Simultaneous Determination of Four Water-Soluble Vitamins in Fortified Infant Foods by Ultra-Performance Liquid Chromatography Coupled with Triple Quadrupole Mass Spectrometry

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Abstract

A novel ultra-performance liquid chromatography electrospray ionization tandem triple quadrupole mass spectrometry method for the simultaneous determination of four water-soluble vitamins, including vitamin B5 (VB5), vitamin B8 (VB8), vitamin B9 (VB9), and vitamin B12 (VB12) in fortified infant foods is developed and validated. A reverse phase UPLC separation system consisting of a Waters ACQUITY UPLC BEH C-18 column (2.1 mm × 100 mm i.d., 1.7 µm) and a binary gradient acetonitrile–water mobile phase is applied for the separation of the four water-soluble vitamins. Formic acid is spiked into the mobile phase to enhance the ionization efficiency. Tandem MS–MS analysis is performed in multi-reaction monitoring mode (MRM). Product-ion traces at *m/z* 220.1 → 89.9 for VB5, 245.1 → 227.1 for VB8, 442.3 → 295.2 for VB9, and 678.9 → 147.0 for VB12 are used for quantitation of the corresponding vitamins, and traces at *m/z* 455.5 → 308.0 are used for methotrexate (internal standard). Limits of quantitation (LOQs) are 0.016, 0.090, 0.020, and 0.019 µg/L for VB5, VB8, VB9, and VB12, respectively. Intra- and inter-day precisions for the determination of the four vitamins are better than 6.84% and 12.26% in relative standard deviations, and recoveries for the four vitamins are in the range of 86.0~101.5%. The developed approach is applied for the determination of the trace amounts of the vitamins in fortified milk powers and fortified rice powers.

Introduction

Vitamins are minor but essential constituents of human bodies. Although a balanced diet usually supplies all the vitamins required by the human body, significant subgroups in most populations are still subject to the risks associated with low micronutrient intakes (1). Addition of vitamins and minerals to foods, therefore, may be useful to address this risk.

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According to their solubility, vitamins are divided into two groups: water-soluble vitamins and fat-soluble vitamins. The former group includes diverse compounds (2) such as thiamine (VB1), riboflavin (VB2), pantothenic acid (VB5), VB6 vitamers (pyridoxal, pyridoxine, and pyridoxamine), cyanocobalamin (VB12), L-ascorbic acid and L-dehydroascorbic acid (VC), niacin (nicotinic acid) and its amide (nicotinamide, vitamin PP), folic acid (VB9), and biotin (VB8).

VB5, known as pantothenic acid (Figure 1A), is involved in several biological functions, and its deficiency is associated with metabolic and energetic disorders in humans (3,4). It has widely been used in fortified infant foods with dosages ranging from 1.7~5 µg/100 g.

VB8, known as biotin (Figure 1B), is an essential co-factor for five biotin-dependent carboxylases: acetyl-CoA carboxylase-α, acetyl-CoA carboxylase-β, propionyl-CoA carboxylase, pyruvate carboxylase, and β-methylcrotonyl-CoA carboxylase (5). Recent evidence indicates that pregnant women develop marginal biotin deficiency during normal pregnancy (5,6). It has widely been used in fortified infant foods with dosage between 5~20 µg/100 g.

VB9, known as folic acid (Figure 1C), is essential for normal human cell division and cell growth. A deficiency of VB9 in the diet is closely linked to the presence of neural tube defects in newborns and to an increased risk of megaloblastic anemia, cancer, Alzheimer's disease, and cardiovascular disease in adults (7,8). It has widely been used in fortified infant foods with dosage between 65~300 µg/100 g.

VB12 is an essential nutrient for all cells. It acts as a co-enzyme for normal DNA synthesis and promotes normal fat and carbohydrate metabolism. In addition, vitamin B12 promotes growth and cell development, and plays an integral role in the development and maintenance of the myelin sheath surrounding nerve cells (9). It has widely been used in fortified infant foods with dosage between 0.3~1.8 µg/100 g.

The rapid and reliable analysis of vitamins in foods repre-

sents a relevant objective for food manufacturers; however, the determination of vitamins in food represents a complex analytical problem for several reasons (10): (*i*) due to their different chemical structures and properties, it is extremely difficult to develop a "general" method suitable for the simultaneous determination of several vitamins in foods; (*ii*) foods themselves are very complex matrixes; (*iii*) of which vitamins are only micro-constituents; and (*iv*) in the case of fortified foods, the natural vitamin content should be accurately known in order to make additions in compliance with terms set by national regulations.

