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Ultra-performance liquid chromatography tandem mass-spectrometry (UPLC–MS/MS) for the rapid, simultaneous analysis of thiamin, riboflavin, flavin adenine dinucleotide, nicotinamide and pyridoxal in human milk

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ABSTRACT

A novel, rapid and sensitive ultra-performance liquid-chromatography tandem mass spectrometry (UPLC–MS/MS) method for the simultaneous determination of several B-vitamins in human milk was developed. Resolution by retention time or multiple reaction monitoring (MRM) for thiamin, riboflavin, flavin adenine dinucleotide (FAD), nicotinamide and pyridoxal (PL) has been optimized within 2 min using a gradient of 10 mM ammonium formate (aq) and acetonitrile. Thiamin-(4-methyl-^{13}C-thiazol-5-yl-$^{13}C_3$) hydrochloride, riboflavin-dioxo-pyrimidine-$^{13}C_4$,$^{15}N_2$, and pyridoxal-methyl-d_3 hydrochloride were used as internal standards. A sample-like matrix was found to be mandatory for the external standard curve preparation. $^{13}C_3$-caffeine was added for direct assessment of analyte recovery. Intra- and inter-assay variability for all analytes ranged from 0.4 to 7.9% and from 2.2 to 5.2%, respectively. Samples were subjected to protein precipitation and removal of non-polar constituents by diethyl ether prior to analysis. Quantification was done by ratio response to the stable isotope labeled internal standards. The standard addition method determined recovery rates for each vitamin (73.0–100.2%). The limit of quantitation for all vitamins was between 0.05 and 5 ppb depending on the vitamin. Alternative approaches for sample preparation such as protein removal by centrifugal filter units, acetonitrile or trichloroacetic acid revealed low recovery and a greater coefficient of variation. Matrix effect studies indicated a significant influence by matrix constituents, showing the importance of stable isotope labeled internal standards for analyte quantitation in complex matrices.

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1. Introduction

Poor maternal intake of B-vitamins during lactation can result in maternal depletion, lower concentrations in breast milk and subsequent infant deficiency, with associated health risks such as beri–beri, ariboflavinosis, pellagra and neurological problems [1,2]. Recommended intakes of B-vitamins for infants are generally set as adequate intakes (AI), which are calculated as the mean intake by infants exclusively fed with human milk from well-nourished mothers during the first 6 months [3]. This calculation is based on the mean volume of breast milk consumed (0.78 L/day), and a concentration of each vitamin in milk based on reported values. Unfortunately, the concentrations of B-vitamins have been measured in only a few breast milk samples, using outdated methods. Protocols applied include microbiological, chromatographic, and spectroscopic methods and are generally optimized for analyzing each B-vitamin individually. Some require laborious techniques and up to several milliliters of sample [3–9]. For example, Sakurai et al. measured fat- and water-soluble vitamins, including the B-vitamins, in human milk from Japanese women using high performance liquid chromatography (HPLC) coupled with fluorescence detection or microbiological techniques for analysis [10]. Due to the fact that each vitamin is analyzed separately, the analyses carried out are sub-optimal regarding time, sample volume and expense.

In contrast, UPLC–MS/MS offers improved resolution, speed, and sensitivity for analytical determinations [11], allowing rapid and simultaneous analysis of analytes. This analytical platform has been used widely for the assessment of analytes such as pharmaceuticals, long chain fatty acids, underivatized amino acids and opiates in various matrices [12–15]. B-vitamins have also been targeted by UPLC–MS/MS mostly in food samples or pharmaceutical preparations [16–18]; however, there have been few analyses of infant foods, and those are usually limited to infant formulas and fortified milk or rice powder [19,20]. While the vitamins in infant formulas are usually present in their free form and

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are not bound to matrix constituents, the complexity of human milk exacerbates the analysis of the endogenous nutrients significantly. While some B-vitamins such as folate and vitamin B-12 are bound to proteins [21,22], others are present in their free or phosphorylated forms. Stuetz et al. reported thiamin and thiamin-monophosphate as the main forms of vitamin B-1 in human milk [23]. Roughead and McCormick described riboflavin and FAD as the prevalent forms of vitamin B-2 [6], while pyridoxal appeared to be the principal form of vitamin B-6 with possible contributions of pyridoxine, pyridoxamine, pyridoxal-phosphate or pyridoxamine-phosphate [24–26]. On the other hand, niacin occurs in human milk as nicotinamide and its enzymatic forms nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) [10,27]. Therefore, sample preparation of human milk depends strongly on the vitamins in question and limits the number of different vitamins that can be analyzed simultaneously.

In this paper we describe a new, rapid and simultaneous UPLC–MS/MS analysis of thiamin, riboflavin, FAD, nicotinamide and pyridoxal in human milk samples that can be easily adopted for high-throughput clinical studies. Moreover, we report effects observed during sample preparation and analysis due to the unique complexity of the matrix.

