



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 AB 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 various required detection ranges.

Introduction

Vitamin B is a group of water-soluble vitamins that play important roles in cell metabolism. The absence of individual B vitamins in a diet 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

Table 1. Daily required values (DV) of different B vitamins for a human adult as obtained from the FDA¹

Vitamin		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



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 highly polar compounds (Figure 1) whose pK_a range from 0.5 to 10.2, making their analysis challenging.

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

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, B12 and folic acid (vitamin B9).. Instrument detection levels for these vitamins using this method have been shown to be less than a 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, by adjusting the MS/MS voltages accordingly, 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. In this new iteration of the method very little sample preparation

has been used to enable a high throughput suitable for routine food testing.

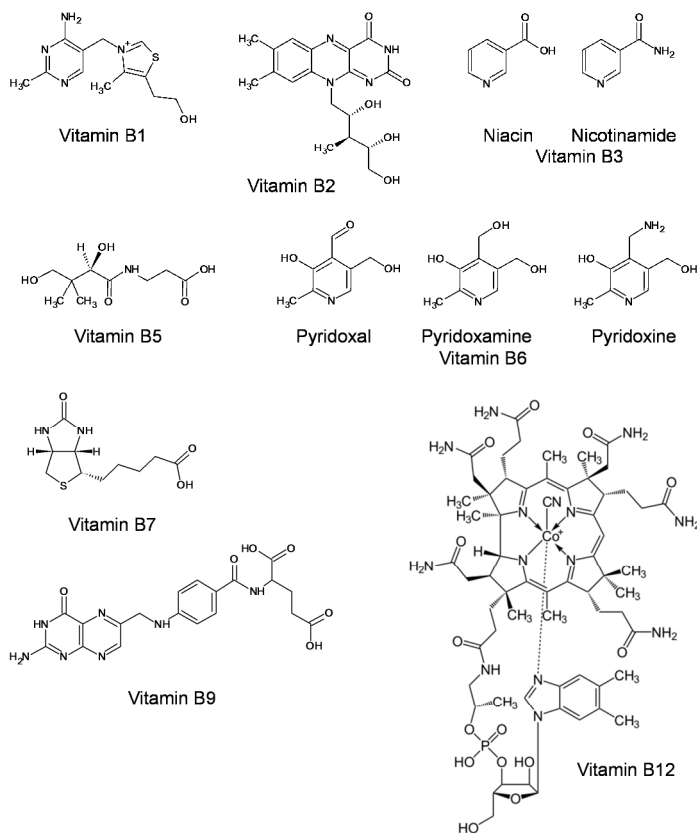


Figure 1. Chemical structures of B vitamins

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 pairing reagent. The sample preparation was kept as simple as possible to reduce possible 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 with 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 eleven minute gradient from acidified water to 100% methanol containing 0.1% formic acid (Table 2). The column temperature was maintained at 50°C 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 make sure that retention times were stable between injections.

Table 2. Gradient conditions used for the separation of B vitamins

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

MS/MS Detection

Analysis was performed on an AB 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 conditions 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 monitor a total of 28 transitions and acquire data with the best reproducibility and accuracy.

Table 3. Quantifier and qualifier MRM transitions and retention times (RT) for the detection of B vitamins

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
IS B1	1.5	268	125
IS B2	5.1	380	173
IS B3 niacin	1.2	127	80
IS B5	2.7	223	93
IS B7	4.6	249	231
IS B9	4.9	446	176

Some of the collision energies were modified to lower the responses and extend the linear ranges (Figure 2). This was done to accommodate the differences in fortification levels in infant formula and response factors of the B vitamins.

All results were processed in PeakView® software version 2.0 and MultiQuant™ software version 3.0.

Results and Discussion

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 as required using the information obtained by ramping the CE. The CE ramps were automatically generated during method development using the 'Compound Optimization' feature in Analyst® software. An example of this is shown in Figure 2.