For the determination of water-soluble vitamins, various methods such as microbiological assays (11), volumetric assays (12), spectrometric assays (13), capillary electrophoresis (14), thin-layer chromatography (15), high-performance liquid chromatography (HPLC) (16–19), gas chromatography (20), and HPLC–mass spectrometry (LC–MS) (10,21) have been reported. Among these methods, LC–MS is a promising method for the simultaneous determination of multiple vitamins in food or multivitamin tablets because of its high sensitivity and specificity. Previous reports (10,21–24) have demonstrated that the LC–MS–MS provides sufficient sensitivity and selectivity for the determination of VB1, VB2, VB6 vitamers (pyridoxal, pyridoxine, and pyridoxamine) and nicotinic acid. However, the sensitivity for VB5, VB8, VB9, and VB12 is limited and needs further improvement.

In the last few years, column packings with particle sizes in the range of 1–2 µm have been employed in liquid chromatography to improve its separation efficiency. According to the Van Deemter equation, as the particle size decreases to less than 2 µm, the separation efficiency is basically not influenced by increasing the flow rate of mobile phase. Nevertheless, the pressure required to pump the mobile phase through a column packed with such small particles is much higher than the uperlimit (below 400 bar) that standard HPLC hardware can withstand. Recently, ultra-performance liquid chromatography (UPLC), which can be operated at ultra-high pressure (for instance 1000 bar), has been applied (25,26). The advantage of UPLC is that quantitative response is improved and the analysis time can be shortened by raising the flow rates, thus allowing a higher sample throughput. However, the UPLC operated in high mobile phase flow rate needs to be coupled to a detector with high response rate. Mass spectrometry systems with triple quadrupole analyzers operated at fast scan rate can meet this requirement (26).

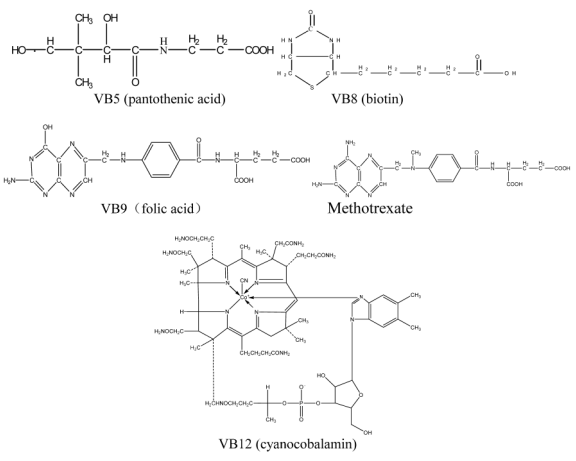

Figure 1. Chemical structures of VB5, VB8, VB9, VB12, and methotrexate.

In this work, the applicability of UPLC–ESI-MS–MS with multiple reaction monitoring (MRM) mode was evaluated for the simultaneous determination of VB5, VB8, VB9, and VB12 in fortified infant foods.

Experimental

Samples, chemicals and standards

VB5, VB8, VB9, and VB12 were purchased from Sigma-Aldrich (purity ≥ 99%, St. Louis, MO). Methotrexate (purity ≥ 99%, internal standard) was from Fluka (St. Louis, MO). Acetonitrile and methanol of HPLC-grade were purchased from Merck (Darmstadt, Germany) and used without further purification. Formic acid, ammonium hydroxide, and ammonium acetate were purchased from Merck (Darmstadt, Germany). Ultra-pure water from a Millipore system (Millipore, Bedford, MA) was used throughout the work. All reagents used were of analytical grade or better.

Ten brands of fortified milk powders and nine brands of rice powders were purchased from local supermarkets.

Solutions preparation

Aqueous stock solutions (approximately 0.1 mg/mL) were prepared for VB5, VB8, VB9, VB12, and methotrexate (internal standard). They were stable for at least 1 month when stored in the dark at 0°C. An aqueous mixed working solution containing the four vitamin standards at the concentration of 1 µg/mL was prepared weekly. Calibration standards ranging from 1–200 ng/mL, each of them containing 20 ng/mL methotrexate as internal standard, were prepared by step-wise dilution of the working solution with water.

Food sample preparation

One gram of each food sample was accurately weighed into a 50 mL centrifuge tube, and 1 mL methotrexate solution (10 ng/mL) and 9 mL ammonium acetate solution (9 mmol/mL) were sequentially added. After thoroughly shaking the mixture for 5 min over a magnetic stirring plate, it was extracted for 15 min, using an ultrasonic bath. After adding 10 mL of chloroform and shaking again for 1 min over a magnetic stirring plate, the mixture was centrifuged for 10 min at 10,000 rpm. Eventually, the supernatant was filtered through a 0.22-µm filter and the filtrate collected for UPLC–MS–MS analysis.