2. Methods and materials

2.1. Chemicals and materials

Thiamin hydrochloride, riboflavin, nicotinamide, flavin adenine dinucleotide, pyridoxal hydrochloride, thiamin-(4methyl-^{13}C-thiazol-5-yl-$^{13}C_3$) hydrochloride riboflavin-dioxopyrimidine-$^{13}C_4$,$^{15}N_2$, pyridoxal-methyl-d_3 hydrochloride and human serum albumin were purchased from Sigma–Aldrich (St. Louis, MO). Caffeine-trimethyl-$^{13}C_3$ from Cambridge Isotope Laboratories (Andover, MA) and ammonium formate was purchased from Hampton Research (Aliso Viejo, CA). Water, methanol, acetonitrile (all LC–MS grade) and α-D-(+)-lactose were obtained from Fisher Scientific (Fair Lawn, NJ). Amber screw thread vials (15 × 45 mm) and caps were purchased from Fisher Scientific, while HPLC screw-cap amber vials and inserts were obtained from Supelco (Bellefonte, PA). LC-vial caps (PTFE/silicone) were purchased from Waters (Milford, MA), and ultrafree centrifugal filters Durapore® PVDF 0.1 μm and Amicon Ultra-0.5 centrifugal filter (NMWL 10,000) from Millipore (Billerica, MA).

2.2. Human milk sample, and NIST SRM 1849 infant/adult formula

Pooled breast milk (0–4 weeks p.p., ~600 mL) was provided by an apparently healthy Caucasian donor (age 32 y) in the Sacramento, CA area. Aliquots were stored at −80 °C in amber tubes. Random samples were obtained from women in ongoing studies in Cameroon, China, India, Malawi and the US and stored at −80 °C in amber tubes until analysis.

NIST Standard Reference Material (SRM) 1849 Infant/Adult Formula was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). 10 g of the reference material was dissolved in 1 L of LC–MS grade water. Aliquots were stored at −80 °C in amber tubes until use.

2.3. UPLC–MS/MS

The UPLC–MS/MS system consisted of an ACQUITY Ultra-Performance-LC (Waters, Milford, MA) coupled to a 4000 QTRAP LC/MS/MS (AB Sciex, Foster City, CA). The analysis was carried out using an ACQUITY UPLC® HSS T3 column (2.1 × 50 mm, 1.8 μm) protected by an ACQUITY UPLC® HSS T3 VanGuard™ pre-column (2.1 × 5 mm, 1.8 μm; Waters, Milford, MA) at 40 °C. During all runs samples were maintained at 10 °C within the instrument until injection. 10 μL of the sample was injected by partial loop every 4.75 min. Ammonium formate (10 $mM_{(aq)}$, solvent A) and acetonitrile (solvent B) served as mobile phases at a flow rate of 0.3 mL/min allowing the elution of all analytes within 2 min and an additional 2 min for column rinse using the following gradient: 0 min (95% A), 1 min (65% A), 2 min (45% A), 2.1–3 min (5% A), 3.1–4 min (95% A). Needle wash was performed after every injection with 600 μL methanol/water (20/80, v/v; strong wash) and 200 μL acetonitrile (weak wash). Acquisition settings were optimized by infusion of a 100 ng/mL solution of the vitamers in water/acetonitrile (50/50, v/v) at a flow rate of 10 μL/min. The mass spectrometer was operated via positive ion mode electrospray ionization (ESI). The vitamins were detected in multiple reaction monitoring (MRM) with unit resolution at both Q1 and Q3. The chromatographic cycle was divided into two periods. The ion transitions and optimized parameters are shown in Table 1. CAD gas (20 psi), ion spray voltage (5000 V), turbo gas temperature (500 °C), ion source gases 1 and 2 (40 psi), entrance potential (10 V), collision cell exit potential (10 V), dwell time (100 ms) and settings for the interface heater (on) were identical for all periods.

2.4. Preparation of standards

$^{13}C_4$-Thiamin hydrochloride, $^{13}C_4$,$^{15}N_2$-riboflavin and $^{2}H_3$-pyridoxal hydrochloride were added to the samples as internal standards for quantification. While the labeled thiamin was used as the internal standard for vitamin B-1 and labeled pyridoxal for all B-6 vitamers, due to the unavailability of stable isotope labeled FAD and nicotinamide, labeled riboflavin was used to quantify both B-2 vitamers and nicotinamide. Labeled caffeine was added prior to analysis to control for possible system fluctuation. Stock solutions for each vitamin and the internal standards were prepared in subdued light in amber glass vials using LC–MS grade water (thiamin hydrochloride: 72.7 μg/mL, riboflavin: 33.5 μg/mL, FAD: 68.0 μg/mL, nicotinamide: 120 μg/mL, pyridoxal: 80 μg/mL; internal standards: thiamin-$^{13}C_4$ hydrochloride: 110 μg/mL, riboflavin-$^{13}C_4$, $^{15}N_2$: 100 μg/mL, pyridoxal-methyl-d_3 hydrochloride: 100 μg/mL; caffeine-$^{13}C_3$: 100 μg/mL). Only B-2 and niacin vitamins stock solutions were stored at −80 °C while the remaining stock solutions were kept at 0 °C in darkness. The internal standards were combined to an aqueous master mix of 1 mg/L of which 10 μL was added to each sample for a final concentration of 100 μg/L.