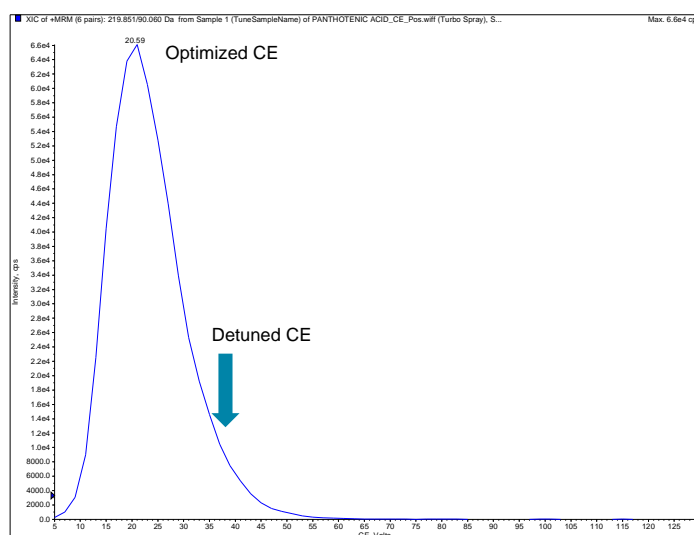


Figure 2. This is a typical ramp of the collision energy (CE) for a vitamin B5 fragment ion. Using this approach the more sensitive vitamins that showed a non-linear response at higher concentrations were detuned for a lower response by choosing non-optimal collision energies.

An example of signal reduction by detuning CE is shown in Figure 3 and the overall effect of adjusting the vitamins is shown in Table 4.

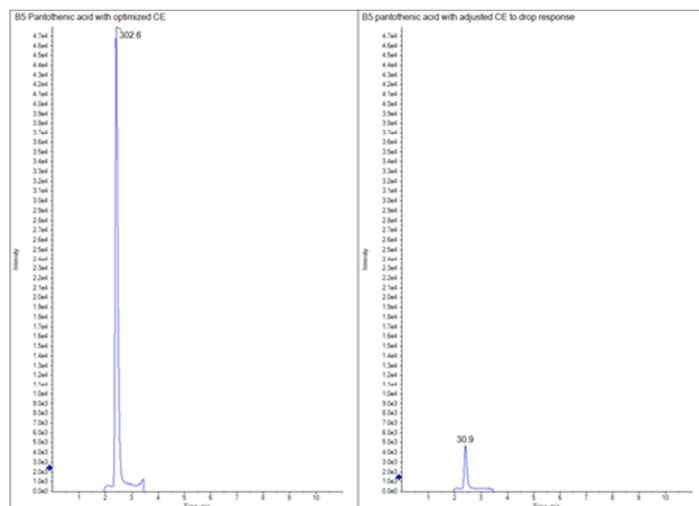


Figure 3. The effect of changing the collision energy on the response of vitamin B5

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 CE for some of the vitamins, a 5 ng/mL solvent standard (Figure 4) clearly shows that all the vitamins are easily detected at this level.

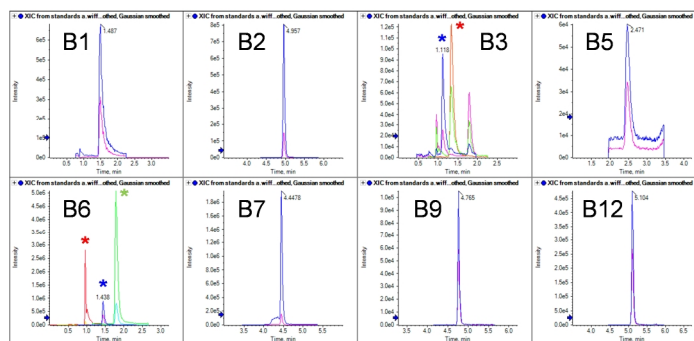


Figure 4. Example of a 5 ng/mL solvent standard of B vitamins, quantifier and qualifier ions are shown

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 from 0.01 to 100 mg/kg. Linear fit with 1/x weighting was used for all target compounds resulting in coefficients of regression (*r*) between 0.994 and 0.999. Internal standards were used to achieve the best quantitative results (Table 5).