LC–MS

Separation, identification, and quantitation of the four water-soluble vitamins were performed with a coupled liquid chromatography–tandem mass spectrometry system consisting of an Acquity Ultra-performance liquid chromatography (Waters, Milford, MA) and a Quattro Ultima Pt (Micromass, Manchester, UK) tandem mass spectrometer. An Acquity UPLC

BEH C18 column (2.1 mm × 100 mm i.d., 1.7 µm) was used for LC separation. The column oven was maintained at 35°C, the flow rate of the mobile phase was 0.2 mL/min, and the injection volume was 10 µL. The binary mobile phase used consisted of acetonitrile and water containing 0.1% formic acid. The acetonitrile was linearly increased from 0% to 20% in 5 min, then increased to 100% in 0.2 min and held for 1 min, finally brought back to 0%, and held for 4 min to the next injection. The mass spectrometer was operated in positive mode electrospray ionization in MRM mode. The experimental parameters for MS are listed in Table I.

The capillary voltage was maintained at 3.50 kV. The cone voltage was 55 V. The multiplier voltage was 650 V. The nebulizing, desolvation, and cone gas were supplied with nitrogen. The nebulizing gas was adjusted to the maximum, the flow of the desolvation gas and cone gas were set to 400 L/h and 50 L/h, respectively. Source temperature and desolvation gas temperature were held at 120°C and 350°C. The RF lens 1 and 2 were set at 12.5 and 12.5. The ion energy 1 and ion energy 2 were 0.6 and 1.4. The entrance and exit were 10 and 12.5. The collision gradient was 1.0. During tandem mass spectrometric analysis, UHP argon was used as the collision gas and the pressure of collision chamber was kept at 3.0×10^{-3} mbar.

The Precursor ion and product ion (characteristic ion) traces were monitored by tandem MS–MS. Collision energy (CE) was optimized for each product ion trace. The traces monitored and the corresponding CE values are listed in Table II.

Data evaluation

VB5, VB8, VB9, and VB12 were identified based on both retention time and characteristic ion peaks in mass spectrum. Quantitative analyses were carried out by internal standard calibration.

Table I. Calibration Curve Parameters, LOD, and LOQ for Four Vitamins

Compound	Standard curve	Correlation coefficient (*r*)	LOD* (µg/L)	LOQ† (µg/L)	Linear range
VB5	$Y = 0.8212x + 0.1755$	0.997	0.005	0.016	0.1~10
VB8	$Y = 1.6963x + 0.2863$	0.998	0.030	0.090	0.1~10
VB9	$Y = 0.6275x + 0.1161$	0.999	0.006	0.020	0.1~10
VB12	$Y = 0.4911x + 0.03896$	0.999	0.006	0.019	0.1~10
methotrexate			0.015	0.05	

* LOD = Limit of detection (S/N = 3).
† LOQ = Limit of quantitation (S/N = 10).

Table II. Mass parameters for the Four Vitamins and Methotrexate

Compound	Molecule weight	Parent ion	Daughter ion	Dwell(s)	Collision energy (eV)
VB5	220.1	220.1 $[M+1]^+$	89.9*	0.04	12
			202.0	0.04	11
VB8	245.1	245.1 $[M+1]^+$	227.1*	0.04	10
			172.0	0.04	19
VB9	442.3	442.3 $[M+1]^+$	295.2*	0.04	10
			177.0	0.04	16
VB12	1355.4	678.9 $[M/2+1]^+$	147.0*	0.04	30
			359.3	0.04	20
methotrexate	455.5	455.5 $[M+1]^+$	308.0*	0.04	20
			174.8	0.04	36

* Quantificational ion.

Results and Discussion

Sample pretreatment

The determination of vitamins in food represents a complex analytical problem for several reasons: (*i*) foods themselves are very complex matrixes; (*ii*) of which vitamins are only microconstituents; and (*iii*) vitamins are easily destroyed by strong acids or alkali. Sample pretreatment is pivotal to quantitation of vitamins in foods, the procedures include removing of protein and isolation of the vitamins by extraction. Extraction solvent and time of extraction should be subject to optimization.

