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Selective Quantitative Determination of Water Soluble Vitamins in Various Food Matrices Using the ACQUITY UPLC H-Class System and ACQUITY QDa Mass Detector

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APPLICATION BENEFITS

- Selectivity of mass detection ensures unambiguous detection of vitamins at low levels, enabling simpler sample preparation protocols with dilution of sample extracts.
- The ACQUITY® QDa® Mass Detector has been designed for integration with UPLC® and UHPLC systems to provide robust reliable orthogonal detection to UV spectroscopy allowing new users to quickly take advantage of the additional selectivity of mass detection.
- The ACQUITY QDa Mass Detector can be incorporated into existing liquid chromatography workflows in order to vastly increase the speed and selectivity over other LC detectors.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY QDa Mass Detector

ACQUITY UPLC HSS T3 Column

KEYWORDS

WSV, vitamins, QDa, mass detection, B vitamins, vitamin C

INTRODUCTION

Many food and beverage products are routinely fortified with vitamins to enhance their nutritional value and to help address any deficiencies in dietary requirements. In order to meet legal requirements, manufacturers must label their products according to the regulations of the country in which the product is consumed. Examples of these regulations include European Commission (EC) 1925/2006 on the addition of vitamins and minerals, and Title 21 Code of Federal Regulations (C.F.R.), Part 101 on food labeling in the United States.

Rapid, reliable, and cost-effective methods are required by food manufacturers and ingredient suppliers in order to verify product consistency and ensure that label claims are met. This can be a challenging task with the combination of complex matrices and low fortification levels of some vitamins. In addition, many of the methods currently employed stipulate that the vitamins are either analyzed separately, or in small groups. Established techniques include microbiological assays, colorimetric and fluorimetric analysis, titrimetric procedures and HPLC methodologies.[1] LC-MS offers the opportunity to consolidate methods along with the ability to improve detector selectivity and reduce limits of quantification. Waters® ACQUITY QDa Mass Detector offers laboratories the opportunity to capture the benefits of mass detection without the challenges associated with the adoption of mass spectrometers.

In this application note, 12 water soluble vitamins (WSVs) were analyzed in dietary supplements and beverage samples using the ACQUITY UPLC H-Class System with the ACQUITY QDa Mass Detector.

EXPERIMENTAL

Table 1 lists the water soluble vitamins included in this study along with the observed retention times, single ion recording (SIR) *m/z*, and cone voltage.

Table 1. Retention times, SIR channels, and cone voltages for the water soluble vitamins studied.

Analyte	RT (min)	SIR *m/z*	Cone voltage (V)
Ascorbic acid (C)	0.91	177	2
Thiamine (B1)	1.01	265	5
Nicotinic acid (B3)	1.27	124	15
Pyridoxal (B6)	1.75	168	5
Nicotinamide (B3)	2.48	123	15
Pyridoxine (B6)	2.50	170	10
Ca_Pantothenate (B5)	5.88	242	15
Cyanocobalamin (B12)	7.17	678	2
Folic acid (B9)	7.22	442	5
B2-5-Phosphate	7.35	457	5
Biotin (B7)	7.50	245	10
Riboflavin (B2)	7.74	377	15

Table 2. Standards concentrations for B vitamins and vitamin C.

Standard	Individual B complex vitamins (mg/L)	Vitamin C (mg/L)
1	1.00	50.0
2	0.75	37.5
3	0.50	25.0
4	0.25	12.5
5	0.10	5.0
6	0.075	3.75
7	0.050	2.5
8	0.025	1.25
9	0.010	0.5
10	0.005	0.25
11	0.001	0.05

Standard preparation

Individual 1 mg/mL WSV stocks were prepared in water. In the case of vitamins B2, B7, and B9, 200 µL of 1 N NaOH were added to affect dissolution. Vitamin C was dissolved in a low pH acetate buffer to enhance stability. From these individual stocks, a mixed stock was prepared by adding 1.25 mL of the vitamin C stock and 0.025 mL of the other stocks and diluting to 25 mL with water. This mixed stock (50 ppm vitamin C, 1 ppm of the other analytes) was further diluted to provide 11 individual calibration standards, listed in Table 2.

