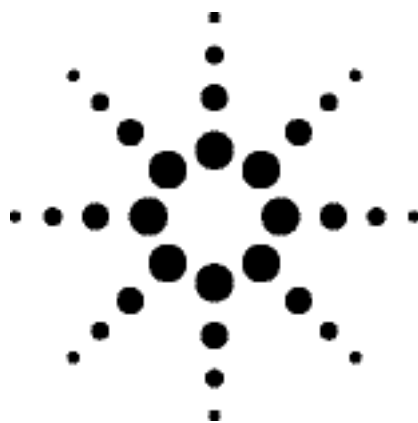




Quantitative Analysis of Water-Soluble B-Vitamins in Cereal Using Rapid Resolution LC/MS/MS

Application

Food Analysis

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Abstract

An Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) is used to analyze several water-soluble B-vitamin compounds in breakfast cereal. A simple gradient elution is carried out on a Rapid Resolution High Throughput SB-Aq column (particle size 1.8 μm). All compounds elute in less than 7.5 minutes, and with the exception of pyridoxine, good linearity over more than three orders of magnitude, from 0.5 to 500 ppb, is demonstrated with good peak area reproducibility at the 0.5 ppb level, which is the lowest level of quantitation considered.

Introduction

Water-soluble vitamins are very polar and have poor retention on reverse-phase columns. The pres-

ence of ion pair reagents such as heptafluorobutyric acid in the mobile phase has been shown to improve the separation and retention of these compounds. However, the drawback of using such ion pair reagents is the high background levels that are generated inside the mass spectrometer. Therefore, we have developed a rapid and sensitive method with ammonium formate in the mobile phase solvent using a column with a bonded phase designed to retain hydrophilic compounds.

The Agilent 1200 Series liquid chromatography (LC) system used in this work was designed to take advantage of sub-2-micron particle columns for rapid, high-resolution separations. Included in the LC design were decreased delay volume, increased pressure range, and increased column temperature. This LC system was coupled to the Agilent 6410 Triple Quadrupole Mass Spectrometer (QQQ) by way of the G1948B electrospray ionization source. Target compound separation was achieved on a ZORBAX AQ 1.8-micron column using a water and methanol gradient with ammonium formate.

Typical LC/MS methods for water-soluble vitamins have shown analysis times as high as 30 minutes with heptafluorobutyric acid ion pairing reagent in the mobile solvent. We have developed a rapid and sensitive method for the LC/MS/MS analysis of water-soluble vitamins by employing a high-efficiency 1.8-micron column in a low-dispersion, 600-bar LC/MS configuration that allowed screening and quantitation with a run-to-run cycle time as low as 10 minutes. Linearity of the mass spec-



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trometer response was observed over three orders of magnitude with limits of quantitation in the 0.5 pg/ μ L range for all of the analytes except for pyridoxine. In the case of pyridoxine, good sensitivity was demonstrated, but good linearity was limited to just under three orders of magnitude. Calibration curves and chromatograms for the vitamins between 0.5 and 500 pg/ μ L were generated

for all compounds with the exception of pyridoxine, which was between 0.5 and 250 pg/ μ L.

The structures of the B vitamins are shown below.

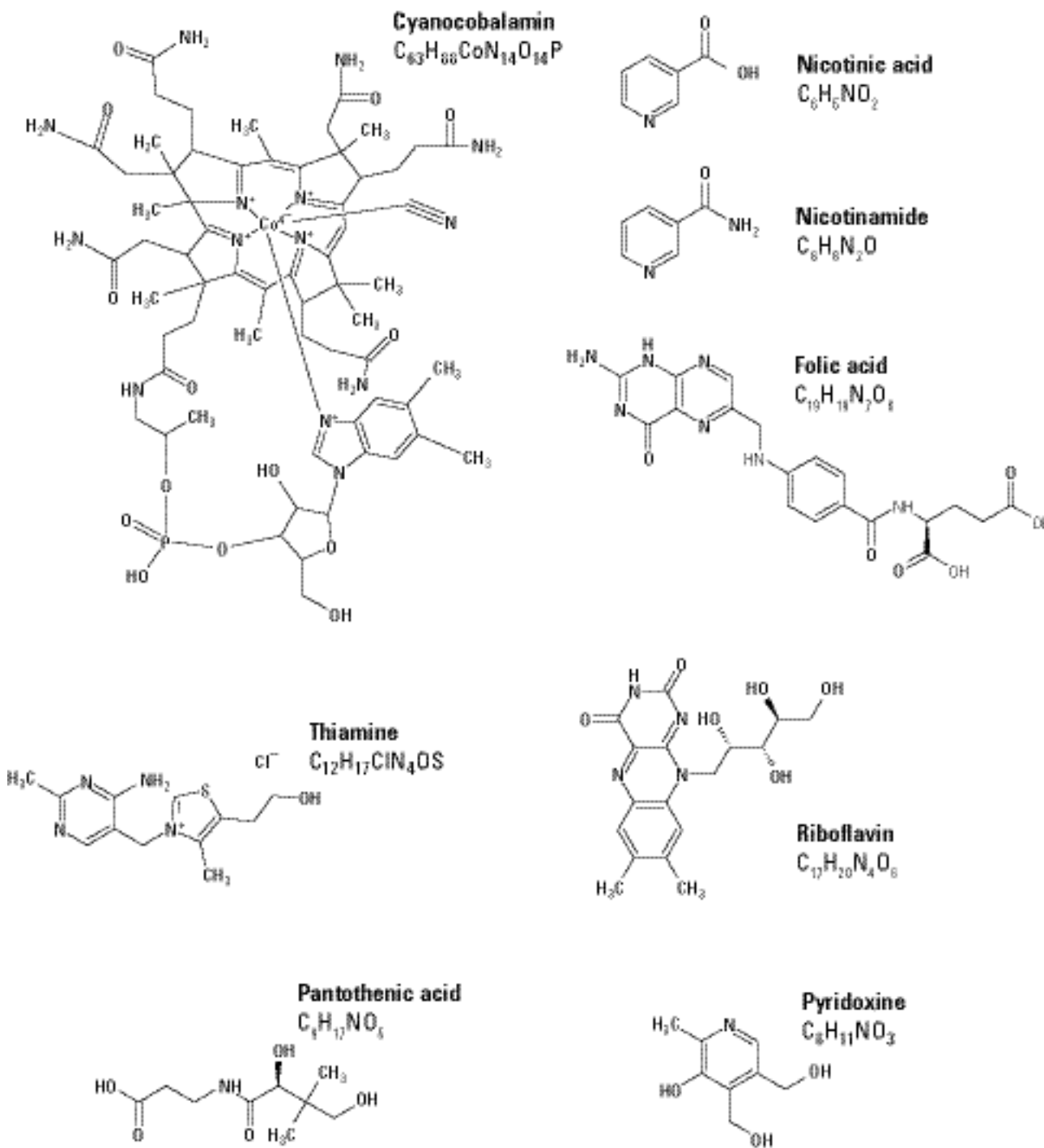


Figure 1. Structures of B vitamins analyzed in this work.