Extraction medium

To obtain complete extraction of the regarded vitamins from food samples, three extraction media including water, 0.1% formic acid aqueous solution, and 10 mmol/L ammonium acetate aqueous solution were tested. It was found (see Figure 2) that 10 mmol/L ammonium acetate aqueous solutions gave the

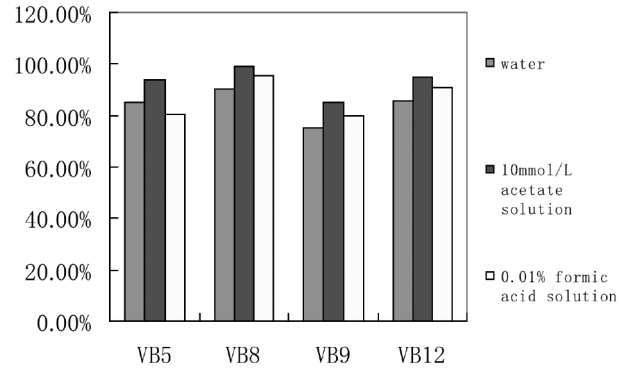

Figure 2. Effect of extracting solvents on recoveries of VB5, VB8, VB9, and VB12.

best recoveries: 93.6%, 99.1%, 85.3%, and 95.0% for VB5, VB8, VB9 and VB12, respectively.

Extraction time

The extraction was carried out using an ultrasonic bath and the ideal duration for the ultrasonic extraction was investigated in the range of 0–30 min. As shown in Figure 3, the recoveries of the four vitamins increased with increasing extraction time up to 10 mins. However, the recoveries decreased when the extraction time was longer than 15 min, presumably due to the instability of the vitamins. Based on these observations, a duration of 10–15 min was understood as ideal extraction time.

Solvent used for eliminating proteins and lipids

Prior to HPLC separation, the co-extracted proteins and lipids should be removed from the prepared solution. Several organic solvents were employed to eliminate the proteins and lipids. Out of the tested solvents (chloroform, acetonitrile, and ethyl acetate), chloroform precipitated proteins and lipids more effectively than acetonitrile and ethyl acetate did. Thus, chloroform was used as a solvent for eliminating proteins and lipids.

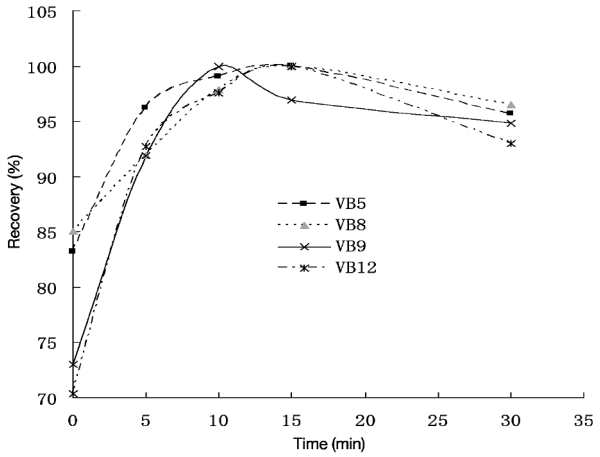

Figure 3. Effect of extraction time on recovery of VB5, VB8, VB9, and VB12.

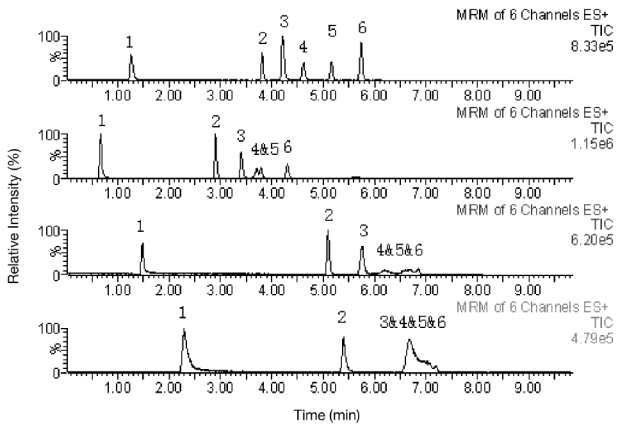

Figure 4. The LC–MS–MS chromatograms obtained with four different LC columns.

LC

Column

In preliminary tests, four ODS C18 columns with different column size and/or different particle size were compared regarding the separation of VB5, VB8, VB9, VB12, and methotrexate (internal standard). The column A is a Waters Atlantis C18 column (2.1 mm i.d. × 150 mm, particle size 5 µm), column B is a Waters Sunfire C18 column (2.1 mm i.d. × 150 mm, particle size 5 µm), column C is a Waters Acquity UPLC BEH C18 column (2.1 mm i.d. × 50 mm, particle size 1.7 µm), and column D is a Waters Acquity UPLC BEH C18 column (2.1 mm i.d. × 100 mm, particle size 1.7 µm).

