Analysis of the Vitamin B Complex in Infant Formula Samples by LC-MS/MS

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OVERVIEW

A rapid, robust, sensitive and specific LC-MS/MS assay using the SCIEX QTRAP® 6500 system has been developed for the simultaneous detection of all major forms of vitamin B complex. The method detects all currently used forms of vitamin B6 and vitamin B3 in infant formula and includes vitamin B12. The sample preparation allows the same extract to be used for Vitamin C detection and the LC-MS/MS conditions have been tuned so that the response for each vitamin is linear over the required detection ranges.

INTRODUCTION

The vitamin B complex is a group of water-soluble vitamins that play important roles in cell metabolism. The absence of individual dietary B vitamins can lead to several conditions, including depression and high blood pressure so they are often added to foods, especially infant formula. Human daily nutritional recommendations for the members of the vitamin B complex vary considerably, for example from 6 µg of vitamin B12 to 20 mg of vitamin B3 (Table 1). The US Food and Drug Administration regulates food labels in the United States and food labeling is required for most prepared foods such as breads, cereals, canned foods, snacks, drinks, and especially for infant formula, which is highly regulated.[1]

Analysis of food samples can be challenging, as the matrices are complex and sensitive methods typically require highly selective sample clean up procedures. Vitamin B is a complex mixture of compounds (Figure 1) whose pKa values range from 0.5 to 10.2, making their extraction and analysis challenging.

Several methodologies exist to look at these analytes in separate classes, but relatively few sensitive and specific analytical methods exist that examine the vitamin B complex as a whole, with high throughput capabilities and minimal sample preparation.

Table 1. Daily required values (DV) of B vitamins for a human adult as obtained from the FDA[1].

Vitamin3		DV (mg)
Thiamine	B1	1.5
Riboflavin	B2	1.7
Niacin	B3	20
Pantothenic acid	B5	10
Pyridoxal	B6	2
Biotin	B7	0.3
Folic acid	B9	0.4
Cyanocobalamin	B12	0.006

Here we present new data acquired by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) from a quantitative method that contains vitamins B1, B2, B3 (two forms), B5, B6 (three forms), B7, B9 and B12. Instrument detection levels for these vitamins using the QTRAP® 6500 have been shown to be ≤1 ng/mL for the neat compounds using positive mode Electrospray Ionization (ESI) and the Scheduled MRM™ algorithm. The required limits of detection vary greatly between each vitamin, but all the B vitamins can be detected in infant formula using the method described herein, even with detection limits having a 10,000-fold range.

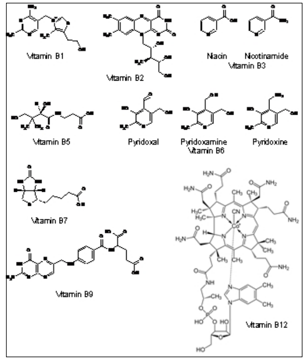
The LC-MS/MS method utilizes a small particle size polar endcapped reversed phase (RP) column and an 11 min gradient. Very little sample preparation has been used to enable a high throughput suitable for routine food testing.

EXPERIMENTAL

Standards
All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and are commonly available. NIST SRM 1849a infant formula reference material (LGC, UK) was used to develop the method and verify the method performance.

Sample Preparation
Sample (1 g) was mixed with 50% acetonitrile in acidified water (containing an antioxidant) and internal standard solution was added. This was then shaken vigorously for 1 minute and roller mixed for 10 minutes (protected from light). After centrifugation the supernatant was filtered and the filtrate diluted 1 in 20 with water containing an ion paring reagent. The sample preparation was kept as simple as possible to reduce vitamin breakdown, with SPE no longer needed for the late eluting B7, B9, and B12 vitamins.

During the development work the effects of light, temperature, and acidity on standard stability were tested and it was found that the use of amber glass with a lower pH and the presence of an antioxidant helped stabilize the extracts.



LC Separation
Samples were separated by LC on a polar endcapped RP column using a Shimadzu UFLC$_{XR}$ system over an 11 minute gradient from acidified water to 100% methanol containing 0.1% formic acid (Table 2). The column temperature was maintained at 50ºC and an injection volume of 20 µL was used. The separation was designed to allow retention of the early eluting vitamins until after the solvent front and to make sure that the late eluting vitamins were baseline resolved to help reduce possible ion suppression. Although the last vitamin (B12) eluted at 5.2 minutes the column was washed and equilibrated for a further six minutes to stabilize retention times between injections.