Experimental

Sample Preparation

A standard mix containing all eight compounds in methanol was provided by ConAgra Foods and diluted in 90% water/10% methanol with 20 mM ammonium formate and 0.1% formic acid to the following concentrations: 500, 250, 100, 50, 5, and 0.5 pg/ μ L. These dilutions were used for the quantitation of unknown samples.

One B-vitamin-fortified sample was also provided using the following sample preparation procedure:

1. Grind and homogenize breakfast cereal in blender
2. Weigh 1 gram of homogenized sample into a 50-mL vial
3. Add 25 mL of 0.1M HCl and heat in water bath at 100 °C for 20 min. This solubilizes the vitamins.

4. Cool to ambient temperature
5. Adjust volume to 1 L with deionized water
6. Filter with 0.45- μ m glass microfiber membrane.

It should be noted that the provided fortified sample was created for testing the sensitivity of the instrument for customer demonstration purposes. A typical unfortified sample extraction consists of 1 g homogenized sample treated with enzymatic digestion, to release naturally occurring vitamins from their conjugated forms, and volume adjusted to 10 mL, which is 1/100th the volume used in the fortified sample analyzed in this work. At the higher concentration, salts and other matrix contributions are seen to cause interference in the analysis of some vitamins. As a result, further dilution may be used to accommodate the matrix effect in these samples.

Table 1. MRM Mode Parameters

Segment	Compound	Transition	Fragmentor (V)	Collision Energy (V)
1	Thiamine	265.2 > 122.0	85	10
	Pantothenic acid	220.2 > 90.0	110	13
	Pyridoxine	170.1 > 152.1	100	10
	Nicotinic acid	124.1 > 80.0	100	27
	Nicotinamide	123.1 > 80.0	100	25
2	Cyanocobalamin	678.6 > 146.7	130	35
	Folic acid	442.2 > 295.1	120	10
	Riboflavin	377.2 > 243.1	110	25

LC/MS Method Details

LC Conditions

Agilent 1100 Series binary pump, degasser, wellplate sampler, and thermostatted column compartment

Column: Agilent ZORBAX RRHT SB-Aq, 3.0 mm $\times$ 100 mm, 1.8 μ m (PN: 828975-314)

Column temperature: 35 °C

Mobile phase: A = 20 mM ammonium formate and 0.1% formic acid in water
B = 20 mM ammonium formate and 0.1% formic acid in methanol

Flow rate: 0.5 mL/min

Injection volume: 10 μ L

Gradient:

Time (min)	%B
0.0	10
8.0	55
8.1	10

 Stop time: 10 min

Needle wash: 75:25 methanol/water (flush port 20 seconds)

MS Conditions

Mode: Positive ESI using the Agilent G1948B ionization source

Nebulizer: 30 psig

Drying gas flow: 10 L/min

Drying gas temperature: 350 °C

V_{cap}: 1850 V

Resolution (FWHM): Q1 = low res; Q2 = low res

Dwell time for all MRM transitions: 200 msec

The precursor ion mass for cyanocobalamin (m/z 678.6) is about half of the expected value in which the empirical formula for this compound is $C_{63}H_{88}CoN_{14}O_{14}P$, as denoted in Figure 1. From a correspondence with an analytical chemist (see Acknowledgments) who has run this compound on an Agilent ion trap mass spectrometer, it is shown that the m/z 678.6 actually represents a doubly charged form of cyanocobalamin.

Figure 2A shows the isotopic profile of the ionized cyanocobalamin and since the $[^{13}C]$ isotope contribution is only 0.5 amu higher in mass from the $[^{12}C]$ isotope at 678.1, the profile represents a

charge state of 2. Furthermore, in Figure 2B, the full-scan MS/MS of cyanocobalamin is shown with higher mass product ions like m/z 997.5 present. The higher mass product ions are singly charged.

Results and Discussion

The calibration curves for all eight compounds are shown in Figures 3A through 3H. Only for the compound pyridoxine is the 500 ppb level needed to be removed for good linearity. No internal standard is included.

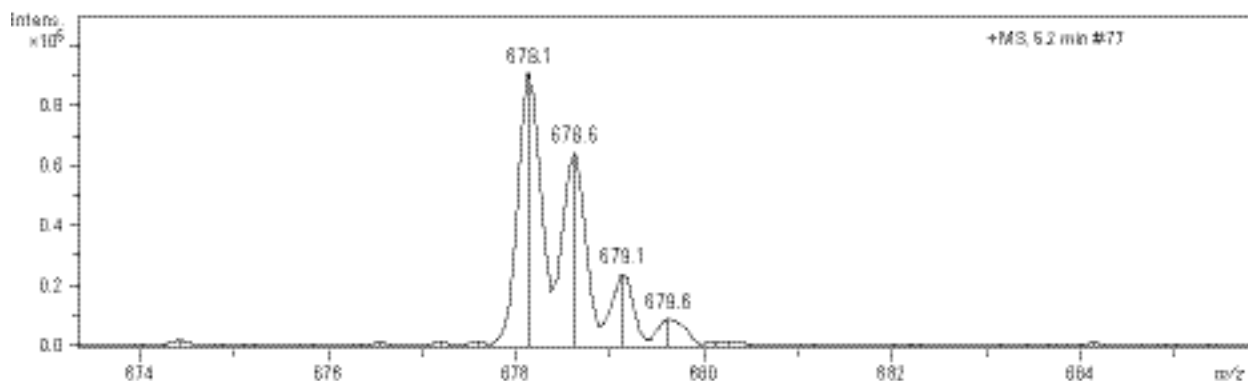


Figure 2A. Doubly charged isotopic profile of cyanocobalamin acquired on Agilent ion trap.

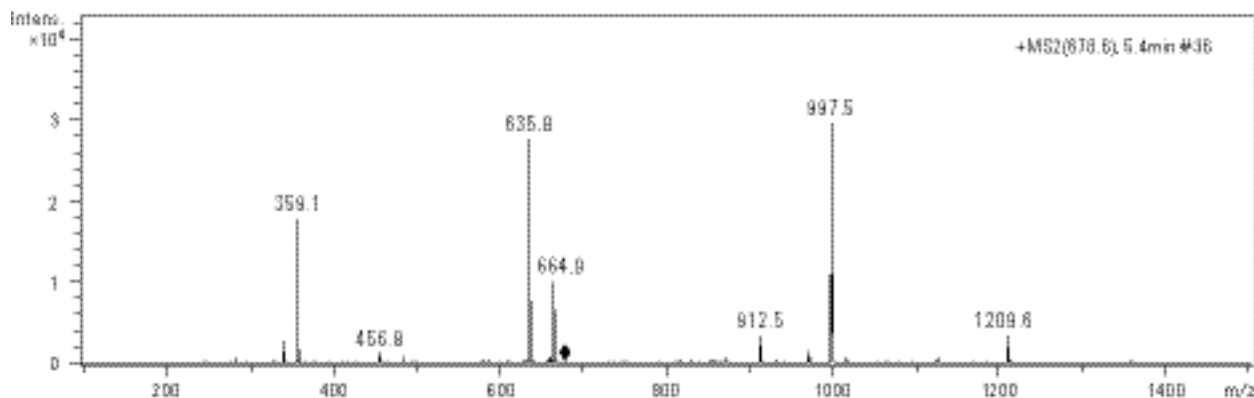


Figure 2B. MS/MS spectrum of the doubly-charged ion of cyanocobalamin.