2.5. Method validation

The method was validated using a pooled human milk sample with unknown B-vitamin content and NIST SRM 1849 Infant/Adult Infant Formula (NIST, Gaithersburg, MD) by standard addition experiments. The stock solutions of the vitamin standards were combined in an aqueous master mix of 1 μg/L for each vitamin. 5 and 10 μL (50 and 100 μg/L respectively, and an additional 10 and 20 μg/L for thiamin, riboflavin and pyridoxal) of this mix were added to the sample to validate recovery rate for each analyte. The analysis of the milk sample and the standard addition samples was carried out in replicates of five for each set and the concentrations were calculated from the equation $y = mx + b$, as determined by weighted ($1/x$; thiamin, riboflavin, PL) or non-weighted (FAD, nicotinamide) linear regression of the standard line. The mean, standard deviation and coefficient of variation (CV) were

Table 1
MS/MS ion transitions monitored (RT, retention time; MW, molecular weight; MRM, multiple reaction monitoring; DCP, declustering potential; CE, collision energy; CUR, curtain gas).

Analyte	RT [min]	MW [g/mol]	MRM		CUR	DCP [V]	CE [eV]
Period 1 (0–1.78 min)							
$^{2}H_3$-Pyridoxal	1.44	206.3	153.4	>97.0	20	80	27
Pyridoxal	1.45	203.6	168.0	>150.0	20	55	21
Pyridoxine	1.51	169.2	170.4	>152.4	20	65	21
$^{13}C_4$-Thiamin	1.52	341.2	269.0	>122.0	20	45	21
Thiamin	1.53	337.3	265.0	>122.0	20	45	21
Nicotinamide	1.55	122.1	123.0	>80.0	20	65	30
FAD	1.63	829.5	786.3	>348.0	20	90	33
Period 2 (1.78–4.0 min)							
$^{13}C_4$,$^{15}N_2$-Riboflavin	1.91	382.4	383.0	>249.0	30	70	36
$^{13}C_3$-Caffeine	1.91	197.2	198.0	>140.0	30	65	27
Riboflavin	1.92	376.4	377.0	>243.0	30	70	36

determined for each set of replicates, which were then used to calculate the recovery rates for each analyte as follows:

$$R[\%] = \frac{(C_{\text{measured}} - C_{\text{endogenous}}) \times 100}{C_{\text{added}}}.$$

Due to the unavailability of human milk samples with known amounts of the B-vitamins, accuracy was determined using 20 μL of the NIST SRM 1849 Infant/Adult Formula with known amounts of thiamin, riboflavin, nicotinamide and pyridoxine. Furthermore, standard addition experiments were also conducted using the NIST formula to compare recovery rates between the matrices.

2.6. Matrix effects

Matrix effects were studied in human milk using the stable isotope labeled internal standards for quantitation. Set 1 represented the neat standard solution in water (100 μg/L), while the milk samples for sets 2 and 3 were prepared for analysis as described but adding the standards either pre- (set 3) or post-preparation of the samples. All sets were carried out in replicates of five. Matrix effect (ME), recovery (RE) and overall process efficiency (PE) were calculated according to Matuszewski et al. using the following equations [28]:

$$\text{ME}[\%] = \frac{\text{set } 2}{\text{set } 1} \times 100$$

$$\text{RE}[\%] = \frac{\text{set } 3}{\text{set } 2} \times 100$$

$$\text{PE}[\%] = \frac{\text{set } 3}{\text{set } 1} \times 100 = \frac{(\text{ME} \times \text{RE})}{100}$$

Furthermore, PE was determined in the calibration curve matrix for all vitamins and internal standards in triplicate of 100 μg/L.

2.7. Quality control

A six-point calibration standard curve (1–250 μg/L) and a reagent blank were processed with each batch of the validation experiments or samples. These standards as well as the reagent blank were prepared in a protein/lactose matrix based on average values in human milk (~1.5% human serum albumin, ~7% lactose [29]) to ensure stable recovery of the analytes. The validated human milk samples were processed as controls in triplicate with each set of samples and analyzed every 15th injection to follow possible degradation of analytes.

The analytes were quantified by the ratio response of the analytes to the respective internal standards as a control for extraction efficiency, volumetric changes and ion suppression. $^{13}C_4$-Thiamin was used to quantify thiamin and $^{2}H_3$-pyridoxal for pyridoxal, while $^{13}C_4$,$^{15}N_2$-riboflavin, was used for quantification of riboflavin, FAD and nicotinamide. Stable isotope-labeled standards for the latter two vitamins were unavailable; thus the available standards were tested for suitability. Riboflavin showed the best results for reproducibility when used for quantification of FAD and nicotinamide and least amount of interferences by matrix effects, recovery, and process efficiency and therefore was chosen as the internal standard for these two vitamins. The internal standards were measured in ratio response to $^{13}C_3$-caffeine to calculate their recoveries, and to track sample specific ion suppression, volumetric changes and shot-to-shot drift in instrument sensitivity.