It was found (Figure 4) that columns A and B, two frequently used columns for LC–MS, could neither separate the four vitamins and methotrexate effectively, nor generate reproducible peak areas in parallel runs, while column C could not separate methotrexate and VB12. Only column D could separate the vitamins and methotrexate, and support constant retention times and peak areas in parallel experiments. Therefore, column D was used for further studies.

Mobile phase

A binary mixture of acetonitrile and water was used as the mobile phase for the separation of the vitamins. To achieve the

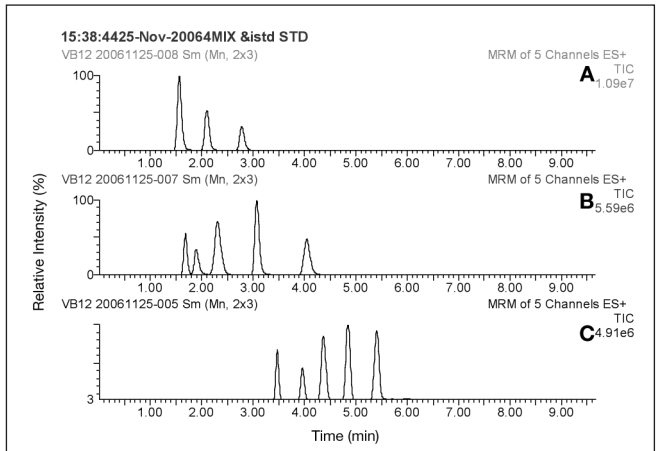

Figure 5. Effect of elution conditions on the vitamin separation.

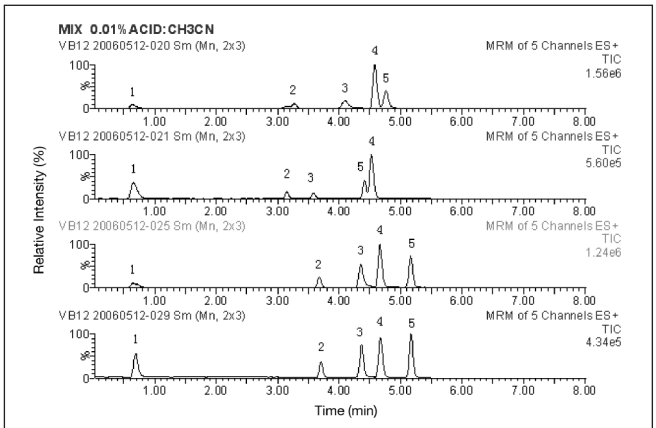

Figure 6. Effect of additives in mobile phase on vitamin separation.