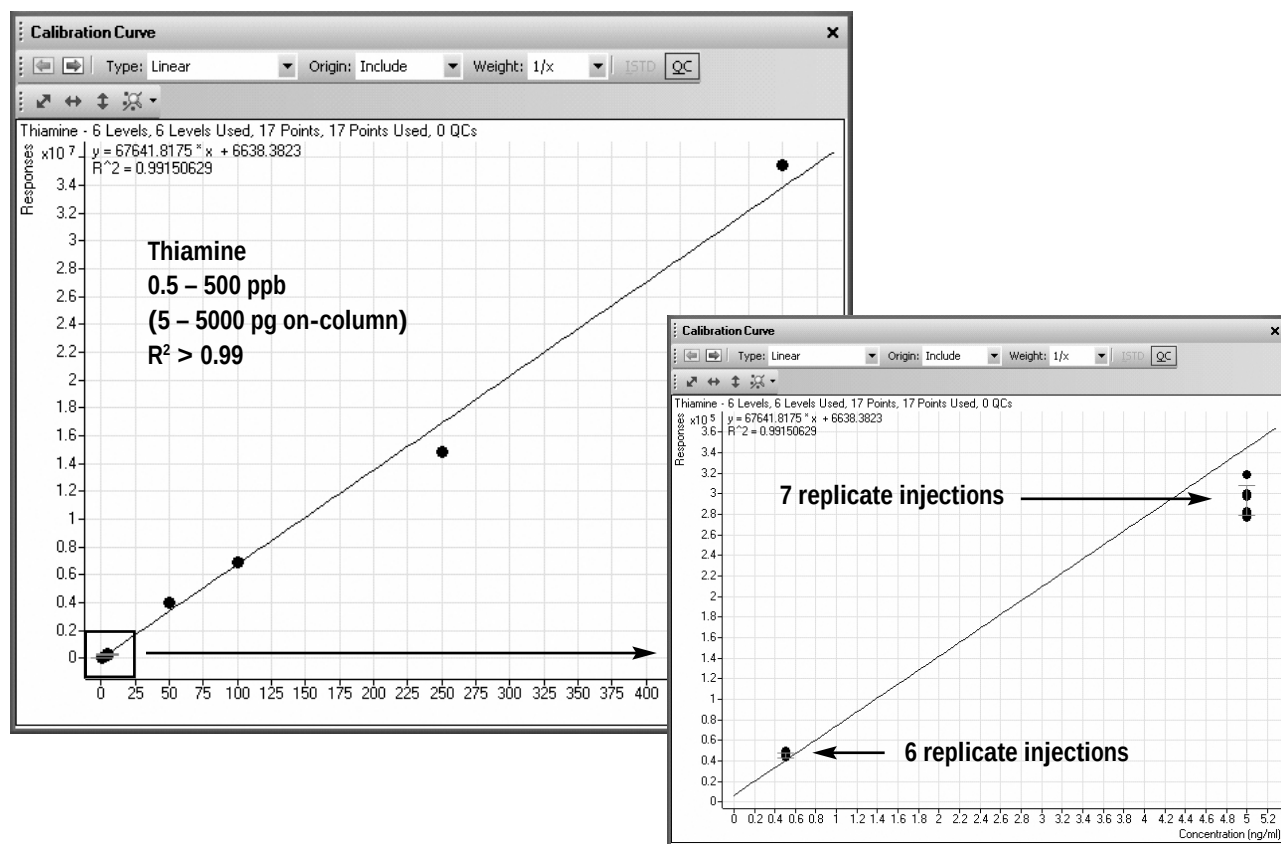


Figure 3A. Linearity of thiamine over three orders of magnitude.