Sample preparation

A packet (8.50 g) of a powdered vitamin beverage was dissolved in 100 mL water and filtered through a 0.2-µm PVDF filter. This sample was then prepared at two additional dilution levels: 1:250 and 1:10. These three dilution levels were injected to cover the different concentrations of vitamins in this sample.

A multi-vitamin supplement tablet was crushed using a mortar and pestle. The powder (1.34 g) was quantitatively transferred to a beaker to which 100 mL water was added. This mixture was sonicated for 15 minutes then stirred and filtered through a 0.2-µm PVDF filter. Three additional dilutions of this sample were prepared in water: 1:1000, 1:100, and 1:20. These dilutions and the initial dissolved tablet solution (undiluted) were analyzed in order to cover the different concentrations of vitamins in this sample.

Two different vitamin water samples were prepared by diluting 1:20 with water and filtering through a 0.2-µm PVDF filter.

EXPERIMENTAL

UPLC conditions

UPLC system:	ACQUITY UPLC H-Class
Run time:	17.5 min
Column:	ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 100 mm (p/n 186003539)
Column temp.:	30 °C
Mobile phase A:	10 mM ammonium formate, 0.1% formic acid in water
Mobile phase B:	10 mM ammonium formate, 0.1% formic acid in methanol
Injection volume:	5 µL

	Time (min)	Flow rate (mL/min)	%A	%B
1.	Initial	0.45	99	1
2.	3.0	0.45	99	1
3.	3.1	0.45	95	5
4.	5.1	0.45	80	20
5.	7.1	0.45	2	98
6.	9.0	0.45	2	98
7.	9.1	0.45	99	1
8	17.5	0.45	99	1

Table 3. UPLC gradient for the separation of water soluble vitamins.

Detector conditions

Detector 1:	ACQUITY UPLC PDA
Wavelength:	Scanning 210 to 400 nm; Analog channel at 270 nm
Scan rate:	10 pts/sec
Detector 2:	ACQUITY QDa
Ionization mode:	ESI+
Run time:	8.0 min
Probe temp.:	600 °C
Capillary voltage:	0.8 kV
Mass range:	*m/z* 50 to 800 (centroid) and select SIRs*
Sampling freq.:	5 Hz
Cone voltage:	Full scan data: 15 V

*See Table 1 for cone voltage of individual SIR channels.

SIR *m/z* were assigned based on previous work.[2]

RESULTS AND DISCUSSION

A chromatogram showing an overlay of all 12 water soluble vitamins used in this study is shown in Figure 1, where all compounds eluted within eight minutes. Using this method, there were two co-eluting pairs (nicotinamide and pyridoxine at ~2.5 minutes and cyanocobalamin and folic acid at ~7.25 minutes). The use of mass detection means that it is no longer necessary to ensure baseline separation of all the analytes. The discrimination offered with mass detection means that these compounds can be accurately measured using their mass-to-charge ratio (*m/z*). This is demonstrated in Figure 2 where the linearity of selected vitamins are shown, including vitamins that co-eluted. Figure 2D and 2F show the calibration curves of folic acid (*m/z* 442) and cyanocobalamin (*m/z* 678), respectively. The selectivity offered with mass detection means that these compounds can be determined quantitatively, even though they co-elute. Figure 2 also shows example calibration curves of vitamins that can be challenging to analyze by UV. For example, biotin (Figure 2A) and calcium pantothenate (Figure 2H) are vitamins that show low responses using UV detection. Those compounds are often analyzed at low wavelengths to obtain a sufficiently sensitive response.[3] At such low wavelengths, the specificity of the analysis may be compromised. Mass detection ensures that the analysis is both specific and sensitive.