2.8. Sample preparation

Sample preparation was carried out under subdued light and on ice to protect the analytes against degradation. 20–50 μL breast milk or NIST SRM 1849 was diluted with LC-MS grade water to a final volume of 100 μL. After the addition of the internal standards (10 μL), methanol (MeOH; 4× volumes) was added and the sample mixed for 2 min before centrifugation for 10 min at 6 °C (10,500 × g; SORVALL® Legend RT refrigerated benchtop centrifuge, Asheville, NC) [30]. 500 μL of the supernatant was transferred to a 4 mL amber glass vial to evaporate the solvent to dryness under a gentle nitrogen-stream. The residual was reconstituted in 120 μL LC–MS grade water containing ^{13}C-caffeine (100 μg/L). The non-polar constituents of the matrix were then extracted with 120 μL diethyl ether followed by incubation at 4 °C for 15 min to allow for possible precipitation. After centrifugation for 10 min at 6 °C (10,500 × g), 100 μL of the supernatant was filtered through a 0.1 μm Durapore® PVDF spin filter and transferred into a 250 μL glass insert in a 2 mL amber glass vial, capped and analyzed.

Additional sample preparation methods tested included protein removal by acetonitrile, trichloroacetic acid and Amicon Ultra-0.5 centrifugal filters (NMWL 10,000). Prior to protein-precipitation using acetonitrile, hexane was used to remove the non-polar constituents. After centrifugation for 10 min at 13,000 rpm (4 °C), the supernatant was collected and evaporated and reconstituted as described above. Samples treated with trichloroacetic acid were subjected to a more laborious preparation including a second ammonium sulfate precipitation and phenol liquid/liquid extraction as previously described [6]. Amicon Ultra-0.5 centrifugal filters were also tested for efficiency of protein removal. Choosing an appropriate molecular weight cut-off allows the vitamins as small molecules (MW < 1000) to elute through the filter unit and to be recovered in the flow through, while the protein fraction with a high molecular weight (MW > 10,000) is retained in the filter unit.