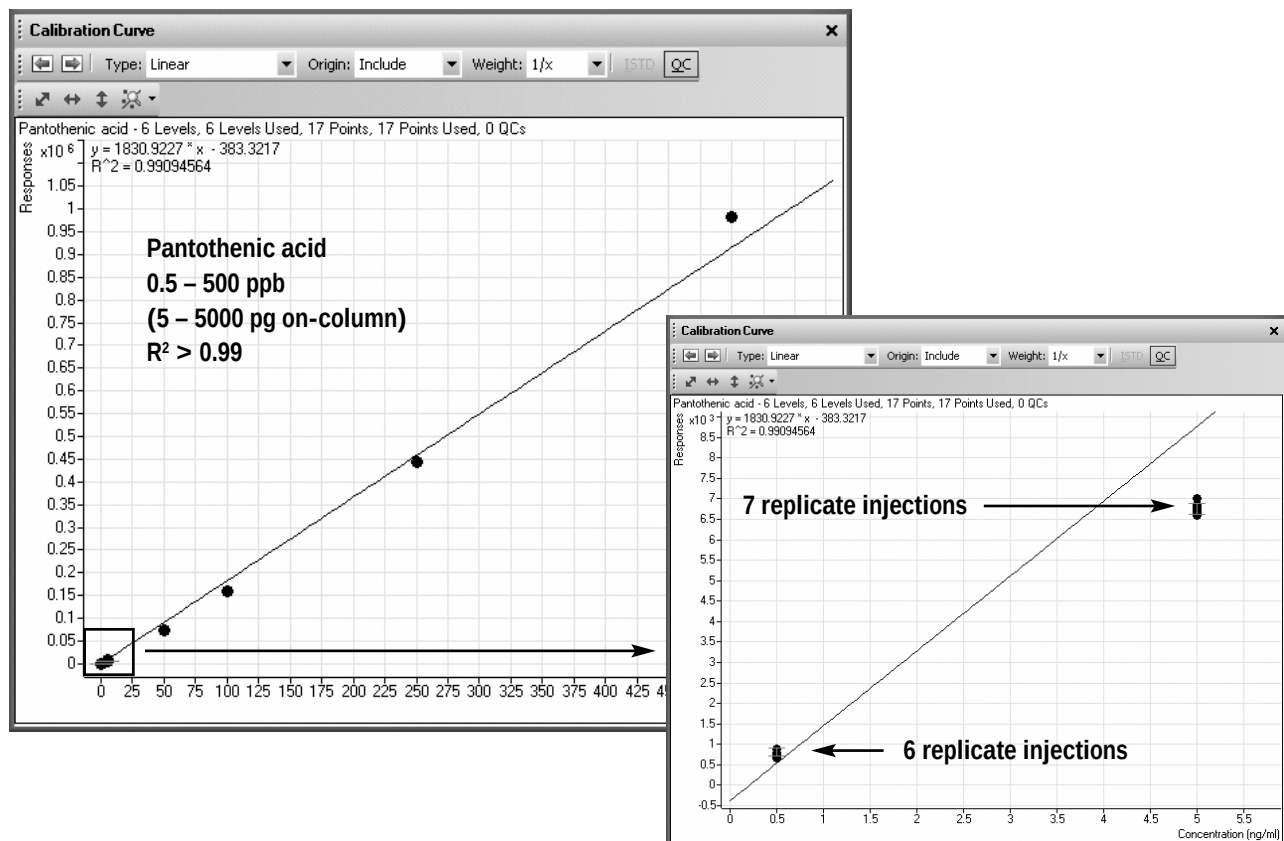


Figure 3B. Linearity of pantothenic acid over three orders of magnitude.

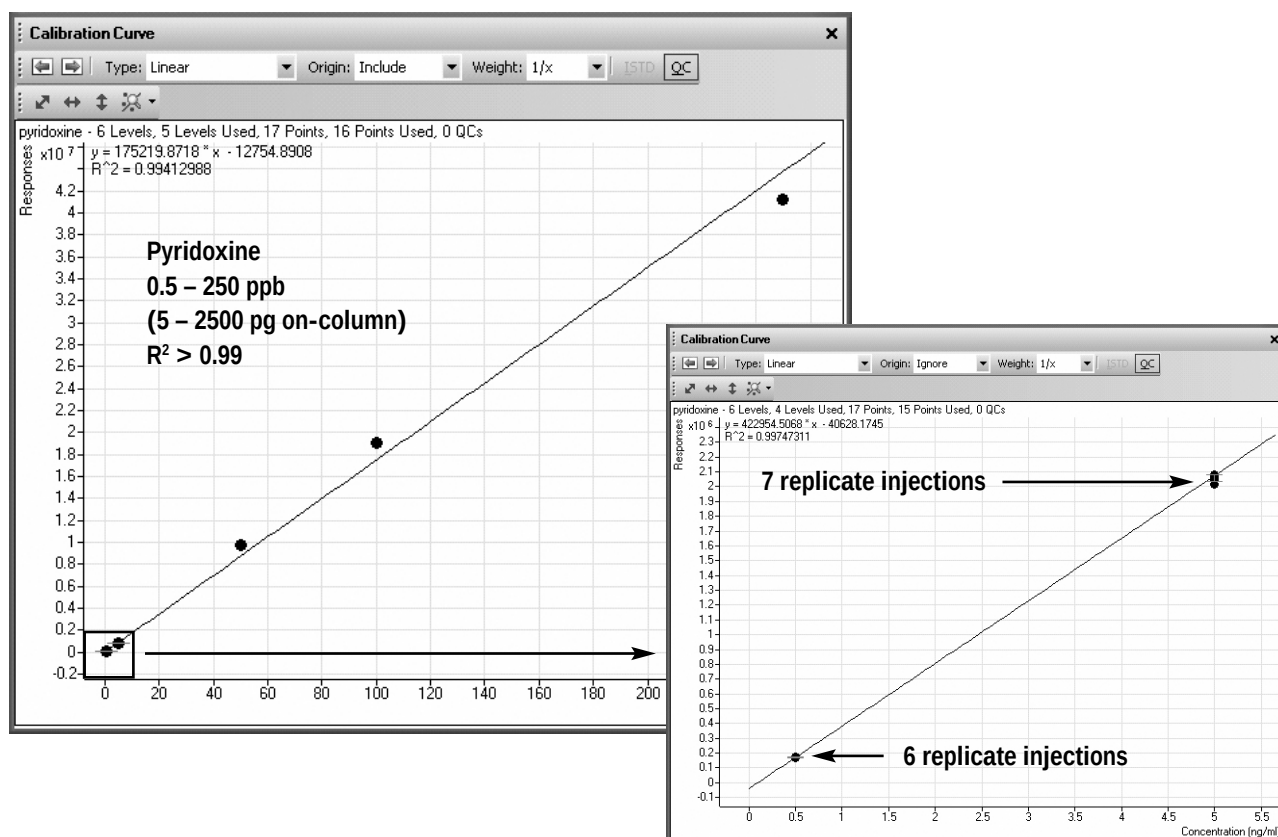


Figure 3C. Linearity of pyridoxine acid over nearly three orders of magnitude.