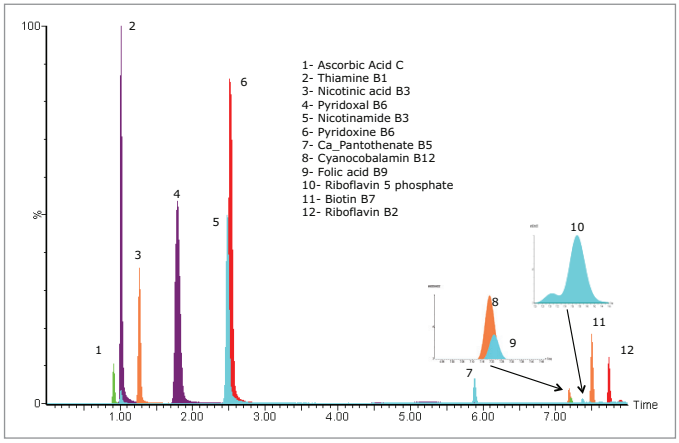

Figure 1. Overlay of SIR chromatograms of 12 water soluble vitamins separated in eight minutes.

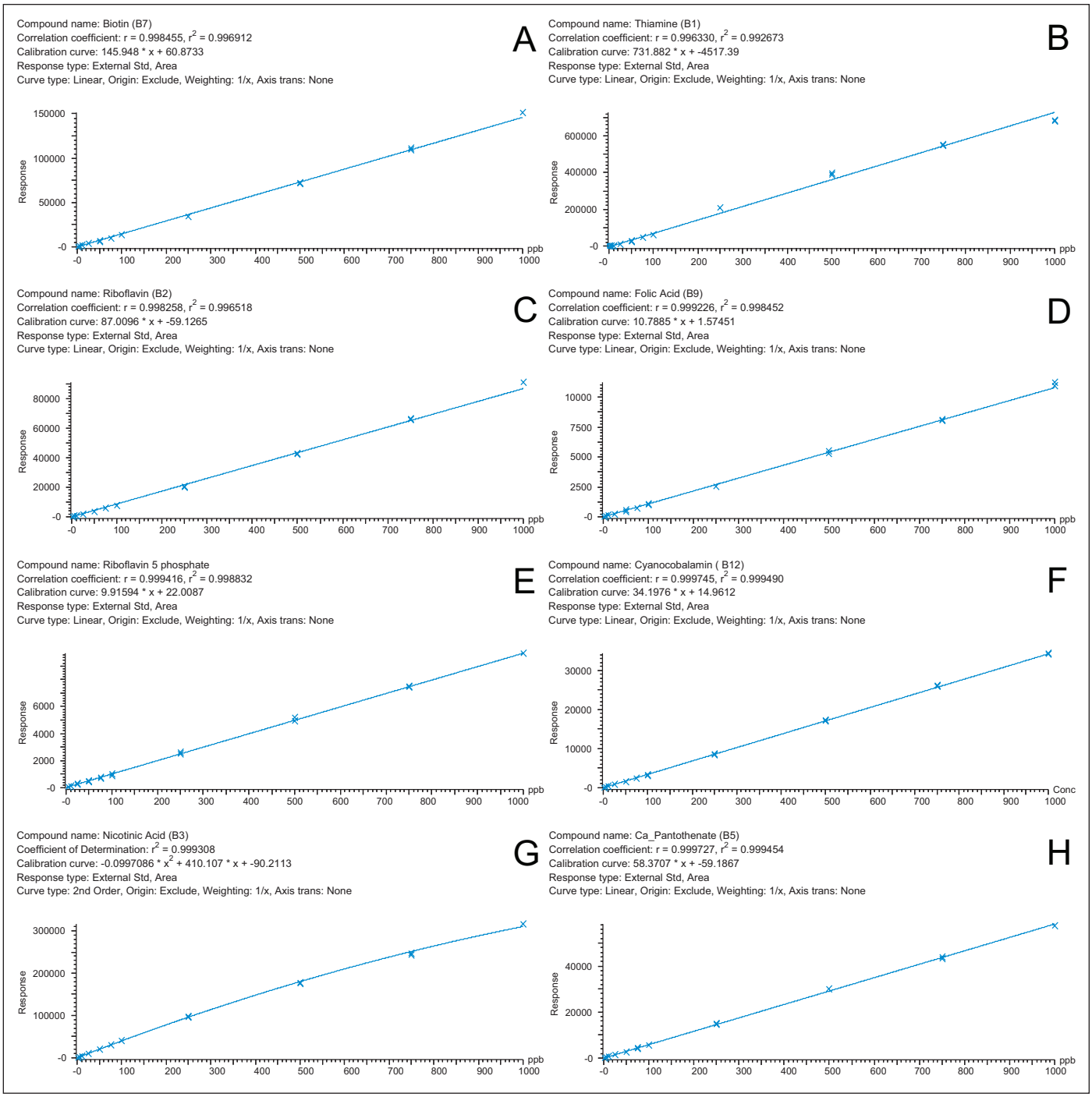

Figure 2. Calibration curves for selected water soluble vitamins.

Mass detection offers the opportunity to detect vitamins at lower levels than can be achieved with UV detection. In Figure 3, the SIR chromatograms of vitamins pyridoxine, pyridoxal, nicotinic acid, and nicotinamide at 5 ppb (5 µg/L) are shown, along with the UV chromatogram (Figure 3A, 270 nm). As shown in Figure 3A, the vitamins could not be detected by UV at this level. The lower limits of quantification that can be achieved with mass detection is important for the quantification of vitamins at low levels. Improved sensitivity also helps to deal with the wide variety of matrices that are encountered by allowing sample extracts to be diluted. In this work, vitamin supplements and drinks were analyzed simply by diluting the sample (in the case of a tablet, an initial step to crush the tablet was required).

