

1 **Determination of selected water-soluble vitamins (thiamine, riboflavin, nicotinamide and**
2 **pyridoxine) from a food matrix using hydrophilic interaction liquid chromatography coupled**
3 **with mass spectroscopy**

4

5

6 Kate Porter and John K Lodge*

7

8 Department of Applied Sciences, Faculty of Health and Life Sciences, Ellison Building,
9 Northumbria University, Newcastle Upon Tyne NE1 8ST, United Kingdom

10

11 * corresponding author

1 **Abstract**

2 Water-soluble vitamins are essential dietary components with a multitude of important
3 functions that require quantification from food sources to characterise the nutritional status
4 of food. In this study, we have developed a hydrophilic interaction chromatography (HILIC)
5 based method coupled to single-quadrupole mass spectrometry (MS) for the analysis of
6 selected water-soluble vitamins. Due to their involvement in energy release from
7 macronutrients, the quantification of thiamine (B₁), riboflavin (B₂), nicotinamide (B₃) and
8 pyridoxine (B₆) offers significant value in food analysis.

9 A commercially available vegetable soup was selected as the food matrix for this study and
10 utilised to develop an efficient extraction procedure for the vitamins of interest. Vitamins
11 were extracted using meta-phosphoric acid coupled with a reducing agent, DL-dithiothreitol
12 (DTT) to produce the parent compound. The extracted vitamins were then analysed using an
13 LC-MS system with electrospray – atmospheric pressure ionization (ES-API) source, operated
14 in positive single ion monitoring (SIM) mode. The MS provided good linearity within the
15 investigated range from 5-400 ng/mL with coefficient of determination (r^2) ranging from 0.98-
16 0.99. Retention times (0.65-9.04 mins) were reproducible and no coelution between vitamins
17 was observed. Limit of detection (LOD) varied from 2.4-9.0 ng/mL and limit of quantification
18 (LOQ) was from 8-30 ng/mL, comparable to previously published studies. The extraction
19 method provided good intra-day (%CV 1.56-6.56) and inter-day precision (%CV 8.07-10.97).
20 Standard injections were used as part of quality control measures and provided excellent
21 reproducibility (%CV 0.9-3.4). The overall runtime of this method was 19 mins, including
22 column reconditioning. Using this method, the quantity of thiamine (67 ± 7 ng/g), riboflavin
23 (423 ± 39 ng/g), nicotinamide (856 ± 77 ng/g) and pyridoxine (133 ± 11 ng/g) was determined
24 from a complex food matrix.

25 In conclusion, we have developed a rapid and reliable, HILIC-single quad MS method utilising
26 SIM for the low-level quantification of four B vitamins in a vegetable soup matrix in under
27 20 mins. This method has shown excellent linearity, intra- and inter-day reproducibility and is
28 directly applicable to other plant-based food matrices.

29

30 **Key Words**

31 Water-soluble vitamins; LC-MS; HILIC; analysis; food matrix

32 **Abbreviations**

33 HILIC, hydrophilic interaction chromatography; MS, mass spectrometry; MRM, Multiple
34 Reaction Monitoring; RP, reversed-phase; LOD, limit of detection; LOQ, limit of quantification;
35 DTT, DL-dithiothreitol; SIM, single ion monitoring; ES-API, Electrospray – Atmospheric
36 pressure ionization; CV, coefficient of variation; SD, standard deviation; B₁, thiamine; B₂,
37 riboflavin; B₃, nicotinamide; B₆, pyridoxine.