MS/MS Detection
Analysis was performed on an SCIEX QTRAP® 6500 system. The source conditions were a standard set up of Curtain Gas™ interface of 35 psi, IonSpray™ source voltage = 5500V (positive polarity), gas 1 = 50 psi and gas 2 = 60 psi, source temperature = 550 ° C, and collision gas = 10 psi. The MRM transitions used are shown in Table 3, with the resolution kept at unit for both Q1 and Q3. Two MRM transitions were monitored for each compound to use the ratio of quantifier and qualifier transition for compound identification. The Scheduled MRM™ algorithm was used to acquire data for a total of 28 transitions to ensure the best reproducibility and accuracy.

All results were processed in PeakView® software version 2.2 and MultiQuant™ software version 3.0.2.

Table 2. LC gradient conditions.

Step	Time (min)	Flow (µL/min)	A (%)	B (%)
0	0.0	500	100	0
1	2.0	500	100	0
2	2.5	500	75	25
3	5.0	500	57	43
4	5.5	500	2	98
5	5.6	500	2	98
6	6.0	1000	2	98
7	6.2	1000	2	98
8	6.3	1000	100	0
9	10.0	1000	100	0
10	10.5	500	100	0
11	11.0	500	100	0

Table 3. MRM transitions and retention times (RT).

Compound	RT (min)	Q1 (amu)	Q3 (amu)
B1 1	1.5	265	81
B1 2	1.5	265	122
B2 1	5.1	377	172.2
B2 2	5.1	377	198.1
B3 niacin 1	1.2	124	53
B3 niacin 2	1.2	124	80
B3 nicotinamide 1	1.5	123	80
B3 nicotinamide 2	1.5	124	81
B5 1	2.7	220	98
B5 2	2.7	220	90
B6 pyridoxal 1	1.6	168	94
B6 pyridoxal 2	1.6	168	67
B6 pyridoxamine 1	0.9	169	134
B6 pyridoxamine 2	0.9	169	106
B6 pyridoxine 1	1.9	170	134
B6 pyridoxine 2	1.9	170	152
B7 1	4.6	245	227
B7 2	4.6	245	97
B9 1	4.9	442	176
B9 2	4.9	442	120
B12 1	5.2	678.4	147
B12 2	5.2	678.4	359

RESULTS

Due to the extended dynamic range requirements and the large differences in limits of detection required for this class of vitamins, some responses had to be adjusted in order to maintain a linear response across the required concentration range. To this end, the collision energies (CE) were adjusted to decrease the vitamin responses by ramping the CE. The CE ramps were automatically generated during method development using the 'Compound Optimization' feature in Analyst® data acquisition software. An example of this is shown in Figure 1. Adjusted CE values are listed in Table 4.

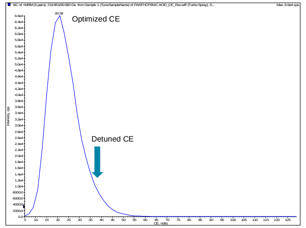


Figure 1. A typical ramp of the collision energy (CE) for a vitamin B5 fragment ion. Using this approach the more sensitive vitamins that showed a non-linear response at higher concentrations were detuned by choosing non-optimal collision energies.

Table 4. The effect of adjusting the collision energy (CE) on reducing the overall response for different vitamins.

Compound	CE (optimal)	CE (adjusted)	Response Decrease
B1	21	53	10x
B2	49	78	10x
B3 niacin	31	55	20x
B3 nicotinamide	29	50	10x
B5	21	38	10x
B6 pyridoxine	19	31	10x

Even though the responses were decreased by changing the CE for some of the vitamins, a 5 ng/mL solvent standard (Figure 2) clearly shows that all the vitamins were easily detected.

Linearity was studied using solvent standards taken through the same sample preparation procedure as the reference material (equivalent to 0.1 to 100 mg/kg in matrix) for all the vitamins except B12, where the range was 0.01 to 100 mg/kg. Linear fit with 1/x weighting was used for all target compounds resulting in coefficients of regression (r) ≥ 0.994. Internal standards were used to achieve the best quantitative results (Table 5).