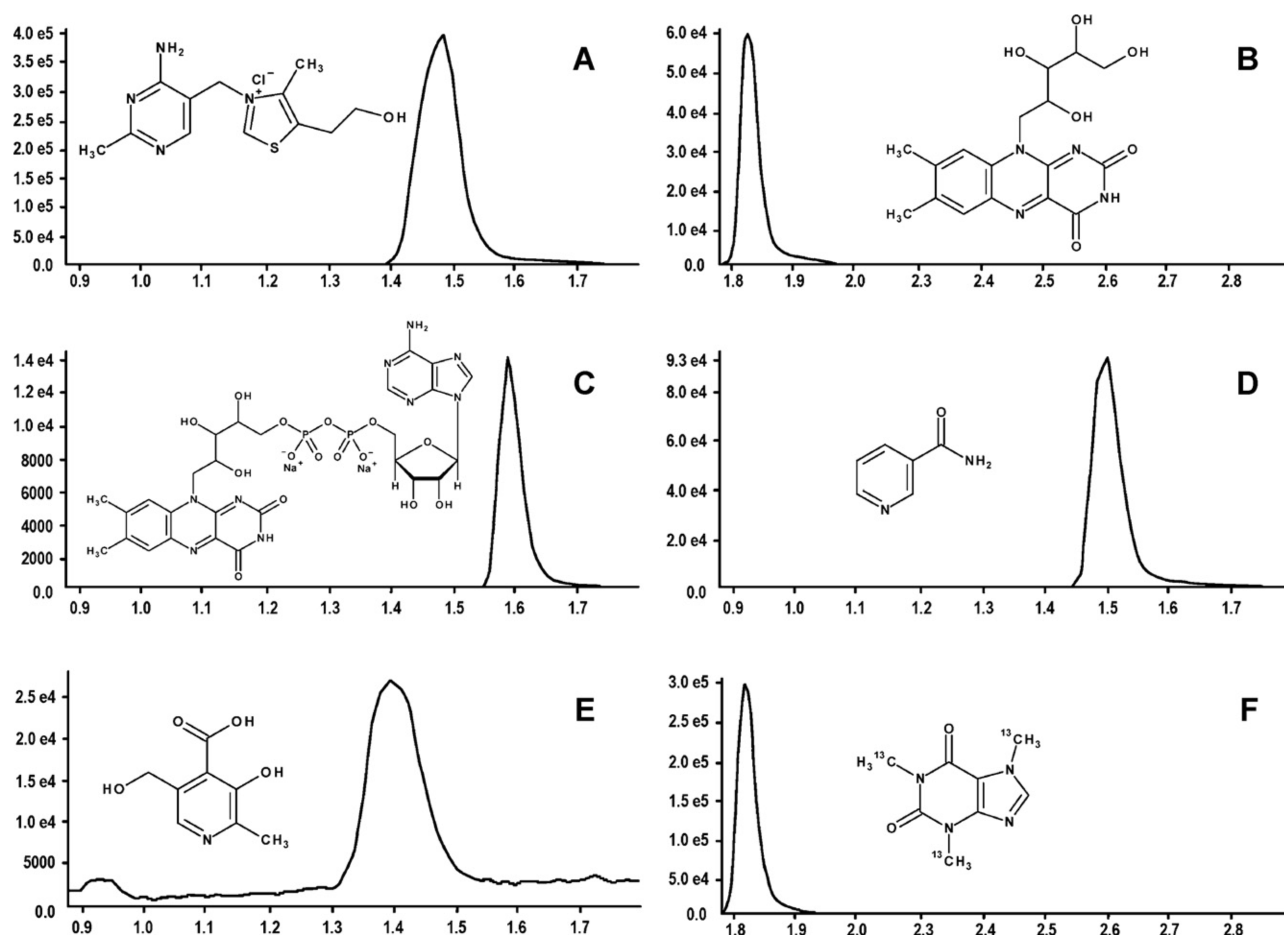

Fig. 1. Extracted ion chromatogram of the analytes and system control standard (A; thiamin, B; riboflavin, C; FAD, D; nicotinamide, E; pyridoxal, F; $^{13}C_3$-caffeine).

3. Results and discussion

3.1. Chromatography

Using reversed-phase chromatography, water, 10 mM ammonium formate, 0.1% ammonium formate, 10 mM ammonium acetate and 10 mM formic acid were tested for the aqueous buffer component of the mobile phase, while methanol and acetonitrile were examined as the organic constituent. Best peak shape, resolution and signal-to-noise ratio was achieved using 10 mM ammonium formate (A) and acetonitrile (B) with an initial ratio of 95:5 (A:B; see Fig. 1); a higher initial aqueous content of the mobile phase as well as a shallow increase of acetonitrile throughout the analysis resulted in peak broadening and split peaks and subsequent lower resolution and signal-to-noise ratios of the analytes and internal standards. Optimal chromatographic separations without UPLC over-pressuring occurred at a flow rate of 0.3 mL/min, the gradient described above and a column temperature of 40 °C.

For all vitamins quantified either chromatographic resolution or MS resolution was achieved. No cross-talk among the chosen mass transitions was observed, even when identical product ions were acquired. Other B-vitamin standards such as vitamin B-12, folic acid or biotin were also detectable with the developed chromatographic method as well as pyridoxamine and pyridoxine, further vitamers of the B-6-complex (data not shown). Since both B-12 and folic acid are bound to protein in human milk and therefore not detectable with the developed method, and given that pyridoxal is the principal form of vitamin B-6 in human milk and neither pyridoxamine nor pyridoxine was detected in human milk samples analyzed, no further emphasis was given to these compounds. However, since the NIST SRM 1849 Infant/Adult Formula contains pyridoxine and not pyridoxal as vitamin B-6, recovery rates and accuracy for pyridoxine was determined in this particular case.

3.2. Water- versus matrix-based calibration curve

To evaluate the need for a matrix-like calibration curve, standard addition experiments in human milk were quantified using a water- and a matrix-like calibration curve. Because vitamins occur naturally in human milk, a matrix-like calibration curve was prepared using average concentrations of human serum albumin to represent the protein fraction in human milk and lactose as the principal sugar in the matrix [29]. A six-point calibration curve (1–250 μg/L for each vitamin) and a reagent blank were prepared in either LC–MS grade water or the matrix-like solution. The internal standards for quantitation were added prior to sample preparation. Five replicates for each standard addition as well as for the unspiked sample were prepared for each set. The concentration of each vitamin was quantified from each standard curve in ratio response to the respective internal standard, and recovery rates were determined based on the amount of standard added. While thiamin, riboflavin, FAD and pyridoxal showed reasonable recovery rates for both standard curves (∼73–101%), nicotinamide revealed an extremely low recovery rate when quantified with the water-based calibration curve (∼14%), while pyridoxine, the B-6 vitamin present in the NIST infant formula and therefore included in the test, showed artificially high recovery rates (∼195%). All

Table 2
Recovery rates in [%] and standard deviations for different methods of protein precipitation (filter: $n = 3$, ACN, TCA: $n = 5$, MeOH: $n = 20$).

Vitamin	Filter	ACN	TCA	MeOH
Recovery [%] and SD				
Thiamin	72.0 ± 7.51	91.5 ± 3.75	99.2 ± 8.92	100.2 ± 2.55
Riboflavin	5.0 ± 2.96	70.8 ± 1.06	83.8 ± 3.87	98.5 ± 4.18
FAD	0.9 ± 0.34	5.5 ± 0.50	114.6 ± 20.0	73.0 ± 3.60
Nicotinamide	26.4 ± 1.46	3.8 ± 16.4	105.1 ± 33.4	77.1 ± 4.00
Pyridoxal	85.1 ± 0.97	107.5 ± 5.37	103.7 ± 0.92	99.9 ± 2.18

Table 3
Sensitivity of B-vitamins in calibration curve matrix compared to AI values. *X*-fold: *x*-fold higher sensitivity (LOQ) of analysis compared to values used to set AIs.