1 1. Introduction

2

3 Water-soluble vitamins and their derivatives are important dietary components essential for
4 health due to their wide range of functions within central metabolism. The B group vitamins,
5 for example, function to release energy from macronutrients, play crucial roles in redox
6 reactions and act as enzyme co-factors [1]. The accurate quantification of water-soluble
7 vitamins in foodstuffs is of significant value and importance. However, simultaneous
8 determination and quantification of these vitamins from various food matrices represents a
9 substantial challenge. In part, this is due to the diverse structural forms and different chemical
10 compounds that make up these vitamins, many of which occur side by side in various foods.
11 Naturally occurring vitamins often occur covalently bound to enzymes as cofactors, or as
12 phosphorylated forms bound non-covalently to proteins and carbohydrates [2]. Thiamine (B₁)
13 occurs in plant-derived foods chiefly in its free form but is present in animal foods as mono-,
14 di-, or triphosphates and their esters, protein complexes, and as thiamine disulfides and their
15 pyrophosphoric acid esters [1, 3]. This renders precise quantification of vitamin levels in food
16 a difficult process, as conventional methods require the parent compound in its free form to
17 compare against pure standards. Another limitation is related to the extraction process
18 employed to release bound vitamins, as B group vitamins can be labile and degrade in the
19 presence of light, heat and pH conditions [2, 4-6]. Due to the reasons presented above
20 highlights the difficulty to develop one method to analyse all vitamins in a given sample and
21 the reason several methodologies exist for each vitamin [7-10].

22 Analysis of individual vitamins in foods were traditionally performed using microbial assays,
23 however technological advances in liquid chromatography (LC) has provided methods
24 offering increased accuracy and precision [11, 12]. Reversed-phase (RP) or hydrophilic
25 interaction chromatography (HILIC) has been the separation method of choice for several
26 years, but with different methods for detection and quantitation. For example, UV/vis
27 detection [13-23], fluorescence detection [18, 24, 25], or electrochemical/coulometric [8, 26-
28 28] have been employed, and we have previously reported a comparison of such
29 methodologies [1]. However, the increased sensitivity offered by mass spectroscopy (MS) is
30 of particular value for vitamin quantification when present in trace amounts and when
31 complex food matrices are required for analysis. MS detection has been utilised for analysis
32 of water-soluble vitamins in various food matrices including infant/nutritional formula,
33 supplements, beverages and fortified produce [2, 10, 12, 21, 29-35]. Santos *et al.* (2012) [21]
34 validated a LC-MS/MS RP method simultaneously analysing seven free form water soluble
35 vitamins in 12 different green leafy vegetables. Gentili *et al.* (2008) [2] developed a LC/ESI-
36 MS/MS RP method to determine B vitamins in four plant-based matrices. The authors utilised
37 a solid-liquid extraction to determine the quantity of B vitamins, from which the vitamin
38 content in maize flour (3.27-0.020 µg/g), green kiwi (2531-0.012 µg/g), golden kiwi (7758-
39 0.19 µg/g) and tomato pulp (116.3-0.10 µg/g) were determined. However, the majority of

1 these methods for quantifying B vitamins in food matrices utilise MS with RP separation, with
2 limited papers utilising HILIC.

3 Chromatographic separation of water-soluble vitamins can be challenging using standard RP;
4 they are highly polar compounds with different extents of hydrophilicity and due to this can
5 be poorly retained using RP separation mechanisms [30, 31]. HILIC is an alternative to RP
6 separation, receiving popularity due to it being best suited for analysis of polar analytes
7 improving selectivity and compatible with MS detection [15]. Goldschmidt and Wolf *et al.*
8 (2010) [30] study into different chromatographic methods involving RP and HILIC for analysis
9 of B vitamins found RP to be problematic for retention of thiamine, nicotinamide and
10 pyridoxine. Due to these vitamins being more hydrophilic they were more effectively retained
11 using HILIC separation. However, as highlighted by Fatima *et al.* (2019) [36], a very limited
12 number of validated methods utilising HILIC separation coupled to MS detection have been
13 developed to analyse water soluble vitamins in complex food matrices. A study by Chatterjee
14 *et al.* (2017) [37] developed a LC-MS/MS method targeting nine water-soluble vitamins in fish
15 using HILIC separation for increased sensitivity. The authors optimised an enzymatic
16 hydrolysis method to subsequently extract B vitamins; including nicotinamide, nicotinic acid,
17 pyridoxine, pantothenic acid, biotin, thiamine, riboflavin and cyanocobalamin from fish flesh.
18 However, this method primarily utilised the advantages offered by Multiple Reaction
19 Monitoring (MRM) of a triple-quadrupole MS for identification and quantification of each
20 analyte. This method may not translate to reliable quantification on a single-quadrupole MS
21 without access to MRM. Single-quad MS instruments are widespread and comparatively
22 cheaper than triple-quad MS systems; therefore, a method specifically developed for
23 quantification on a single-quad MS may be more widely applicable for routine vitamin
24 analysis.

