiamine and pantothenic acid in manufactured infant formulas and adult nutritional products is described. This method was subjected to single-laboratory validation and was found to be accurate, precise, and fit for purpose.

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Table 1. Chromatographic gradients

Time, min	Flow rate, mL/min	Mobile phase composition	
		A, %	B, %
0	0.4	100	0
2.0	0.4	95	0
5.0	0.4	60	40
6.0	0.4	5	95
8.0	0.4	5	95
8.5	0.4	100	0
10.0	0.4	100	0

Oven temperature = 40 °C

Mobile phase A = Ammonium formate (20 mmol/L)

Mobile phase B = Methanol (100%)

Table 2. Mass spectrometry compound parameter

Analyte ^a	Precursor ion, m/z	Product ions, m/z	DP ^b , V	EP ^c , V	CE ^d , V	CXP ^e , V	Dwell time, ms
NLB1	265.1	122.1 ^f	31	10	19	10	100
		144.0 ^g			17	14	50
SILB1	269.1	122.1 ^h			19	10	100
NLB5	220.1	90.0	56	10	17	10	100
		72.0			23	40	50
SILB5	224.1	94.0			17	10	100

^a NLB₁=Thiamine; SILB₁=¹³C₄-thiamine; NLB₅=Pantothenic acid; SILB₅=¹³C₃, ¹⁵N₁-pantothenic acid

^b DP=Declustering potential

^c EP=Entrance potential

^d CE=Collision energy

^e CXP=Collision cell exit potential

^f Quantifier ion

^g Qualifier ion

^h Internal standard

Table 3. Mass spectrometry instrument settings

Instrument parameter	Value
Ionization mode	ESI ^b
Curtain gas	45 psi
Nebulizer gas GS1	40 psi
Heater gas GS2	40 psi
Collision gas	N ₂
Source temperature	350°C
Ion spray voltage	5500 V