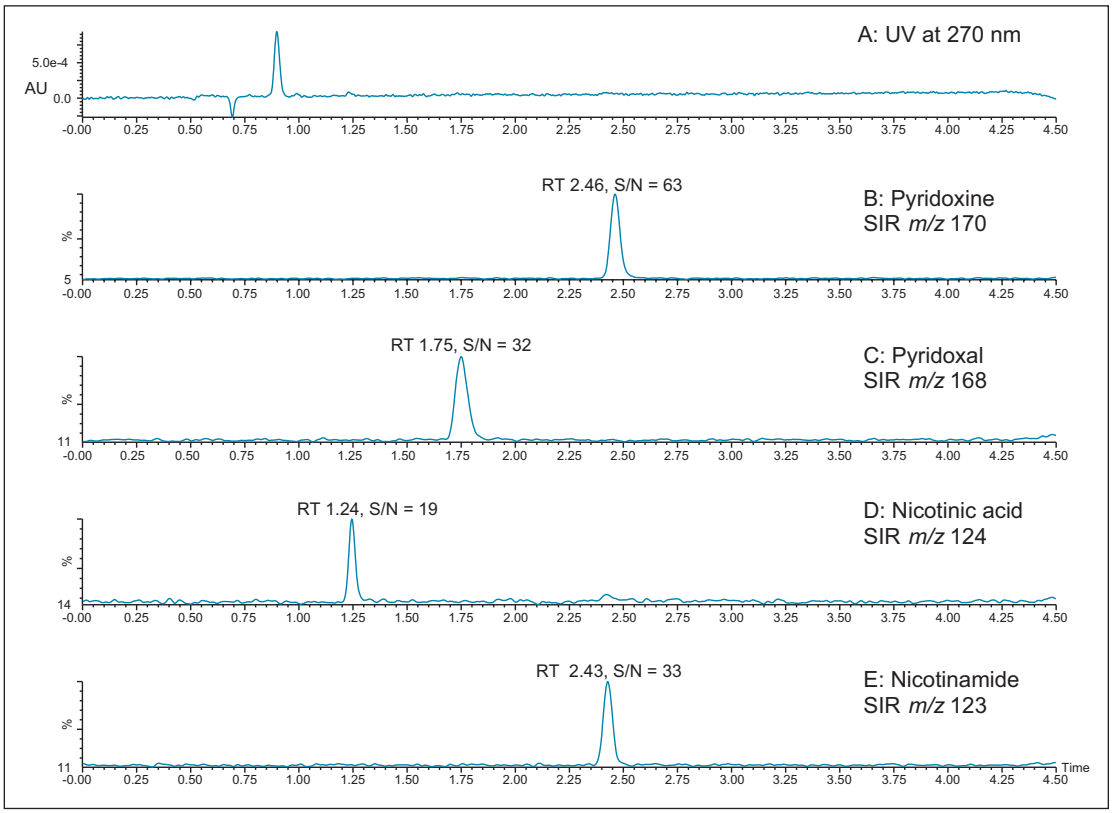

Figure 3. UV chromatogram at 270 nm and SIR chromatograms of a standard mix of vitamins at 5 µg/L. SIR channels for four vitamins are shown. B: pyridoxine, C: pyridoxal; D: nicotinic acid; and E: nicotinamide.

Figure 4 shows the detection of vitamin B5 (calcium pantothenate) in two vitamin water samples. As shown in the UV chromatogram, vitamin B5 could not be detected by UV without additional sample preparation. Vitamin B1 (thiamine) is another vitamin that is difficult to detect using UV. Figure 5 shows an example of the detection of vitamin B1 and vitamin C (ascorbic acid) in a diluted powdered vitamin beverage. Although vitamin C could be detected in the UV chromatogram, vitamin B1 was not detected. Vitamin B1 however, was clearly detected using SIR with the ACQUITY QDa Mass Detector, as shown in Figure 5A.