25 Alongside, extraction procedures employed to extract vitamins from food matrices are often
26 time consuming, involving many steps and chemical preparations. For example, Nurit *et al.*
27 (2015) [12], employed enzymatic hydrolysis consisting of 6 different solutions and an 14 h
28 incubation stage to extract vitamins. Therefore, in this study it was important to develop a
29 simple analytical method suitable for high throughput analysis. We report here a method
30 that combines a relatively simple extraction and combination of HILIC chromatography and
31 single-quad MS detection that offers a practical and affordable approach to the quantification
32 of B vitamins from food matrices.

33

2. Materials and Methods

2.1 Chemical and Reagents

All water-soluble vitamins were acquired from Sigma-Aldrich (Gillingham, Dorset): nicotinamide (98%), (-)-riboflavin (98%), pyridoxine hydrochloride (99%) and thiamine hydrochloride (99%). Methanol, ammonium acetate and 35% ammonia solution, all HPLC grade, were purchased from Fisher Scientific (Loughborough, Leicestershire). LC/MS grade acetonitrile and acetic acid (Optima grade) were additionally purchased from Fisher Scientific. Meta-phosphoric acid was obtained from Acros Organics (Fisher Scientific). HPLC grade DL-dithiothreitol (DTT) was purchased from Sigma-Aldrich (Gillingham, Dorset). A Milli-Q Integral three water purification system was used (Merck Millipore, Watford, Hertfordshire) which purified water to 18.2 MΩ. 0.2 μm nylon syringe filters were used for the preparation of final LC-MS samples (Chromacol, Fisher Scientific).

2.2 Preparation of Standards (Thiamine, Nicotinamide, Riboflavin and Pyridoxine)

Stock solutions of individual vitamins were prepared on ice and in low light as follows: thiamine, nicotinamide and pyridoxine were prepared in a 0.12% acetic acid solution at 5 mg/mL. Riboflavin was dissolved in 0.28 M ammonia solution at a concentration of 0.5 mg/mL adjusted to pH 7.0 with diluted acetic acid. All stock solutions were placed in a sonicated water bath (Bandelin Sonorex, Berlin) for 5 mins, aliquots of the vitamin stocks were taken and frozen immediately in liquid nitrogen and stored at -80 °C for 1 month. Aliquots of stock were defrosted daily for working standards and diluted in mobile phase B; 10 mM ammonium acetate (pH 5) in 95:5 (V:V) acetonitrile: 18.2 MΩ deionised water. Working standards and multi-vitamin standards were diluted within a 5-400 ng/mL calibration range, filtered through a 0.2 μm nylon filter; all were prepared in glass amber vials.

2.3 Liquid Chromatography Mass Spectroscopy Analysis

Analysis of targeted water-soluble vitamins was completed on an Agilent Technologies (Stockport, Cheshire) 1260 Infinity LC system, features consisted of a: quadruple channel pump, auto sampler with a 100 μl injection loop, column oven, UV detector, coupled with an Agilent 6120 single quadrupole MS detector equipped with a electrospray – atmospheric pressure ionization (ES-API) source.

Chromatography conditions were: Flow rate of 0.8 mL/min, auto sampler set to 7 °C and column oven set to 30 °C. Targeted vitamins were separated on an Agilent (Stockport, Cheshire) ZORBAX HILIC Plus Silica column (100 mm x 2.1 mm, 3.5 μm). Chromatographic separation was achieved following a published method [1] using mobile phase A: 10 mM ammonium acetate (pH 5.0) in 95:5 (V:V) water: Acetonitrile and mobile phase B: 10 mM ammonium acetate (pH 5.0) in 95:5 (V:V) Acetonitrile: water. Column equilibrated at set instrumental conditions at 60% eluent B for 2 hours, followed by 100% eluent B for 30 mins.