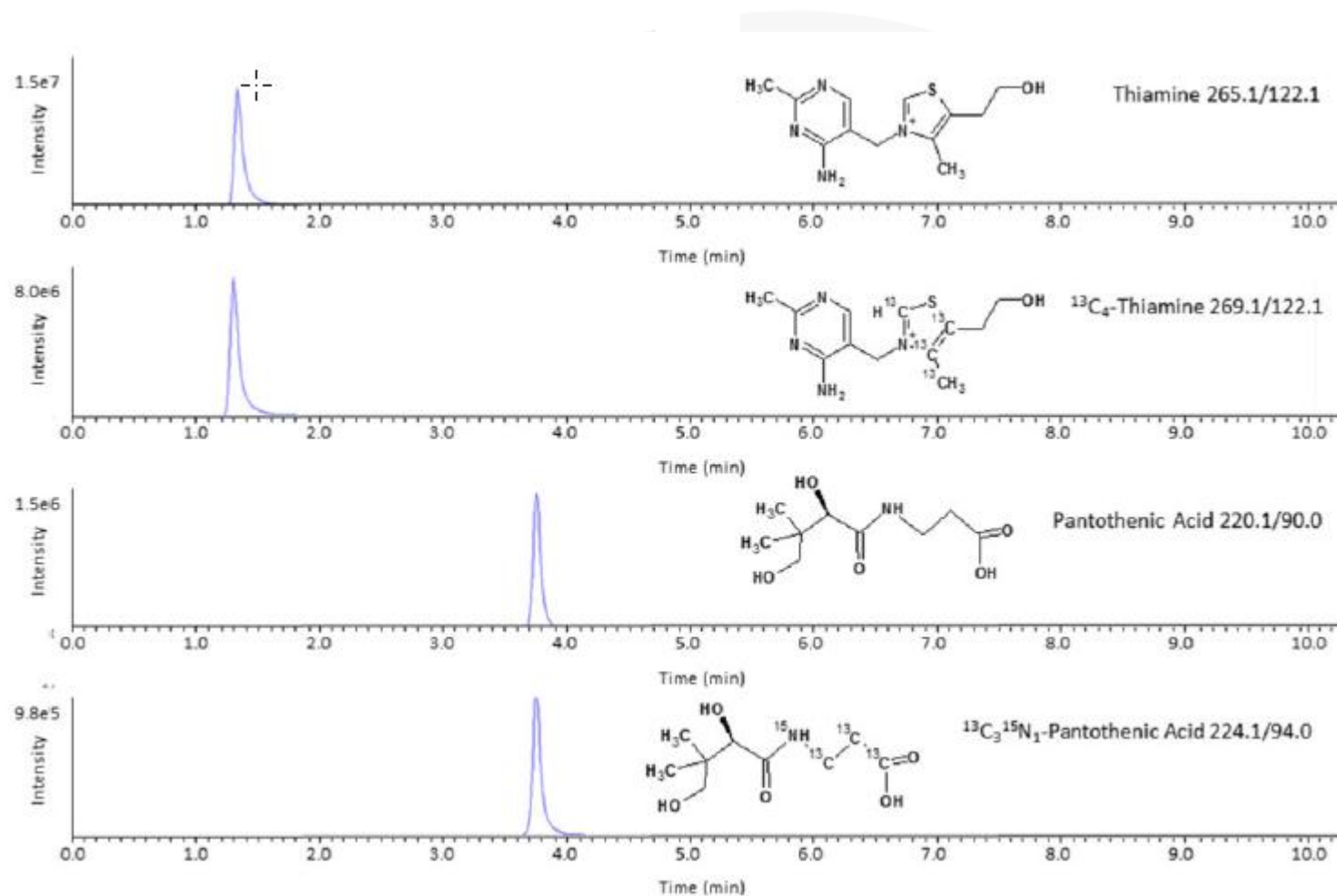


Figure 1. MRM chromatograms of quantifier ions of thiamine, $^{13}\text{C}_4$ -thiamine, pantothenic acid, and $^{13}\text{C}_3\text{ }^{15}\text{N}_1$ -pantothenic acid.

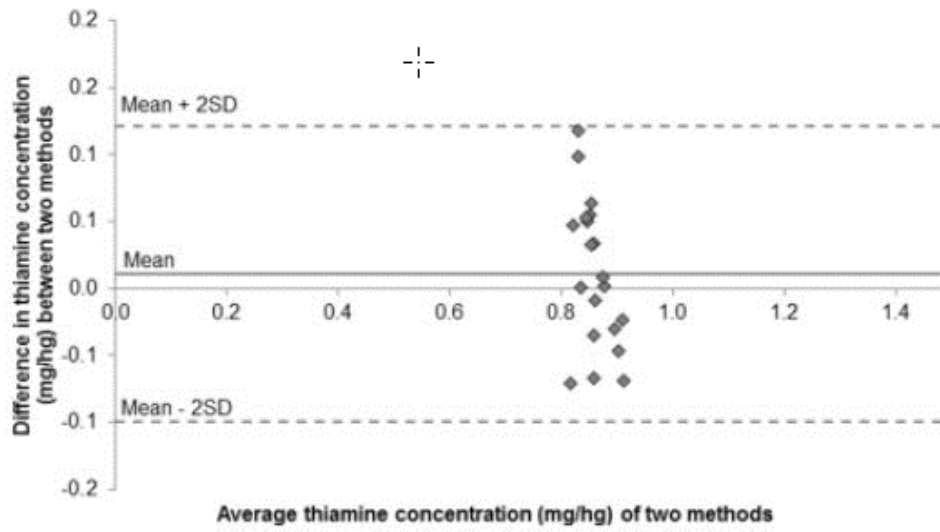


Figure 2. Bland-Altman plot for thiamine, comparing LC-MS/MS and LC-fluorescence methods

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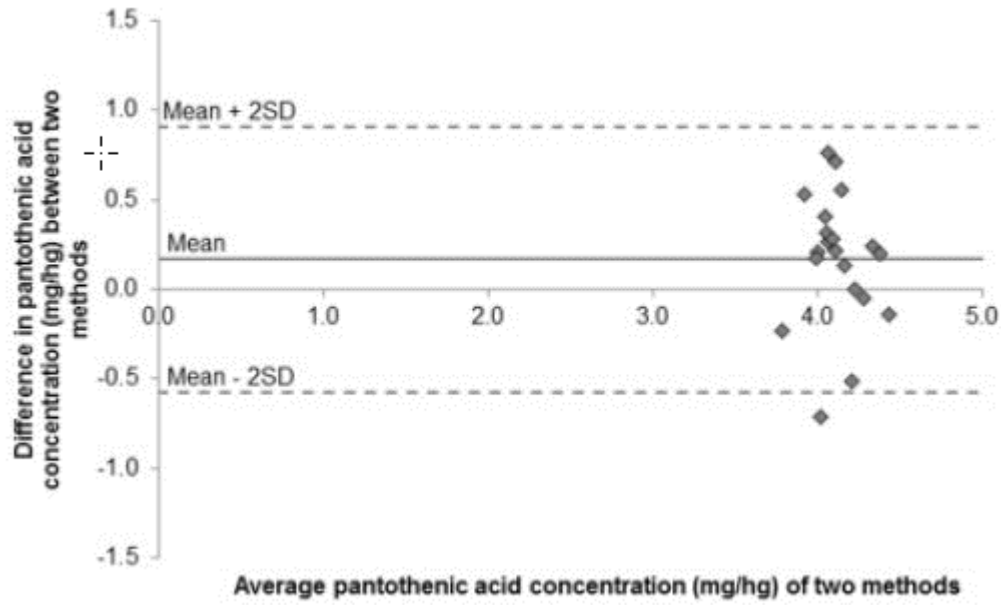