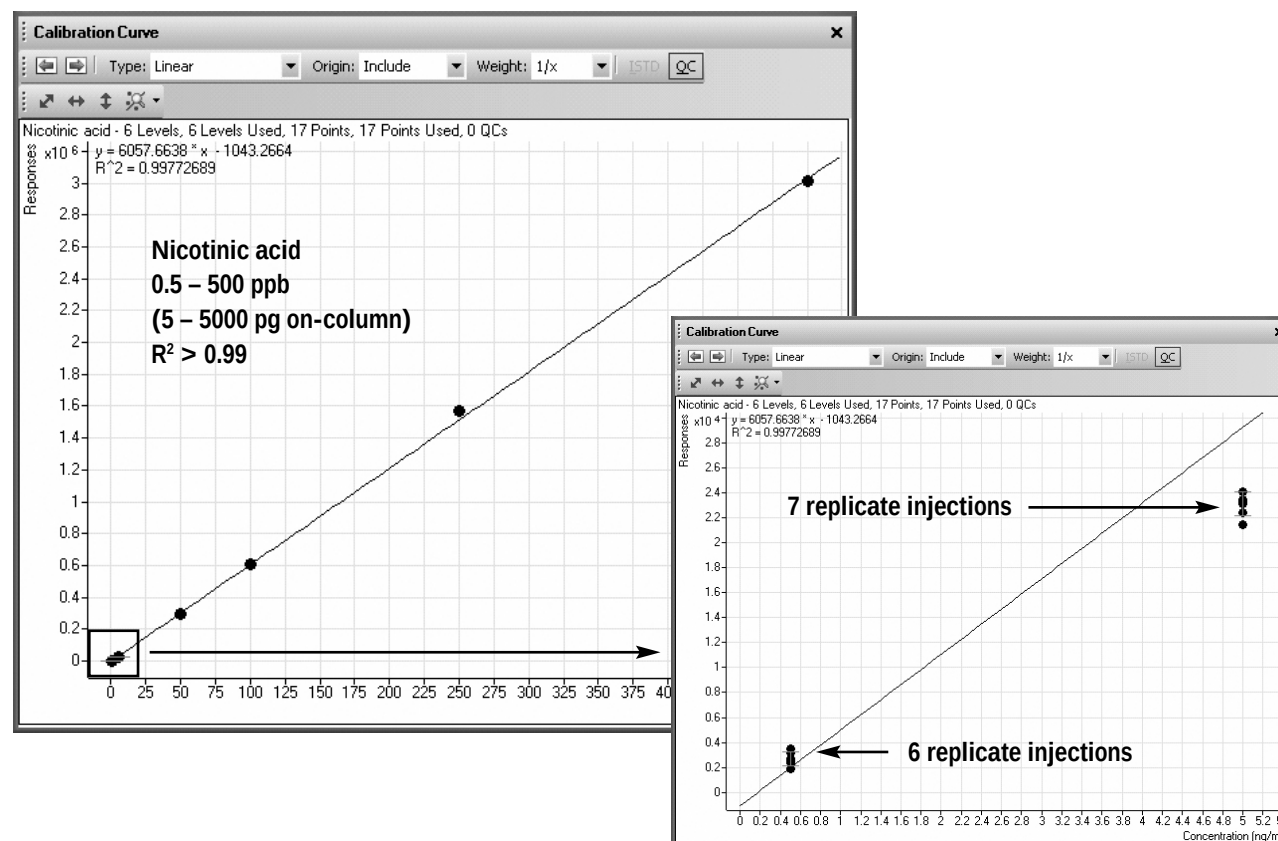


Figure 3D. Linearity of nicotinic acid over three orders of magnitude.

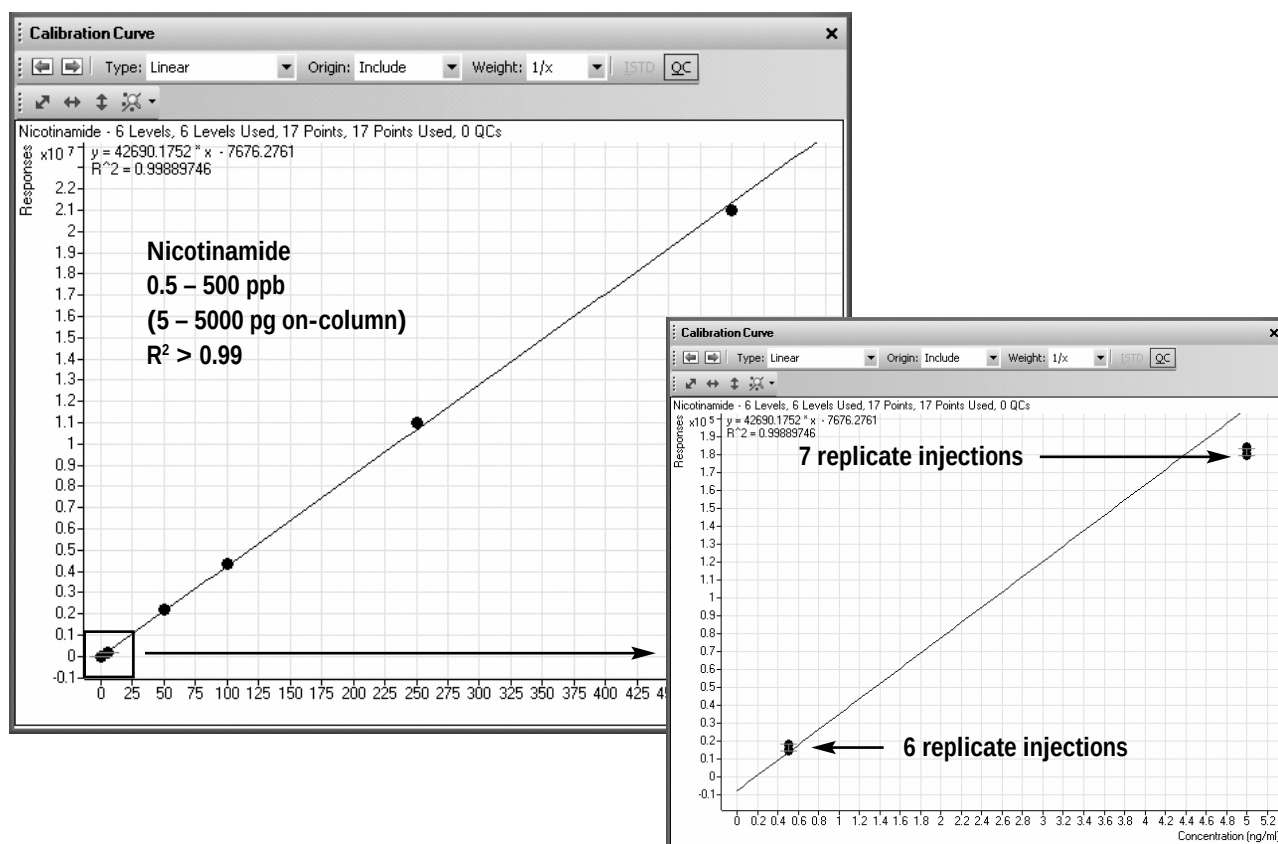


Figure 3E. Linearity of nicotinamide acid over three orders of magnitude.