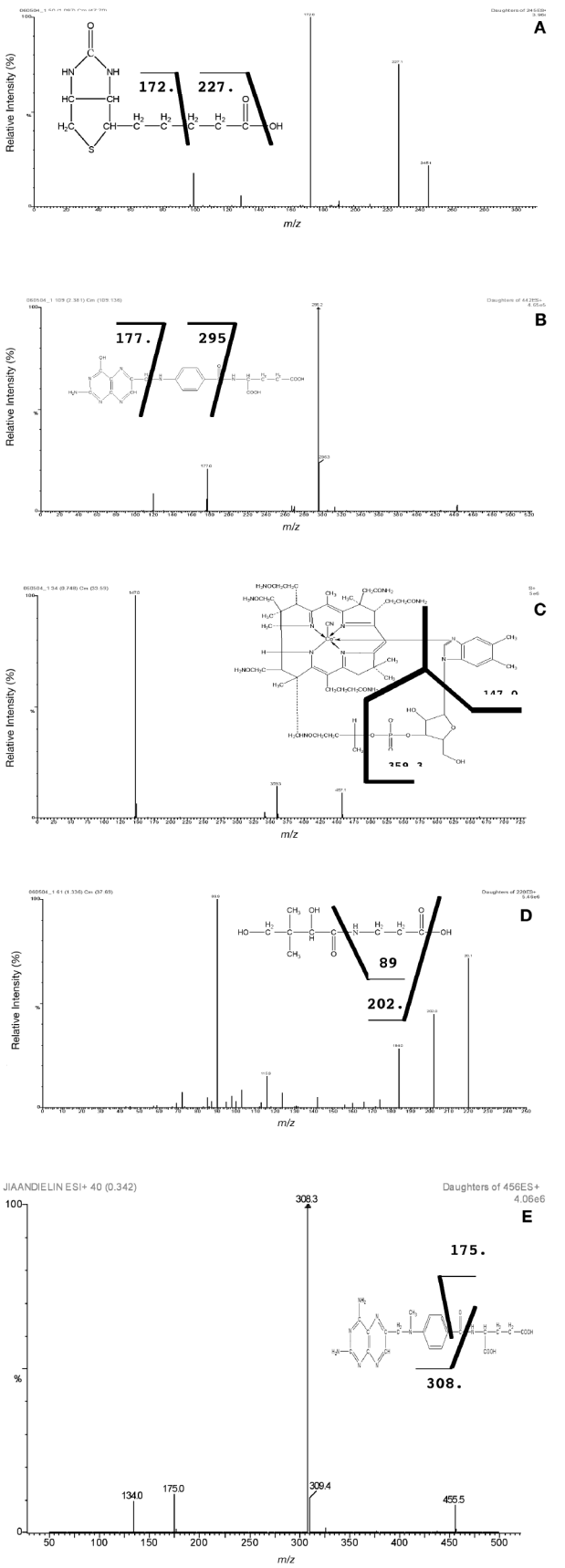

Figure 7. MS–MS spectra of the four vitamins.

best separation of the four vitamins and the internal standard, the water to acetonitrile ratio was optimized by either isocratic elution or gradient elution. Under the isocratic conditions, four vitamins were eluted too fast to be completely separated (see Figure 5A and 5B). Better separation was generated by linear gradient elution (Figure 5C). Thus, the linear gradient elution as described in the Liquid chromatography–mass spectrometry Section was applied.

Because the LC column is coupled with the ESI source, the composition of the mobile phase may also affect the sensitivity of the MS detection. In the present study, ammonium acetate (10mM), ammonia (0.1%), and formic acid (0%, 0.01%, and 0.1%) were employed as additives to improve the ionization efficiency. Results (Figure 6) revealed that addition of 0.1% formic acid to the mobile phase enhanced the MS responses by a factor of 0.8212, 1.6962, 0.6275, and 0.4911 for VB5, VB8, VB9 and VB12, respectively. Therefore, 0.1% formic acid was added to the water used for gradient elution.

MS

Since the levels of the four vitamins in fortified infant foods are rather low, multiple reaction monitoring (MRM), the most sensitive detection mode, was employed. Before optimization of the operational parameters of the tandem MS–MS system, full scans and product scans under both positive and negative ionization mode were carried out for each vitamin by direct sample infusion to select the most abundant mass-to-charge ratio (*m/z*) for further studies. Regarding the negative ionization mode full scan, no relevant ions could be detected in the mass spectrum. The mass spectra (see Figure 7) obtained using positive ionization mode full scan show that the most abundant forms of protonated molecules of VB5, VB8, VB9, VB12, and methotrexate were $[M+H]^+$ (*m/z* = 220.1), $[M+H]^+$ (*m/z* = 245.1), $[M+H]^+$ (*m/z* = 442.3), $[M+2H]^{2+}$ (*m/z* = 678.9), $[M+H]^+$ (*m/z* = 162.1), and $[M+H]^+$ (*m/z* = 455.5), respectively. Therefore, these five protonated molecules were respectively confirmed as precursor ions of the corresponding four vitamins and methotrexate for the following collision induced decomposition (CID) fragmentation.

The abundance of the product ions was affected by the collision energy (CE). Table II lists the characteristic ions and the corresponding collision energy for each vitamin. Showing the individual CID mass spectra for each of the four vitamins, Figure 7 illustrates that the major product ions were at *m/z* 89.9 and 202.0 for VB5 (Figure 7A), at *m/z* 172.0 and 227.1 for VB8 (Figure 7B), at *m/z* 177.0 and 295.2 for VB9 (Figure 7C), and at *m/z* 147.0 and 359.3 for VB12 (Figure 7D). Focused on these precursor ions and product ions, the MS–MS parameters were optimized, as described in the Liquid chromatography–mass spectrometry Section

Internal standard

Due to the lack of isotope-labeled internal standards of the vitamins concerned, methotrexate was selected as the internal standard because it is usually absent in fortified infant foods, its molecular structure is similar to VB9 (MW = 454), and it has a suitable retention time (4.78 min) according to the retention times of the four vitamins in the present LC

system. With the MRM mode, a $[M+H]^+$ ion with *m/z* 455.5 was used as the precursor ion, and traces of *m/z* 174.8 and *m/z* 308.0 (Figure 7) were used for quantitation and supplementary identification, respectively. Recovery tests showed that recoveries of methotrexate were 86.8%, 93.5%, and 98.1%, respectively, similar to those of the concerned vitamins.