Figure 3. Bland–Altman plot for pantothenic acid, comparing LC-MS/MS and biosensor immunoassay methods

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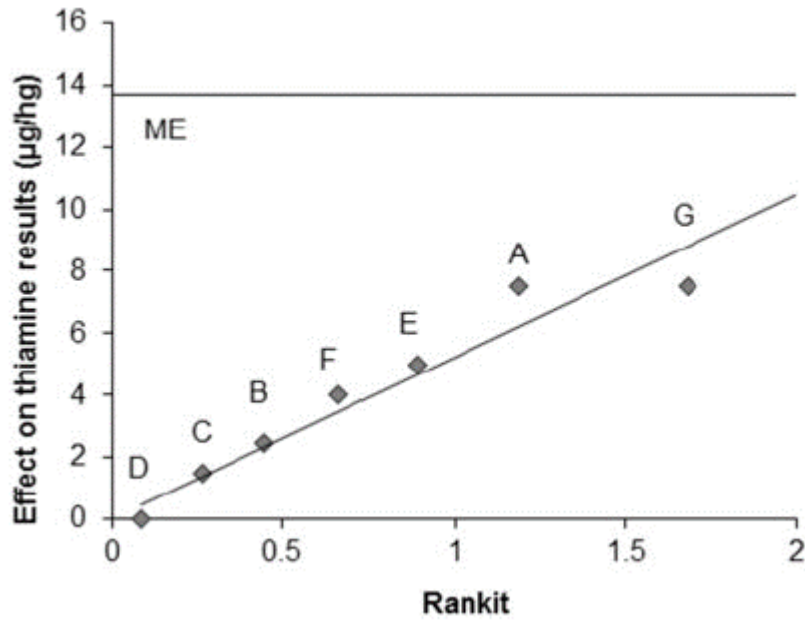


Figure 4. Half-normal plot of thiamine ruggedness experiment. A = sample weight (1.8 and 2.2 g); B = buffer volume (28 and 32 mL); C = shaker time (8 and 12 min); D = centrifuge speed (2500 and 2700 g); E = centrifuge time (35 and 45 min); F = filter type (PTFE and nylon); and G = a dummy factor

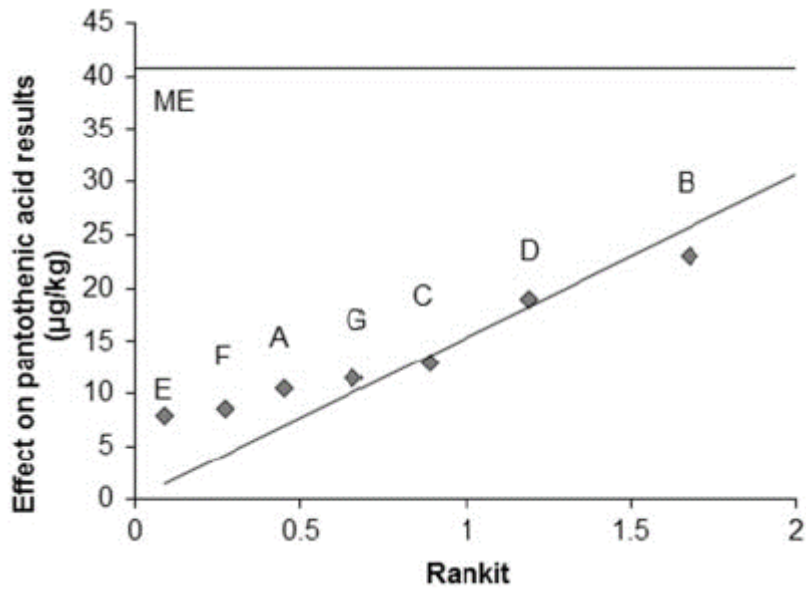


Figure 5. Half-normal plot of pantothenic acid ruggedness experiment. A = sample weight (1.8 and 2.2 g); B = buffer volume (28 and 32 mL); C = shaker time (8 and 12 min); D = centrifuge speed (2500 and 2700 g); E = centrifuge time (35 and 45 min); F = filter type (PTFE and nylon); and G = a dummy factor