Vitamin [μg/L]	LOD	LOQ	AI	*x*-fold
Thiamin	0.01	0.05	200	4000
Riboflavin	0.1	0.5	350[a]	700
FAD	0.1	0.5	350[a]	700
Nicotinamide	0.5	1.0	1800	1800
Pyridoxal	4.0	5.0	130	26

[a] AI value represents total vitamin B-2, riboflavin + FAD

experiments were therefore carried out using the matrix-like calibration curve.

3.3. Effects on recovery due to sample preparation method

Different types of sample preparations have been applied to human milk. Common treatments include protein precipitation using acetonitrile (ACN) or trichloroacetic acid (TCA), or incubation at elevated temperatures and under acidic conditions [6,10,25,26,31]. Since FAD is readily converted into riboflavin below pH 5 [32], any treatment of the sample below that pH was omitted. Additionally, centrifugal filter units commonly used in cell culture work for protein purification, desalting or concentration of the protein fraction were also tested for possible effective protein removal and recovery of the analytes (NMWL 10,000).

Recovery rates were varied depending on the sample preparation method applied (Table 2). Using the centrifugal filter units, riboflavin and FAD both showed low recovery. Further analysis of the protein fraction remaining in the unit indicated <8% of the vitamins remained in that fraction (data not shown), which cannot explain the low recovery rates for the B-2 vitamers. FAD and nicotinamide revealed low recovery using ACN as the precipitation agent, whereas TCA-precipitation resulted in an overall acceptable recovery for all vitamins; however, this sample preparation method is laborious, more expensive and showed generally greater variation compared to using methanol for protein precipitation (Table 2).

3.4. Linearity and limits of detection and quantitation (LOD/LOQ)

The calibration curve for each vitamin consisted of a blank and six standard solutions in a linear range of 1–250 μg/L. The analysis of 20 different calibration curves over a time period of five months showed a mean linear correlation coefficient (r) greater than 0.998 and a mean coefficient of determination (r^2) greater than 0.997 for all vitamins indicating a strong positive correlation between the theoretical concentrations and the analyzed values.

The limit of detection and quantitation (LOD/LOQ) for each analyte was calculated using Analyst software (version 1.4.2, AB Sciex; Foster City, CA) and based on the signal-to-noise ratios of the analytes in the calibration curve matrix (Table 3). The results were compared to the adequate intake recommendations for infants age 0–6 months [3]. All analytes are quantifiable below the concentrations used as the basis to set the AIs (Table 3). Most of the vitamins were quantifiable at levels 700- to 4000-fold lower than those concentrations; pyridoxal revealed a quantification level only 26 times lower than the AI value, most likely due to considerable matrix effects during analysis. Nevertheless, in all cases quantification well below the concentrations assumed in the AIs is possible and accurate for the vitamins in milk from micronutrient-deficient women, and for detecting response to supplementation or fortification.

3.5. Recovery rates, precision and accuracy

Shot-to-shot variance in $^{13}C_3$-caffeine area response over an analytical run of 74 sample injections showed a CV of 12.4%; and the shot-to-shot CV over six different analytical runs of the seven-point calibration curves was between 2.5 and 7.4%. Since only a matrix-like calibration curve was available for quantitation, recovery rates for thiamin, riboflavin, FAD, nicotinamide and pyridoxal in human milk based on the matrix-like calibration curve were determined. Even though the calibration curve is not an exact match to the actual sample matrix, the applied standard addition approach was used to account for the matrix differences between the calibration curve and samples. The recovery was calculated from four runs of the standard addition experiments over 35 days. Five replicates were prepared for the unspiked human milk and each spiked sample for each run. Recovery rates for thiamin, riboflavin and pyridoxal were between 98.5 and 100.2%, while FAD and nicotinamide revealed recovery rates between 73 and 77.1%. Inter- and intra assay variability for all analytes ranged between 0.4 and 7.9% and 2.2 and 5.2%, respectively (Table 2). Interestingly, the two vitamins quantified using $^{13}C_4$,$^{15}N_2$-riboflavin as a substitute internal standard revealed the lowest recovery rates, indicating potential matrix effects, differences in recovery or process efficiency between the internal standard and analyte. However, the recovery rates determined for FAD and nicotinamide were stable and in a reasonable range for quantitation.

The accuracy of the assay was tested by analyzing the NIST SRM 1849 infant/adult formula. Due to the differences in matrix between human milk and infant formula, recovery rates for thiamin, riboflavin, FAD, nicotinamide and pyridoxine were determined by a two-point standard addition experiment using the infant formula as matrix ($n = 5$ for each standard addition and unspiked sample). As expected, the matrix appeared to influence recovery rates for most of the analytes indicating the need to determine recovery rates for matrices of different types. However, the analyzed concentrations of all vitamins were in good agreement with the theoretical values, revealing an accuracy of 95.6–101.4% (Table 4).