Method validation

Linear range and limits of detection

An internal standard calibration curve was obtained using eight standard solutions with concentrations ranging from 0.1~10.0 µg/L for VB5, 0.1~10.0 µg/L for VB8, 0.1~10.0 µg/L for VB9, 0.1~10.0 µg/L for VB12, and 20 µg/L methotrexate. Linear calibration curves were obtained in the tested concentration ranges for all of the four vitamins. Table I lists the linear regression equations and the corresponding correlation coefficients (*r*).

The achieved limits of detection (LOD) (based on a signal-to-noise ratio of 3) and the limits of quantitation (LOQ) (based on a signal-to-noise ratio of 10) were 0.005 and 0.016 µg/L for VB5, 0.030 and 0.090 µg/L for VB8, 0.006 and 0.020 µg/L for VB9, 0.006 and 0.019 µg/L for VB12, and 0.015 and 0.05 µg/L for methotrexate, respectively.

Precision and accuracy

The precision of the developed method was evaluated by repeated analysis of a sample. The sample was pre-treated with the procedure described in the Food sample preparation Section and subjected to UPLC–MS–MS analysis as described in the Liquid chromatography–mass spectrometry Section. For evaluation of the intra-day precision, 11 parallel test solutions were prepared and analyzed in one day, and the relative standard deviations (RSDs) for the 11 measurements were examined. For evaluation of inter-day precision, three parallel test solutions were prepared and analyzed on each of 11 consecutive days, and the RSDs for the measurements performed were examined. The observed results of intra- and inter-day precision for the four vitamins are listed in Table III.

Table III. Intra-day and Inter-day Precision for the Four Vitamins

Intra-day precision (*n* = 11) Item	VB5	VB8	VB9	VB12
Conc.	1.88 mg/100 g	26.02 µg/100 g	91.23 µg/100 g	2.00 µg/100 g
RSD (%)	4.45	6.84	5.20	3.04
Inter-day precision (*n* = 11) Item	**VB5**	**VB8**	**VB9**	**VB12**
Conc.	1.84 mg/100 g	26.00 µg/100 g	91.22 µg/100 g	2.01 µg/100 g
RSD (%)	7.75	11.5	10.1	12.3

The accuracy of the developed method was validated by spiked-recovery tests. A milk powder bought from a local food market was used as a control sample, and the 4 vitamins and the internal standard were spiked into the sample at three concentration levels (Table IV). Then, both the original sample and the vitamin-spiked ones were subjected to the sample preparation procedure described in the Food sample preparation Section and UPLC–MS–MS analysis described in the Liquid chromatography–mass spectrometry Section. As listed in Table IV, satisfying recovery rates ranging from 85.0–105.0% were obtained for the tested vitamins by the developed method.

Table IV. Results of Spike-Recovery Tests (*n* = 6)

Compound name	Content in original sample (µg/100 g)	Spiked (µg/100 g)	Detected (µg/100 g)	Recovery (%)
		2.5	184.6	99.6
		7.5	189.6	100.0
VB5	182.064	20.0	202.1	101.5
		2.5	3.5	99.2
		7.5	8.5	99.9
VB8	1.042	20.0	21.1	100.1
		2.5	3.3	89.6
		7.5	8.5	98.7
VB9	1.091	20.0	21.1	99.6
		0.5	0.5	86.0
		2.5	2.5	98.4
VB12	0.034	7.5	7.5	99.9
		0.5	0.4	86.8
Methotrexate	0.000	2.5	2.3	93.5
		7.5	7.4	98.1

Specificity

Both the blank sample and the methotrexate-spiked one were subjected to the sample preparation procedure described in the Food Sample preparation Section and UPLC–MS–MS analysis described in the Liquid chromatography–mass spectrometry Section for investigation of potential interference with endogenous substances. The results showed that no endogenous peaks that would interfere with the determination of the four vitamins, and the internal standard could be observed (Figure 8). These results suggest that each selected ion was specific for the corresponding analyte.