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

Compound	Internal Standard	LDR (mg/kg)	<i>r</i>
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 CD 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

Examples of the calibration lines for vitamins B5 and B7 are shown in Figures 5a and 5b. This shows, in the case of B3, that linearity of response is obtained after the adjustment of CE and linear responses are obtained for early and late eluting vitamins

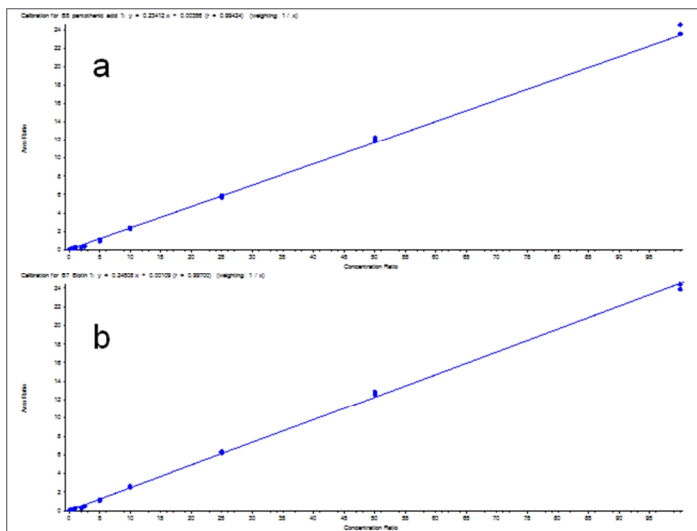


Figure 5. Calibration lines for two vitamins, an early eluting vitamin B5 (a) and a late eluting vitamin B7 (b)

Once each vitamin had their linear response verified for the desired dynamic range, extracts of the NIST 1849A infant formula reference materials were prepared. The results of these extracts are shown in Table 6 and example chromatograms are shown in Figure 6.

Table 6. Results from the repeat analysis of NIST reference material which had been extracted separately three times, with each extract injected seven times (in mg/kg)

Compound	NIST Reference Value	LC-MS/MS Value	CV (%)
B1	12.6	17.1	1.82
B2	20.4	16.5	2.22
B3 niacin	N/A*	N/A	N/A
B3 nicotinamide	109	105	3.01
B5	68.0	81.8	2.36
B6 pyridoxal	13.5	13.9	2.80
B6 pyridoxamine	N/A	N/A	N/A
B6 pyridoxine	N/A	N/A	N/A
B7	1.99	1.96	3.16
B9	2.29	2.45	4.79
B12	N/A	0.078	5.59

* N/A - compound not present in NIST material or not detected in sample

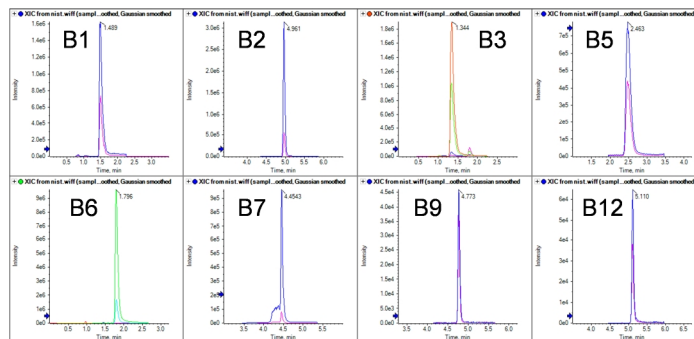


Figure 6. Example of an extract from NIST 1849A reference material showing chromatograms for the fortified vitamins. Vitamin B3 was present as nicotinamide and vitamin B6 as pyridoxine.

Built-in queries of MultiQuant™ software version 3.0 can be used to calculate ion ratios and flag outliers. Ion ratio tolerances for each analyte can be defined in the quantitation method editor (Figure 7). The peak review of an extract of NIST 1849A reference material with ion ratio tolerances is shown in Figure 8.

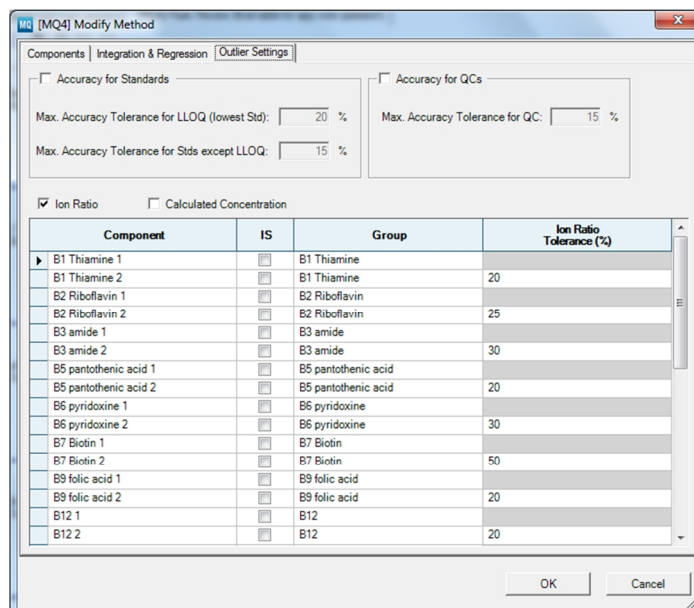


Figure 7. Query settings in the quantitation method editor of MultiQuant™ software to calculate ion ratios