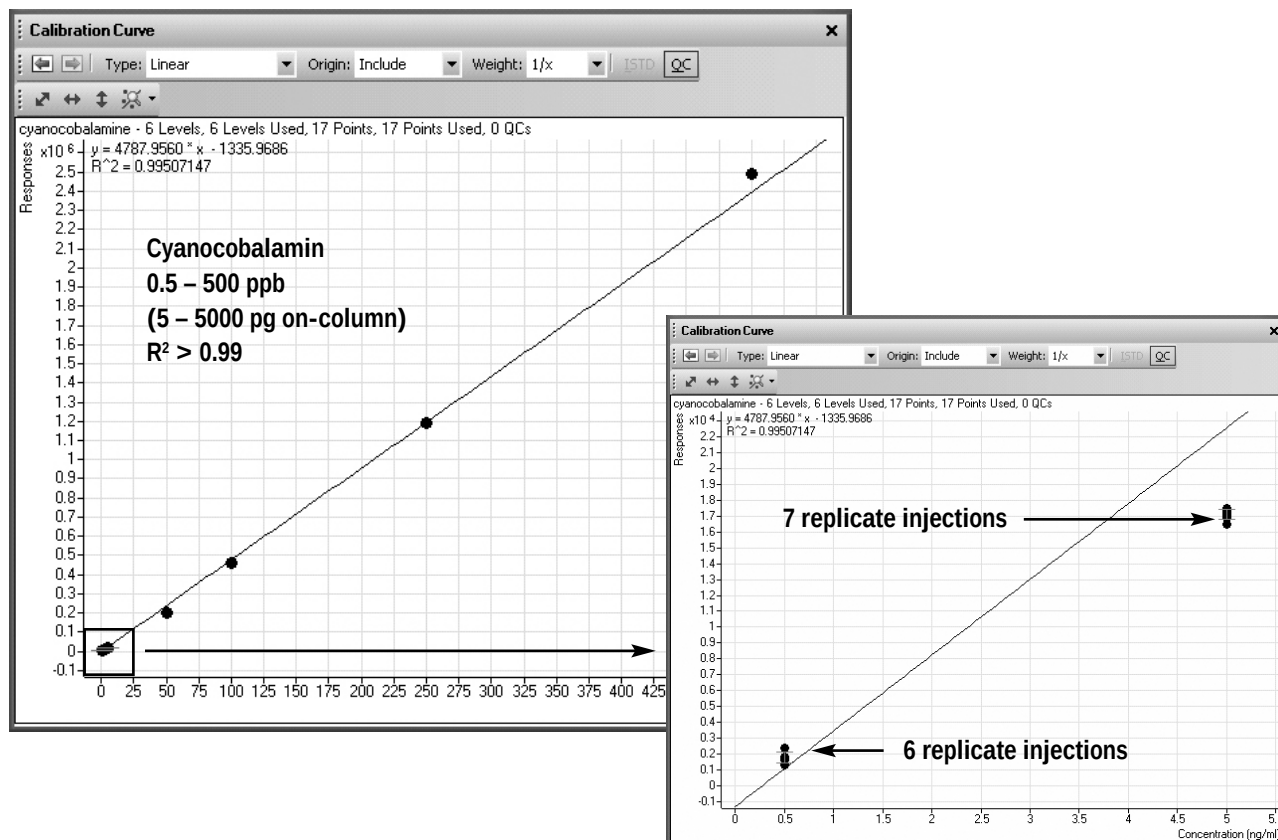


Figure 3F. Linearity of cyanocobalamin over three orders of magnitude.

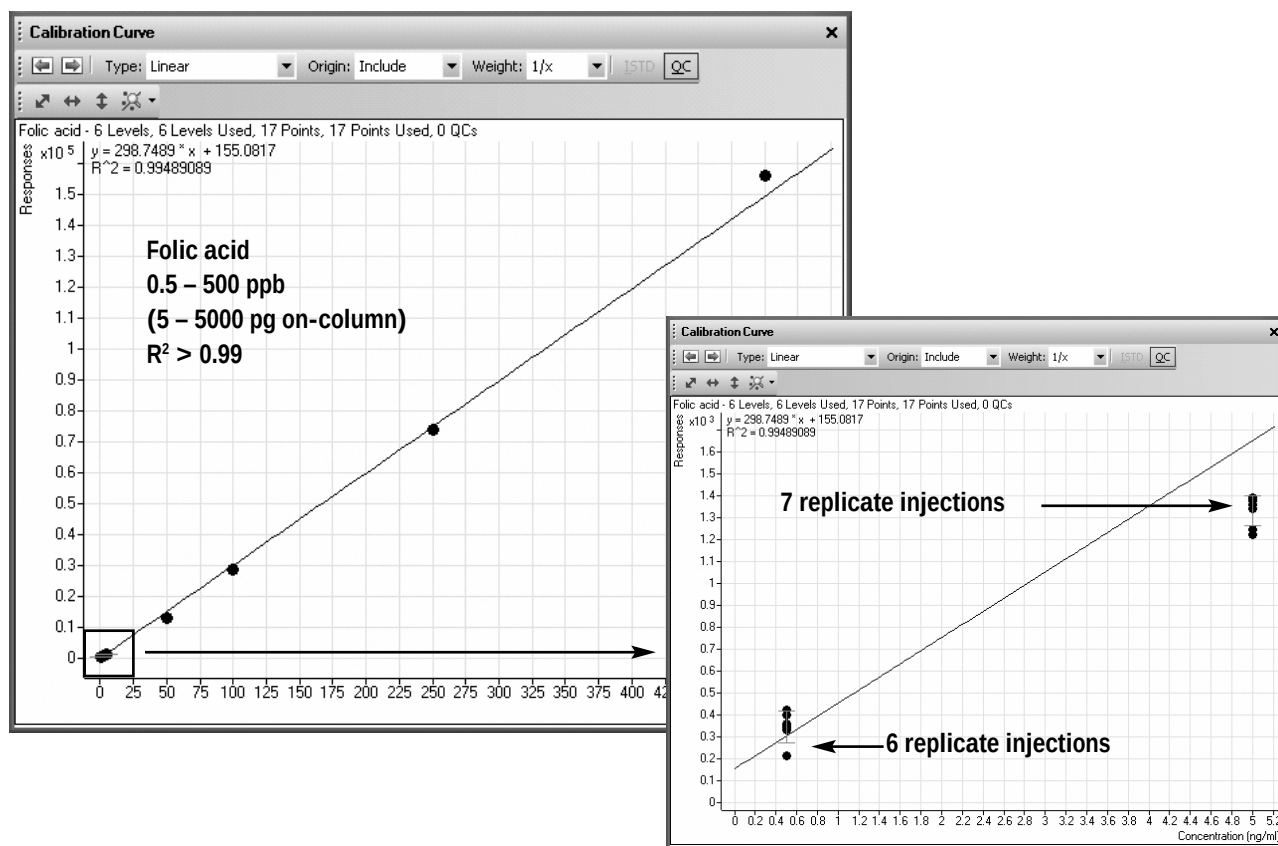


Figure 3G. Linearity of folic acid over nearly three orders of magnitude.