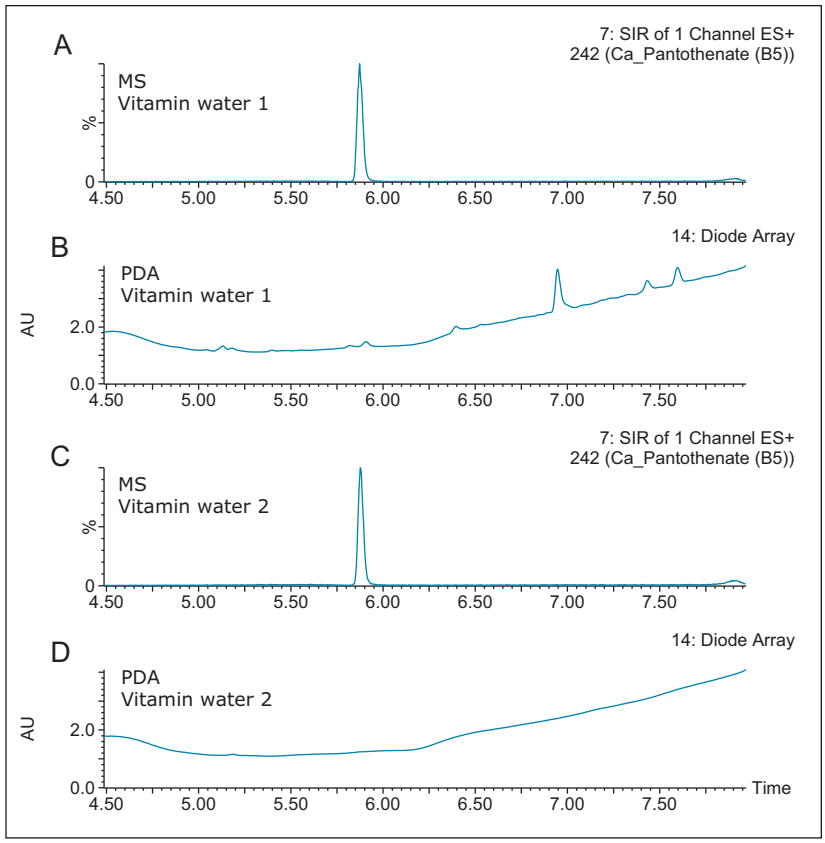

Figure 4. Detection of vitamin B5 in two different vitamin water samples. The peak at 5.9 minutes shows excellent signal-to-noise using mass detection (A and C) but cannot be detected using UV (B and D).

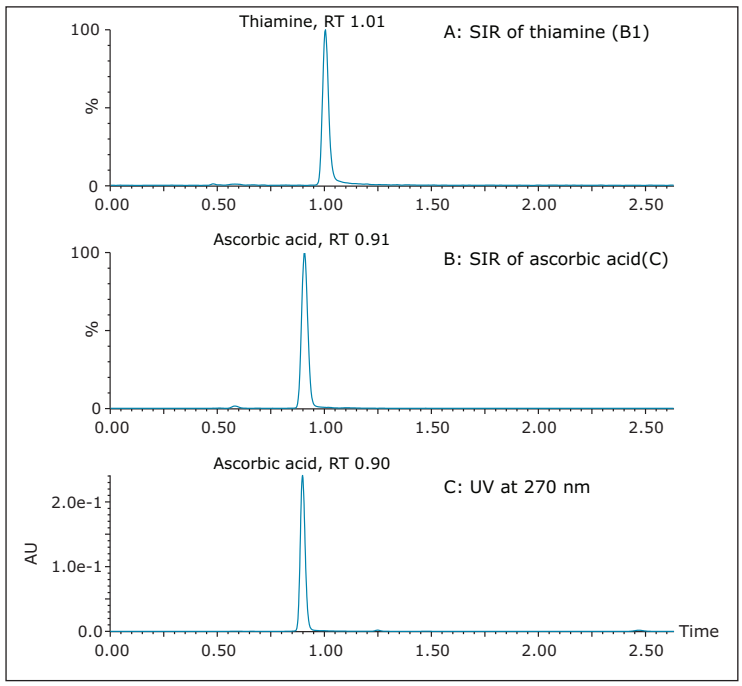

Figure 5. Chromatograms from a 1:250 dilution of a powdered vitamin beverage. A: SIR of vitamin B1 (thiamine), B: SIR of vitamin C (ascorbic acid), C: UV at 270nm; vitamin C was detected in the UV trace while vitamin B1 was not detected by UV.

Cyanocobalamin is a WSV that is fortified at very low levels in supplements and foods and it traditionally requires separate methodologies for its quantification. Two-dimensional chromatography is a routine strategy for the detection of this vitamin.[4] Figure 6 shows an example of cyanocobalamin detected in the multi-vitamin supplement tablet using the UPLC-MS method presented here. At this level, no peak was apparent in the UV chromatogram (Figure 6B). Mass detection offers the ability to detect vitamin B12 using the same method used to detect vitamins that are fortified at much higher levels. The ACQUITY QDa Detector, which can easily be incorporated into existing LC workflows, offers an easier-to-use method than existing multi-dimensional methods.