1 The analytical gradient started in isocratic mode (100% B) over a 4.5 min period, reduced to
2 60% B over 6 mins, returned to isocratic mode (100% B) over 6 mins, run completed at
3 19 mins. 5 µl of sample was injected onto column.

4 Mass spectroscopy parameters were: drying gas flow 12.0 L/min, nebulizer pressure 35 psig,
5 drying gas temperature 350 °C and capillary voltage 3000 V (positive and negative mode).
6 Signal settings set to signal ion monitoring (SIM).

7

8 2.4 Calibration, Precision and Quality Control Measures

9 Calibration curves for targeted vitamins were made for multi-vitamin standards by plotting
10 the multi-vitamin concentration against peak area. The calibration curve for vitamins B₁, B₂
11 and B₆ consisted of 7 points ranging from 5-400 ng/mL, whereas B₃ calibration curve
12 comprised of 6 points from 10 ng/mL to 400 ng/mL. Calibration curves were made fresh each
13 day of analysis and an average taken from multiple injections on separate days.

14 As part of quality control, a multi-vitamin standard at 50 ng/mL was prepared and injected
15 throughout each chromatographic run and confirmed against calibration curve to assess
16 accuracy and investigate deviation during each run. Blank mobile phase samples and blank
17 extraction samples were also injected throughout each chromatographic run to ensure no
18 interferences.

19

20 2.5 LOD and LOQ

21 Limit of detection (LOD) was calculated on the slope and the standard deviation of the
22 response, based on the calibration curve according to published guidelines [38].

$$23 \quad LOD = \frac{3\sigma}{S}$$

24 Where: σ = the standard deviation of the y-intercept

25 S = Calibration curve slope

26

27 Limit of quantification (LOQ) was calculated the same as LOD, but 10x the standard deviation
28 of the y-intercept and expressed as:

$$29 \quad LOQ = \frac{10\sigma}{S}$$

30

31 2.6 Food Sample Extraction

32 Commercially available vegetable soup purchased from a local supermarket, nutritional
33 values as stated on packaging were; carbohydrates 4.2 g (sugars 2.3 g), fat 0.7 g, protein 0.6 g,
34 fibre 1.1 g and salt 0.48 g. Sample preparation for analysis of targeted vitamins was adapted
35 from a published method [1] and all extraction procedures were completed in low light

1 conditions. Soup was homogenised in a blender for 2 mins, approximately 40 g aliquots were
2 taken in 50 ml falcons and rapidly frozen in liquid nitrogen, stored at -80 °C. Upon analysis
3 40 g aliquots were removed from the freezer and defrosted at room temperature under low
4 light conditions. Defrosted aliquots were collectively poured into a sterile stomacher bag and
5 homogenised in a stomacher blender on 'normal mode' for 1 min (Stomacher®- 400
6 circulator, Seward Ltd, Sussex). Aliquots, 5 g, were weighed from the blended sample and
7 rapidly frozen in liquid nitrogen, before placing in a lyophiliser (VirTis SP Scientific, Sentry 2.0)
8 for 24 hrs. For vitamin extraction, 3 mL of a 3% meta-phosphoric acid/ 200 mg/L DL-
9 dithiothreitol (DTT) solution, 1 mL methanol and 0.5 mL of 18.2 MΩ deionised water was
10 added to the dried sample. Vortexed (Vortex-Genie 2, Scientific Industries Inc., USA) using a
11 Mo-Bio 15 ml flacon vortex adapter for 10 mins and centrifuged (Beckman Allegra 6R
12 centrifuge, High Wycombe) at room temperature at 4000 rpm for 15 mins. Subsequently,
13 1 mL of the supernatant was added to 3 mL acetonitrile, vortexed for an additional minute
14 and centrifuged again for 15 mins at 4000 rpm. The supernatant was filtered through a 0.2 µm
15 nylon syringe filter into amber vials and 5 µl of sample was injected onto column.