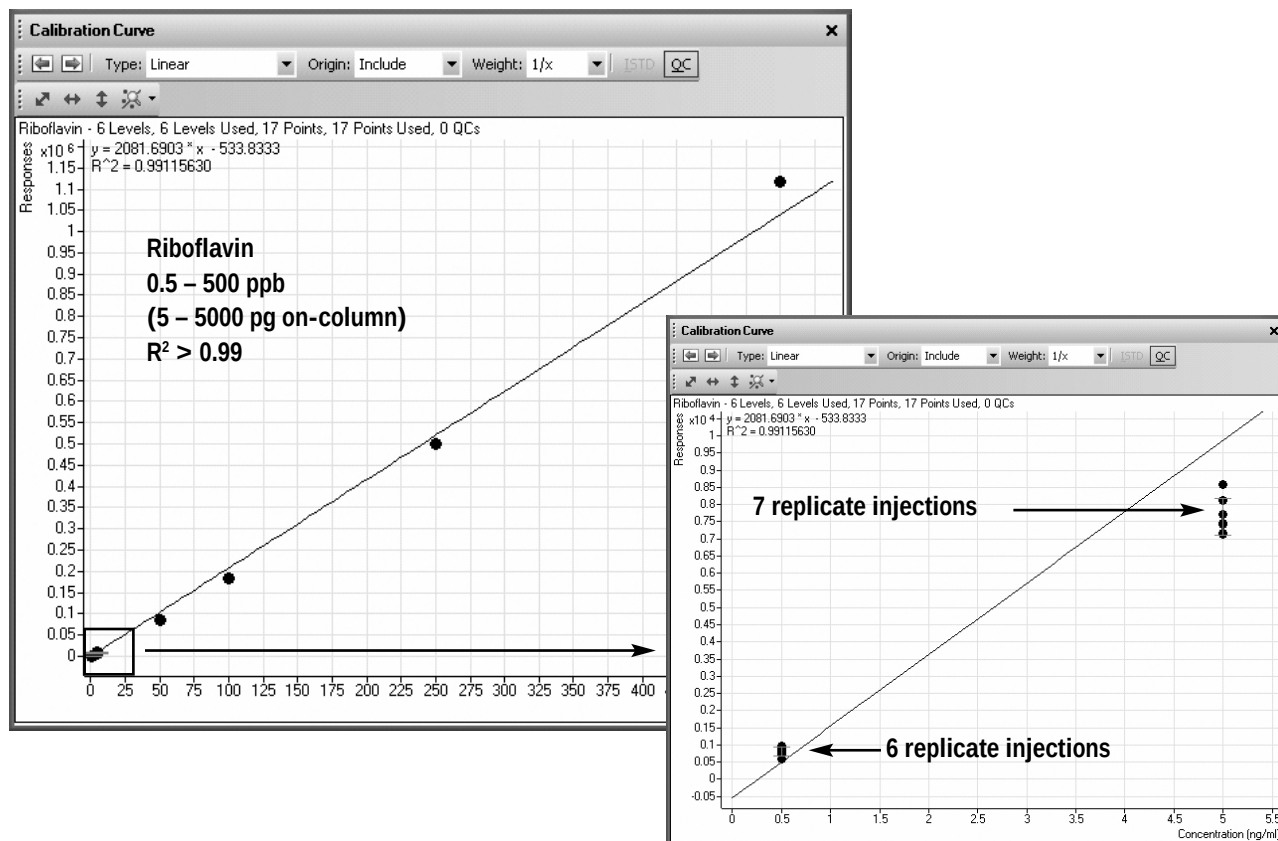


Figure 3H. Linearity of riboflavin acid over three orders of magnitude.

All line fits to data are carried out as linear with the origin ignored and a 1/x weighting.

An example of reproducibility at the 0.5 ppb level for pyridoxine is shown in Figure 4. The peak area %RSD values for all compounds at the 0.5 and 5 ppb level are given in Table 2.

Table 2. Peak Area Reproducibility for Each Compound at the Two Lowest Levels Used for Quantitation

Compound	Level %RSD	
	0.5 ppb	5 ppb
Thiamine	5.9	1.8
Pantothenic acid	14.0	3.3
Pyridoxine	1.7	1.0
Nicotinic acid	9.2	1.8
Nicotinamide	5.5	0.7
Cyanocobalamin	2.7	1.3
Folic acid	20.0	3.9
Riboflavin	4.4	2.7

A fortified cereal extract is also analyzed and quantitated using the diluted standard mix already mentioned. An example of the batch results using the MassHunter Quantitative Analysis is shown in Figure 5. The concentration of nicotinamide present in the sample is calculated to be 116.2 pg/μL.

The concentrations calculated for all compounds in the fortified extract are given in Table 3.

The corresponding chromatographic elution of the eight compounds detected in the fortified extract is shown in Figure 6.

Table 3. Calculated Concentrations for Each Compound in Fortified Cereal Extract

Compound	Calculated concentration (pg / μL)
Thiamine	24.0
Pantothenic acid	1.2
Pyridoxine	15.5
Nicotinic acid	43.5
Nicotinamide	116.2
Cyanocobalamin	0.4*
Folic acid	2.6
Riboflavin	8.6

* Outside quantitation limits.

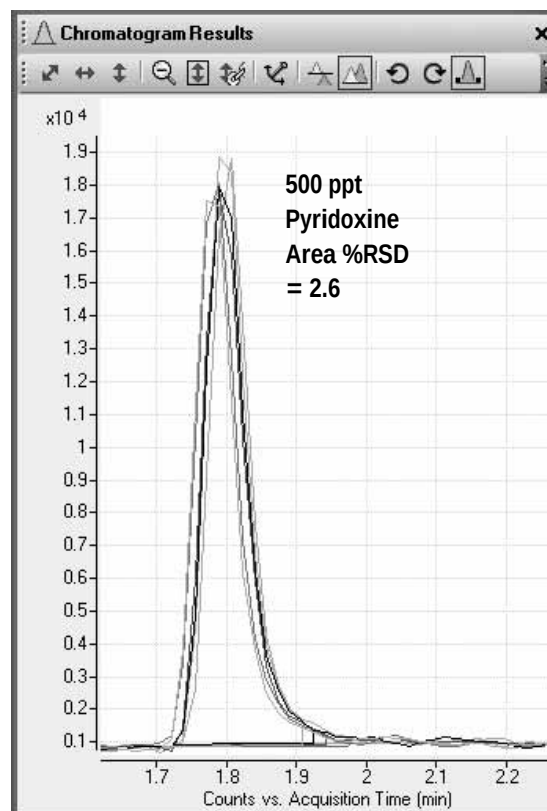


Figure 4. Peak area reproducibility of pyridoxine at 500 ppt level, 6 injections.