Another challenge that is encountered in vitamin analysis is the wide range of concentrations at which the vitamins are fortified. For the example of the multi-vitamin supplement in tablet form that was used in this study, the label stated that the B vitamins ranged from 6 µg for vitamin B12 (cyanocobalamin) to 16 mg for B3 labeled as niacin (nicotinic acid), with other B vitamins within that range. In this work, the same LC-MS method was used for the analysis of all the vitamins, with different dilution factors of the initial extraction in order to account for the different vitamin levels. Figure 7 shows chromatograms from the analysis of the multi-vitamin tablet. Figures 7A and 7B show the SIR channels of riboflavin (B2) in the 1:100 dilution of the sample and the undiluted sample, respectively. Figure 7C shows the SIR channel of cyanocobalamin (B12) in the undiluted sample. No peak was detected in the diluted sample (data not shown). The UV trace of the undiluted sample at 270 nm is shown in Figure 7D, and the riboflavin peak showed a good response for this sample. The quantified amount for riboflavin and cyanocobalamin were 12.5 ppm and 41 ppb, respectively. These amounts corresponded to 96% and 68% of the label claim of the supplement. Although the label claims were not verified for this work, nor did we undertake a recovery study, this short study demonstrated the feasibility of using the multiple dilution strategy within the calibration range specific in Table 2.

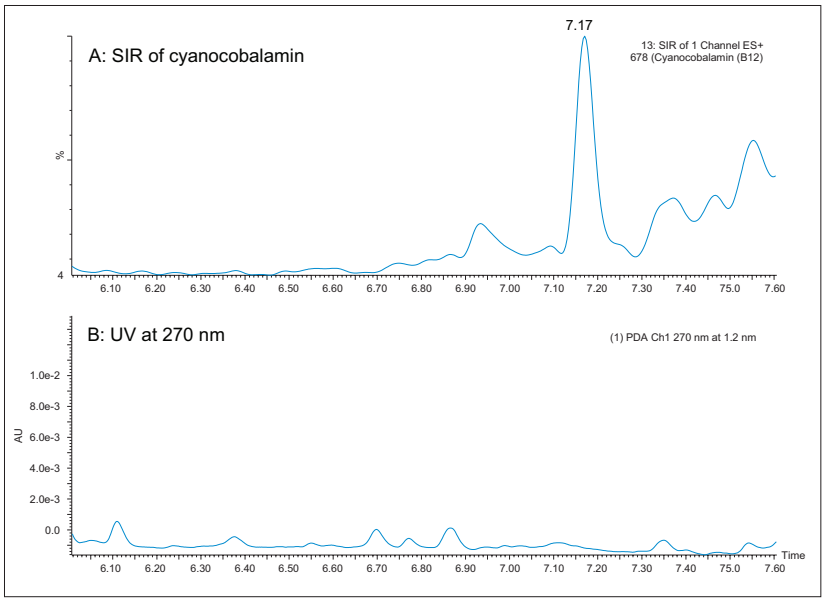

Figure 6. Detection of Vitamin B12 in a vitamin supplement tablet using mass detection (A). The level of the vitamin is below the UV detection limit (B).