16 As part of preliminary studies taken to provide information on the most suitable extraction
17 protocol, removal of the lyophilisation step was investigated. All other steps in the procedure
18 were identical; 5 g aliquots were weighed from the blended sample and the vitamin
19 extraction protocol followed. After addition of extraction solutions, the samples were
20 vortexed for 1 min, rather than 10 mins, as per the lyophilisation sample for reconstitution.

21

22 2.7 Recovery and Precision

23 Sample recovery was performed on the lyophilised sample by spiking 5 g soup sample in
24 quintuplicate with either 0.5 mL mobile phase B (n=5), or 0.5 mL of a multi-vitamin standard
25 at a concentration of 2.5 µg/mL (n=5) prior to placement in the lyophiliser (VirTis SP Scientific,
26 Sentry 2.0), followed by the extraction procedure. Alternatively, recovery was investigated in
27 the non-lyophilised, spiking 5 g with a multi-vitamin standard (0.5 ml) comprising of B₁, B₂, B₃
28 and B₆ at a concentration of 10 µg/mL, with the exception of B₃ at a concentration of
29 20 µg/ml. The multi-vitamin standard concentrations (2.5-20 µg/mL) used for sample
30 recovery were diluted throughout both extraction protocols (lyophilised and non-lyophilised),
31 as outlined in above section (2.6) with meta-phosphoric acid/DTT solution and acetonitrile.
32 This ensured the multi-vitamin standard spiking concentrations fell inside the investigated
33 linear range, by the final stage of extraction. Percentage recovery was calculated based on
34 the quantity of each analyte recovered according to the calibration curve, as a percentage of
35 the known spiked quantity the soup contained. However, when calculating percentage
36 recovery in the non-lyophilised sample, average percentage moisture of the soup (92%) was
37 taken into consideration.

1 The extracted soup sample (one extraction) was injected in quintuplicate for intra-day
2 precision, while inter-day precision was determined by extracting and analysing 5 different
3 soup samples from the same batch.

4

5 2.8 Data Analysis

6 All statistical analysis completed to determine precision and reproducibility of method was
7 calculated based on mean and standard deviation of replicates. The standard deviation was
8 further divided by the mean of the replicates, to give the percentage coefficient of variation
9 (CV).

3. Results and Discussion

3.1 LC-MS Method Development and Optimisation for Separation of Nicotinamide, Pyridoxine, Riboflavin and Thiamine

This study focused on the quantification of selective water-soluble, B vitamins using HILIC chromatography, as we have previously shown [1], but with the added sensitivity and selectivity of MS detection. HILIC offers the advantage of targeting more polar, hydrophilic vitamins that otherwise would be difficult to target using RP and includes B vitamins thiamine and nicotinamide [21, 30]. HILIC chromatography achieves separation of polar analytes/strong hydrophilic complexes by partitioning these between the mobile phase and the stationary phase enriched with a water layer [39, 40]. Although, this is the commonly accepted mechanism other interactions are potentially taking place alongside and include surface adsorption by hydrogen bonding, dipole-dipole interactions and electrostatic interactions [33, 36, 39]. The MS behaviour of the B vitamins was first investigated as part of method development. Although some vitamins were observable in negative mode (e.g. B₆), positive mode ionisation provided better intensities by approximately 10-fold and so all data was acquired in positive mode ionisation. Leporati *et al.* (2005) [10], reported similar findings and found operating in positive mode resulted in greater intensities and highlighted a 100 – 1000 fold increase for B vitamins compared to negative mode. The increased intensity in positive mode reported by Leporati *et al.* (2005) [10] was higher than observed in this paper, but many factors including the selected B vitamin analysed, the type of MS, and the MS operating conditions could play a role in the stated differences. Single ion monitoring (SIM) mode offered significant improvements over scan mode including reduced background noise and improving peak shape for each of the target analytes, increasing both precision and accuracy. SIM parameters are shown in Table 1 and a representative chromatogram of B-vitamin standards is shown in Figure 1. Retention times range from 0.65 min to 9 min with no coelution between vitamins.