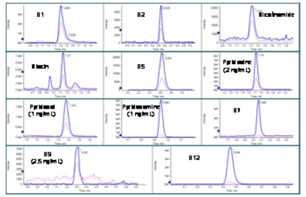


Figure 2. Example chromatograms for a B vitamin 5 ng/mL solvent standard (unless indicated otherwise). Quantifier (blue) and qualifier (pink) transitions are overlaid.

Table 5. Linear dynamic range (LDR) and coefficients of regression (r) for each vitamin.

Compound	Internal Standard	LDR (mg/kg)	r
B1	B1 - D_3	0.1 - 100	0.997
B2	B2 - D_6	0.1 - 100	0.959
B3 niacin	B3 niacin - D_3	0.1 - 100	0.997
B3 nicotinamide	B3 niacin - D_3	0.1 - 100	0.998
B5	B5 - ^{13}C, D_2	0.1 - 100	0.994
B6 pyridoxal	B3 niacin - D_3	0.1 - 100	0.998
B6 pyridoxamine	B3 niacin - D_3	0.1 - 100	0.995
B6 pyridoxine	B3 niacin - D_3	0.1 - 100	0.997
B7	B7 - D_4	0.1 - 100	0.997
B9	B7 - D_4	0.1 - 100	0.996
B12	none	0.01 - 100	0.999

Once each vitamin had their linear response verified for the desired dynamic range, extracts of the NIST 1849A infant formula reference material were prepared. The results of these extracts are shown in Table 6 and example chromatograms are shown in Figure 3. Note that only those compounds with certified mass fraction values are summarized herein. Pyridoxamine and pyridoxal are not evaluated in the NIST 1849a certificate of analysis and B12 does not have a certified mass value (only a reference mass fraction). MultiQuant™ software version 3.0 automatically calculates qualifier ion ratios and flags outliers. The peak review of an extract of NIST 1849A reference material with qualifier ion ratio tolerances is shown in Figure 4.

Table 6. Results from the repeat analysis of NIST 1849a reference material (mg/kg) across SCIEX sites. Experimental values are shown ± 1 standard deviation (n = 3). NIST results are shown ± the uncertainty at 95% confidence.

Compound	NIST 1849a	UK	Singapore	Canada	Average
Thiamine (B1)	12.57 ± 0.98	17.1 ± 0.31	15.0 ± 0.59	12.5 ± 0.23	14.0 ± 0.53
Riboflavin (B2)	20.37 ± 0.52	16.5 ± 0.36	17.5 ± 0.35	23.47 ± 0.49	19.86 ± 0.44
Nicotinamide (B3)	109 ± 10	105 ± 3.15	98.9 ± 2.67	104 ± 5.09	101 ± 5.04
Pantothenic Acid (B5)	68.2 ± 1.9	81.8 ± 1.96	70.8 ± 1.49	66.9 ± 1.01	69.25 ± 1.52
Pyridoxine (B6)	13.46 ± 0.93	13.9 ± 0.39	12.1 ± 0.65	13.07 ± 0.37	12.47 ± 0.56
Biotin (B7)	1.99 ± 0.13	1.96 ± 0.06	1.93 ± 0.09	2.09 ± 0.05	1.99 ± 0.08
Folic Acid (B9)	2.29 ± 0.06	2.45 ± 0.12	2.48 ± 0.09	2.65 ± 0.37	2.55 ± 0.16

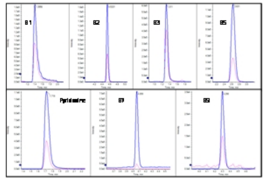


Figure 3. NIST 1849A reference material extract chromatograms for the fortified vitamins. Vitamin B3 is present as nicotinamide and vitamin B6 as pyridoxine.

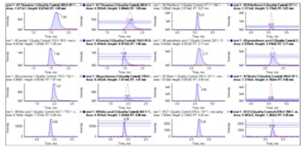


Figure 4. Peak review with ion ratio tolerances of an extract from NIST 1849A reference material.

SUMMARY

An LC-MS/MS method has been developed to detect the vitamin B complex in infant formula. Detection limits and linear dynamic range were shifted into required ranges by adjusting (detuning) collision energies for some of the B vitamins. Using a simple sample preparation has proved a valid approach to detect all of the fortified B vitamins in NIST 1849a infant formula. The NIST 1849A infant formula reference material results demonstrate the validity of this method. Results with excellent accuracy and reproducibility were achieved across different laboratories.

REFERENCES

1 http://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/labelingnutrition/ucm064928.htm

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