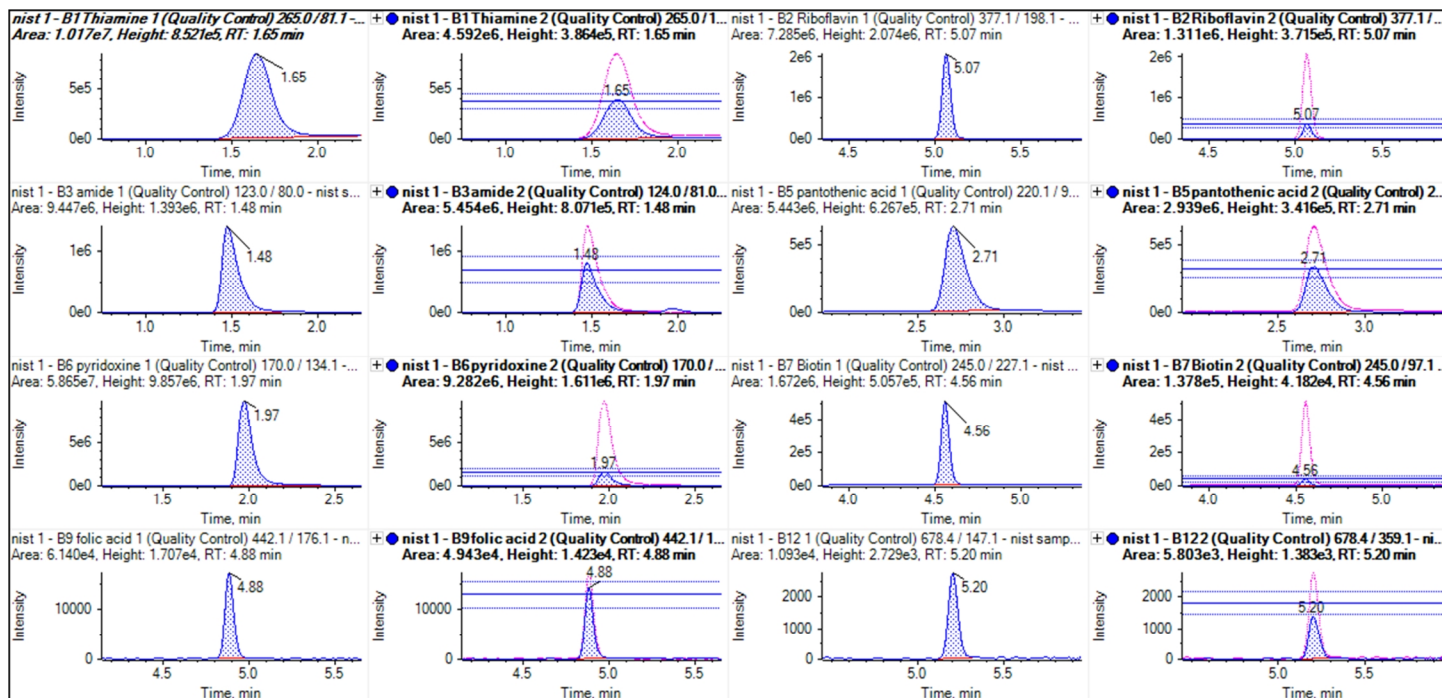


Figure 8. Peak review with ion ratio tolerances of an extract from NIST 1849A reference material

Summary

An LC-MS/MS method has been developed to detect the vitamin B complex in infant formula. Detection limits and linear dynamic range of quantitation were shifted into required ranges by adjusting (detuning) collision energies for some of the B vitamins.

Using a simple sample extraction followed by a 20-fold dilution has proved a valid approach to detect all B vitamins in infant formula. NIST 1849A infant formula reference material was analyzed for method verification. Results with excellent accuracy and reproducibility were achieved.

References

- <http://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/labelingnutrition/ucm064928.htm>

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