3.6. Matrix effects

Matrix effect (ME), recovery (RE) and overall process efficiency (PE) were determined according Matuszewski et al. [28]. While RE for all analytes is between 70 and 88%, PE and ME vary largely among the standards (Table 5). ME was determined using suitable stable isotope labeled internal standards for quantitation. Since no stable isotope internal standard was available for FAD or nicotinamide, ME could not be studied in a human milk matrix. However, using the calibration curve matrix, similar results were obtained for the overall PE (Table 5). In general, the matrix used for the

Table 4
NIST SRM 1849: theoretical concentrations, recovery rates and accuracy of thiamin, riboflavin, nicotinamide and pyridoxine analysis in NIST SRM 1849 [μg/L].

Vitamin	NIST [μg/L]	Recovery [%]	Analysis [μg/L]	Accuracy [%]
Thiamin	158.0	88.5 ± 1.18	153.8 ± 4.74	97.3
Riboflavin	174.0	97.5 ± 3.07	178.6 ± 4.28	102.6
Nicotinamide	975.0	89.5 ± 0.75	932.4 ± 11.8	95.6
Pyridoxine	142.0	71.6 ± 1.96	144.1 ± 1.86	101.4

Table 5
Process efficiency (PE), matrix effect (ME), and recovery (RE) of the stable isotope labeled internal standards in human milk.

Vitamin	PE [%]	ME [%]	RE [%]	PE[a] [%]
$^{13}C_4$-Thiamin	25.2 ± 1.14	36.3 ± 4.93	69.5 ± 3.14	39.5 ± 1.19
$^{13}C_4$,$^{15}N_2$-Riboflavin	53.8 ± 0.77	61.3 ± 2.85	87.9 ± 1.26	59.7 ± 0.80
$^{2}H_3$-Pyridoxal	6.0 ± 0.29	9.0 ± 1.09	68.8 ± 3.21	14.0 ± 0.36
$^{13}C_3$-Caffeine	42.2 ± 1.63	52.4 ± 1.74	80.6 ± 3.90	50.8 ± 4.26
Thiamin	n/a	n/a	n/a	39.5 ± 3.42
Riboflavin	n/a	n/a	n/a	60.6 ± 6.00
FAD	n/a	n/a	n/a	75.0 ± 10.3
Nicotinamide	n/a	n/a	n/a	28.9 ± 2.93
Pyridoxal	n/a	n/a	n/a	13.6 ± 0.13

n/a: not available.
[a] PE, process efficiency in calibration curve matrix.

calibration curve showed a better PE than the actual human milk matrix because the calibration curve matrix is less complex. However, the effects are still significant, especially for pyridoxal, and show the importance of using stable isotope-labeled internal standards when quantification is influenced by matrix effects.

3.7. Stability

Various aliquots of a light-protected pooled human milk sample, stored at −80 °C in amber tubes, were analyzed over the course of five months. The analyses were carried out by two different investigators using refrozen (up to three times) or non-refrozen sample aliquots at random (n = 151). The relative recovery of each vitamin was based on the mean value (Fig. 2). While thiamin, riboflavin and pyridoxal showed comparable values throughout the study (CV = 3.6–11.6%), FAD and nicotinamide revealed a greater variation (CV = 26.4% and 21.0% respectively), indicating that these compounds are less robust under the given condition and require more cautious handling.

To test the stability of the analytes during an analytical run, a control human milk sample was repeatedly injected every 15 injections to evaluate possible degradation of the vitamins in the auto sampler. A typical analytical run consisted of 50 samples; therefore, three control samples were injected in the beginning and subsequently every 15 injections as well as at the end of the analysis. While thiamin, riboflavin, FAD and pyridoxal did not reveal a considerable difference (CV < 10%), nicotinamide appeared to degrade over time. Evaluating 12 runs on 12 days over four months indicated a linear decrease of ~15% within the first 32 injections with a mean linear correlation coefficient (r) > 0.995 and a mean coefficient of determination (r^2) > 0.991, while no significant change occurred between the 32nd and the 50th injection (recovery: 84 and 83% respectively).

3.8. Application to human samples

For purpose of pilot testing and demonstration, the method was applied to 80 human milk samples from Cameroon, China, India, Malawi and the USA (Table 6). Analysis of Variance and Tukey's post hoc test for adjustment for multiple comparisons was performed using SAS® statistical software (SAS Institute, Cary, NC).