Application

The developed method was applied to analyze 10 brands of fortified milk powders

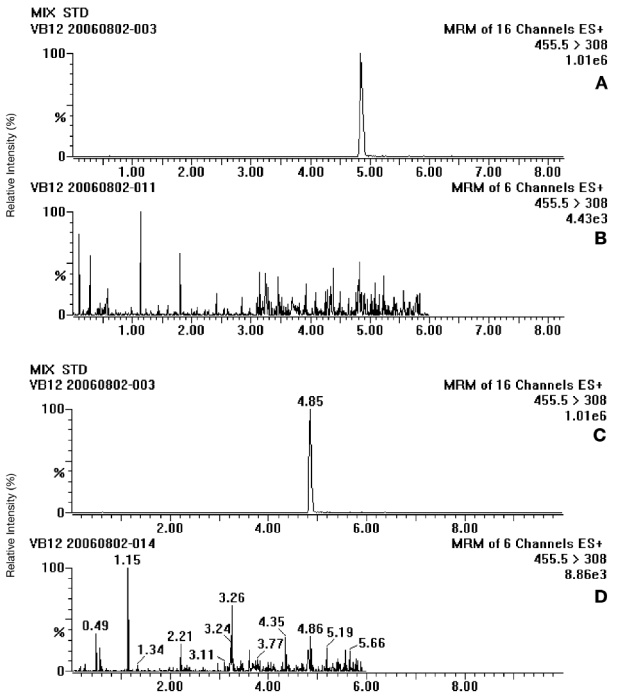

Figure 8. LC–MS–MS chromatograms of the blank sample and a sample spiked with methotrexate.

Table V. Analytical Result of the Vitamin Contents in Fortified Milk Powders and Fortified Rice Powders

Sample	VB5 (mg/100 g)	VB8 (μg/100 g)	VB9 (μg/100 g)	VB12 (μg/100g)
1# milk powders	2.346	35.563	41.017	1.084
2# milk powders	1.284	20.963	20.363	0.853
3# milk powders	1.905	26.102	91.167	2.011
4# milk powders	1.111	47.328	76.522	0.310
5# milk powders	2.284	32.364	63.548	2.388
6# milk powders	1.550	20.853	199.942	0.030
7# milk powders	2.496	51.149	41.611	1.935
8# milk powders	2.333	43.933	35.215	3.035
9# milk powders	2.351	26.206	153.300	0.352
10# milk powders	2.315	35.787	30.480	0.266
1# rice powders	1.644	1.067	164.936	0.867
2# rice powders	0.207	0.576	2.373	–
3# rice powders	0.329	0.484	0.992	–
4# rice powders	1.646	11.136	94.870	1.928
5# rice powders	0.182	1.042	1.090	0.034
6# rice powders	1.341	25.404	34.409	0.933
7# rice powders	0.979	2.343	14.433	0.738
8# rice powders	0.345	0.724	0.859	–
9# rice powders	2.369	8.222	135.401	1.020

and 9 brands of fortified rice powders, which were purchased from a local food market. The obtained results listed in Table IV and Figure 9, are consistent with those claimed by the producers. So, the method can be applied successfully to analyze VB5, VB8, VB9, and VB12 in the fortified rice powders and fortified milk powders.

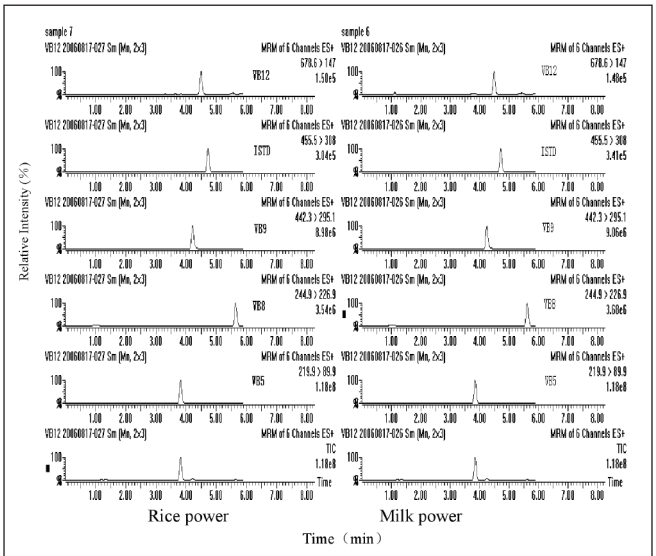

Figure 9. Typical LC–MS–MS chromatograms of rice powder and milk power on different channels.

Conclusion

The newly developed method of UPLC–ESI-MS–MS using the MRM mode has been proved to be an effective, sensitive, selective, and reliable approach for the individual determination of trace or ultra-trace levels of VB5, VB8, VB9, and VB12 in fortified infant foods.

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