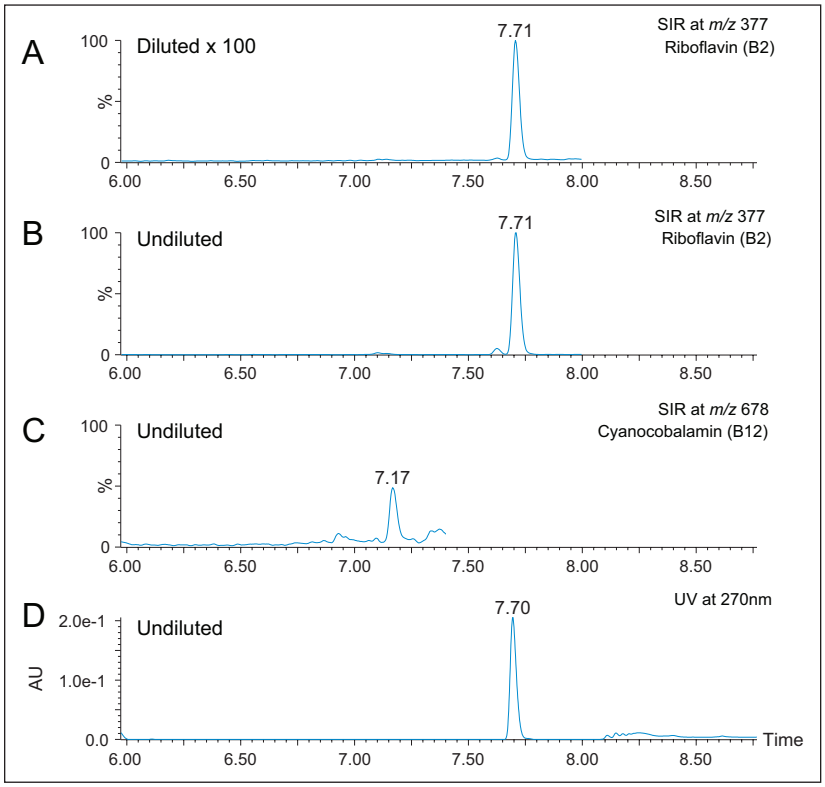

Figure 7. Detection of two B vitamins of very different concentrations in a vitamin supplement tablet. Riboflavin can clearly be detected in a 1:100 dilution of the sample extract (A), whereas B12 is only apparent in the undiluted extract (C). The response of riboflavin in the undiluted extract (B) is outside the calibrated range. At this level it can easily be detected using UV (D).

In order to assess the repeatability of the method for the B vitamins, multiple injections at different vitamin levels were assessed. Results for retention time repeatability, and peak area repeatability are shown in Tables 4 and 5, respectively. In Table 4, 10 injections of two different standards had been combined to give a total of 20 injections. Retention time stability was excellent, even for the early eluting water soluble vitamins, with all RSDs at or below 0.6%. Peak area repeatability was assessed with 10 injections at 0.025 mg/L (Table 5). For the majority of vitamins, %RSDs were well below 10%, with the exception of folic acid and riboflavin 5 phosphate, which were the lower responding analytes mentioned above. Vitamin C was excluded from this study as it is known to degrade over time.

Table 4. Repeatability of retention times for 20 injections using two different standards 0.75 mg/L (10 injections) and 0.025 mg/L (10 injections).

Analyte	%RSD for retention time
Thiamine (B1)	0.6
Nicotinic acid (B3)	0.19
Pyridoxal (B6)	0.23
Nicotinamide (B3)	0.22
Pyridoxine (B6)	0.26
Ca_Pantothenate (B5)	0.04
Cyanocobalamin (B12)	0.03
Folic acid (B9)	0.03
Riboflavin 5 phosphate	0.03
Biotin (B7)	0.02
Riboflavin (B2)	0.03

Table 5. Repeatability of B vitamins peak areas for 10 injections of a standard mix at 0.025 mg/L.

Analyte	%RSD for area
Thiamine (B1)	6.78
Nicotinic acid (B3)	2.35
Pyridoxal (B6)	2.62
Nicotinamide (B3)	2.24
Pyridoxine (B6)	2.65
Ca_Pantothenate (B5)	4.60
Cyanocobalamin (B12)	7.00
Folic acid (B9)	11.53
Riboflavin 5 phosphate	14.29
Biotin (B7)	2.77
Riboflavin (B2)	2.28

CONCLUSIONS

This work shows the capability of the ACQUITY QDa Mass Detector to accurately quantify water soluble vitamins at levels that cannot be achieved with UV. The acquisition of SIR channels allows for sensitive and selective quantification of analytes, even when co-elution occurs. This helps to remove the burden of ensuring all analytes are baseline separated and enables the detection of lower levels of vitamins.

The ACQUITY QDa Mass Detector allows new users to:

- Quantify analytes which have little or no UV response.
- Selectively quantify compounds that co-elute but have different masses.
- Consolidate water soluble vitamin methods into a single LC-MS method.
- Reduce limits of quantification in order to assess easier sample preparation strategies.
- Easily integrate into existing LC workflows, with the choice of Empower® 3 CDS or MassLynx® MS software control.
- Quickly take advantage of the ACQUITY QDa's mass detection capabilities – no special mass spectrometry knowledge required.

References

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