Since the samples were collected at different stages of lactation, possible changes in concentrations of the vitamins based on time of collection need to be considered. While amounts of thiamin, niacin and B-6 increase with duration of lactation, the vitamin B-2 concentrations remains relatively constant [33,34]. Despite the small

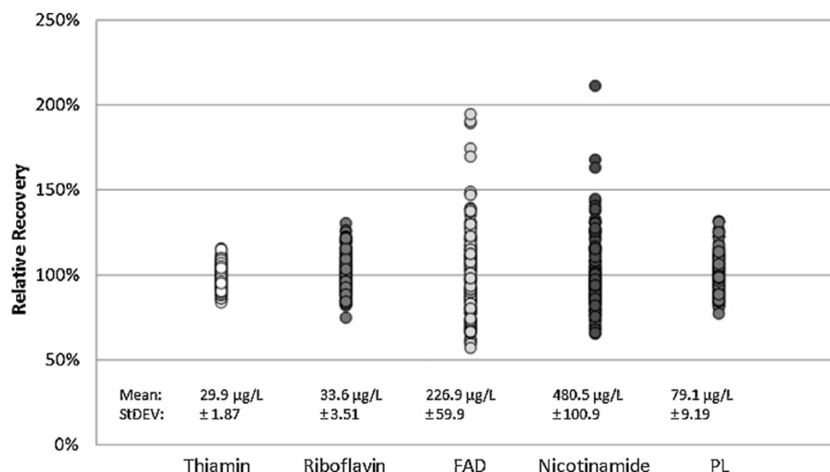

Fig. 2. Relative spread [%] of the results of a pooled human milk sample over 5 months (n = 151). Actual values for each analysis are graphed against the mean value (=100%). Analyses were carried out by two investigators using refrozen and non-refrozen aliquots at random.

Table 6
Median concentrations and range of B-vitamins [μg/L] from human milk samples of diverse geographic origin. Superscripts represent significant differences within variables at $p < 0.05$ (a: Cameroon (13–104 weeks p.p.), b: China (2–35 weeks p.p.), c: India (12–24 weeks p), d: Malawi (2–24 weeks p.p.), e: USA (4–12 weeks p.p.)).

Vitamin [μg/L]	AI	Cameroon (n = 5), a	China (n = 5), b	India (n = 24), c	Malawi (n = 18), d	USA (n = 28), e
Thiamin	200	116^{cde} (86–221)	31 (15–127)	11^{ae} (4–75)	21^{a} (2–152)	37^{ac} (5–66)
Riboflavin + FAD	350	399^{cd} (247–367)	361^{cd} (271–704)	142^{abe} (34–402)	148^{abe} (54–324)	324^{cd} (118–1163)
Riboflavin		52^{d} (52–80)	80^{cd} (30–223)	22^{bde} (5–94)	6^{abce} (0–35)	86^{cd} (32–845)
FAD		347^{cd} (195–557)	281^{cd} (241–481)	120^{abe} (29–308)	142^{abe} (54–289)	238^{cd} (86–818)
Nicotinamide	1800	161^{e} (46–248)	77^{e} (2–385)	334^{e} (100–890)	175^{e} (71–947)	1177^{abcd} (266–3179)
Pyridoxal	130	281de (58–361)	358de (66–692)	129de (65–406)	85^{abce} (27–303)	29^{abcd} (6–82)

number of non-representative samples and disregarding the different stages of lactation, there were significant differences across geographic origins (Table 6). Examining vitamin B-2, samples from Cameroon, China and the USA were not significantly different from each other, nor were those in India compared to Malawi. The USA samples differed significantly from all remaining origins in their nicotinamide concentration. Values for pyridoxal were not significantly different among samples from Cameroon, China and India, but all were significantly higher compared to samples from Malawi and the USA. Therefore, higher amounts of thiamin in the Cameroon samples might be explained by the later stage of lactation; however, the differences in vitamin B-2 concentration and the amounts of niacin in the Cameroon samples compared to those of the USA samples indicate differences based on the geographic origin rather than stage of lactation. Overall, the results show that vitamin content of human milk varies depending on the geographic origin, most likely due to differences in diet and fortification that remains to be explored; moreover, the influence of the lactation stage during sample collection needs to be investigated and its contribution to the variation of concentration determined.

4. Conclusion

This paper describes the first rapid UPLS–MS/MS analysis of thiamin, riboflavin, FAD, nicotinamide and pyridoxal simultaneously in human milk. Unlike methods used commonly in the past, this new approach provides more information from low sample volumes, in less time and with higher sensitivity. Including $^{13}C_3$-caffeine as an internal standard allows the evaluation of the method performance and system stability.

The method was validated by linearity, accuracy, matrix effect studies, LOD/LOQ, precision and stability. Even though matrix effects could not be avoided, the use of suitable stable isotope labeled internal standards for each vitamin could account for those effects. Since human milk contains endogenous vitamins, a matrix-like calibration curve was used for quantitation and the determination of recovery rates. Thiamin, riboflavin and pyridoxal are very stable even under suboptimal handling by different investigators, while FAD and nicotinamide appeared to be more sensitive to the given conditions. However, due to its sensitivity, the method allows the quantitation of all vitamins analyzed even in milk from severely depleted women.

Conflict of interest

The authors do not have associations of a commercial or other nature that might pose a conflict of interest.

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