Batch Table															
Sample:		Sample Type: <A		Compound: 1: Nicotinamide		ISTD:		Time Segment: <A							
Sample							Nicotina..		Nicotinamide Results						
		Name	Type	Level	Acq. Date-Time	Data File	Dil	Exp. Conc.	RT	Resp.	S/N	MI	Calc. Conc.	Final Conc.	Accuracy
		blank	Blank		1/26/2007 1:23 PM	blank1.d	1.0		3.306	507	0.12		0.0299	0.0299	
		0.5ppb	Cal	1	1/26/2007 2:55 PM	500ppt1.d	1.0	0.5000	3.245	45440	14.51		0.4955	0.4955	99.1
		0.5ppb	Cal	1	1/26/2007 3:07 PM	500ppt2.d	1.0	0.5000	3.251	41164	12.48		0.4512	0.4512	90.2
		0.5ppb	Cal	1	1/26/2007 3:18 PM	500ppt3.d	1.0	0.5000	3.236	45058	14.15		0.4916	0.4916	98.3
		0.5ppb	Cal	1	1/26/2007 3:30 PM	500ppt4.d	1.0	0.5000	3.260	44073	14.74		0.4814	0.4814	96.3
		0.5ppb	Cal	1	1/26/2007 3:41 PM	500ppt5.d	1.0	0.5000	3.245	42910	16.45		0.4693	0.4693	93.9
		0.5ppb	Cal	1	1/26/2007 3:53 PM	500ppt6.d	1.0	0.5000	3.249	39420	12.32		0.4332	0.4332	86.6
		5ppb	Cal	2	1/26/2007 4:04 PM	5ppb1.d	1.0	5.0000	3.255	488810	61.80		5.0900	5.0900	101.8
		5ppb	Cal	2	1/26/2007 4:16 PM	5ppb2.d	1.0	5.0000	3.238	483252	70.06		5.0324	5.0324	100.6
		5ppb	Cal	2	1/26/2007 4:27 PM	5ppb3.d	1.0	5.0000	3.258	494014	76.35		5.1439	5.1439	102.9
		5ppb	Cal	2	1/26/2007 4:39 PM	5ppb4.d	1.0	5.0000	3.248	488774	78.38		5.0896	5.0896	101.8
		5ppb	Cal	2	1/26/2007 4:50 PM	5ppb5.d	1.0	5.0000	3.257	489640	83.64		5.0986	5.0986	102.0
		5ppb	Cal	2	1/26/2007 5:02 PM	5ppb6.d	1.0	5.0000	3.259	486089	79.07		5.0618	5.0618	101.2
		5ppb	Cal	2	1/26/2007 5:13 PM	5ppb7.d	1.0	5.0000	3.250	484797	76.87		5.0484	5.0484	101.0
		50ppb	Cal	3	1/26/2007 5:25 PM	50ppb.d	1.0	50.0000	3.255	5413252	52.58		55.1193	55.1193	112.2
		100ppb	Cal	4	1/26/2007 5:36 PM	100ppb.d	1.0	100.0000	3.255	10915097	77.91		113.1320	113.1320	113.1
		250ppb	Cal	5	1/26/2007 5:48 PM	250ppb.d	1.0	250.0000	3.255	25481085	84.13		254.0716	254.0716	105.6
		500ppb	Cal	6	1/26/2007 5:59 PM	500ppb.d	1.0	500.0000	3.255	44995630	573.69		466.2904	466.2904	93.3
		blank	Blank		1/26/2007 6:22 PM	blank3.d	1.0								
		sample	Sample		1/26/2007 6:57 PM	sample1b.d	1.0		3.258	11213094	93.72		116.2200	116.2200	

Figure 5. Quantitation batch results for nicotinamide in sample. Concentration calculated to be 116.2 pg / μ L (highlighted).

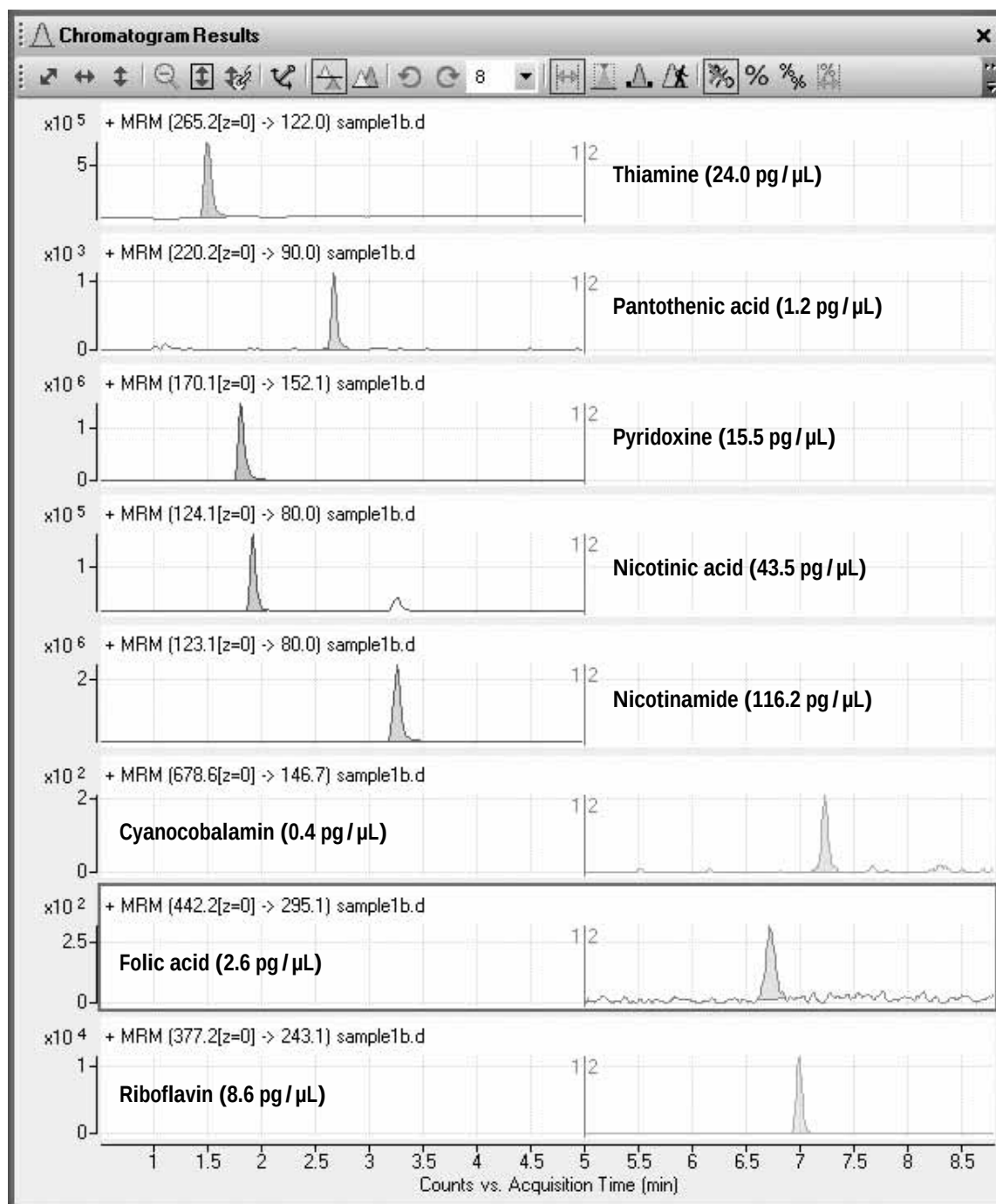


Figure 6. Chromatogram of compounds in fortified extract and calculated concentrations.

Conclusions

The water-soluble B vitamins are successfully analyzed using LC/MS/MS. Good linearity with at least $R^2 > 0.99$ is demonstrated over three orders of magnitude for all compounds, with reproducibility as low as 1.7 %RSD at the lowest level of quantitation for pyridoxine. An extracted fortified sample is successfully analyzed with only the cyanocobalamin concentration falling below the quantitation limit.

Acknowledgements

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