3.1.1 LC-MS Method Validation

Calibration curves constructed from individual, authentic vitamin standards were used for quantification. Representative calibration curves are shown in Figure 2 and characteristics are given in Table 2. All vitamins displayed a linear range from 5 ng/mL to 400 ng/mL which was the investigated range for this study, with coefficient of determination (r^2) values >0.98. In addition to r^2 , the relative standard deviation of the slopes were calculated as proposed by Raposo (2016) [41]. The %RSD_{slope} is a useful variable for confirming linearity of linear regression. In this study, thiamine, riboflavin, nicotinamide and pyridoxine achieved intra-day calibration %RSD_{slope} of 1.7%, 1.5%, 2.9% and 2.9% respectively. LOD and LOQ data are also shown in Table 2, calculated based on the slope of the calibration curve and standard deviation of the response. These values are comparable to other publications utilising ESI-MS detection. In this method riboflavin has a quantification limit of 8 ng/mL, comparable to the

1 2 ng/mL [10] to 60 ng/mL [42] of previously reported methods. Similarly, the observed limits
2 of detection and quantification for pyridoxine were within previously reported values [10, 42].
3 The LOD of nicotinamide was 9.0 ng/mL, comparable to the reported 8 ng/mL [43], but
4 slightly higher than that reported by Leporati *et al.* (2005) of 5 ng/g [10]. Thiamine detection
5 and quantification limit of 6.5 and 21.7 ng/mL respectively is higher than published 3 and
6 7 ng/mL [43], but comparable to the values described by Lebedzińska *et al.* (2007) [8] of
7 9.2 ng/mL (LOD) and 22.0 ng/mL (LOQ) using HPLC-ED UV. However, when compared to our
8 previous work employing UV, fluorescence and coularray detection [1], all B group vitamins
9 in this current study have lower LOD and LOQ limits. For example, thiamine, riboflavin,
10 nicotinamide and pyridoxine LOQ were lower using MS detection by 91%, 52%, 68% and 94%
11 respectively when compared to UV and fluorescence detection. The LOD of investigated
12 vitamins was improved by 92% for thiamine; 69% for riboflavin; 50% for nicotinamide; and
13 94% for pyridoxine. This further highlights that incorporation of MS provides greater
14 sensitivity and is suitable for applications where the vitamins are present in trace amounts in
15 a complex 'dirty' matrix, such as food.

16 An internal standard was not utilised in this study; an effective internal standard should be
17 structurally similar to each of the targeted analytes, absent from the complex food matrix,
18 while having almost identical chromatographic behaviour [12]. Therefore, an internal
19 standard was not feasible due to targeting several structurally diverse B vitamins
20 simultaneously in a complex food matrix. Other previously published methods have omitted
21 the use of an internal standard, in support of external calibration standards or standard
22 addition techniques [2, 10, 35, 37]. Stable isotope versions of the B vitamins are the most
23 adequate internal standards when using MS detection [12, 29, 32], but due to significant cost
24 implications they are not always utilised. Alternatively, in this study a standard injection was
25 used throughout each analytical run as part of quality control measures, to confirm any
26 injection/sensitivity deviation and confirm precision of calibration curve. Table 3 displays
27 precision and reproducibility of injection standard (multi-vitamin standard at 50 ng/mL)
28 based off calibration curves in a run. From this, it can be seen the injection standard behaved
29 as expected with excellent reproducibility (<4%) over the course of the run.

30

31 3.2 Analysis and Validation of Water-Soluble Vitamins in a Soup-Based Food Matrix

32

33 3.2.1 Extraction Method and Precision (Intra-Day and Inter-Day)

34 After optimising the LC-MS behaviour of the vitamins, it was important to investigate the
35 extraction of water-soluble vitamins from a typical food matrix. For this we chose to use
36 commercially available fresh vegetable soup as we wanted to test the methodology on a
37 largely liquid based food matrix as that would have a more homogenous consistency. The
38 most common extraction processes for B group vitamins can be complex, utilising weak acid
39 treatment, coupled with high temperatures (0.1 M HCl at 100 °C-121 °C), further followed by
40 enzymatic hydrolysis, with the objective of extracting bound vitamins [1, 2, 22, 44, 45]. This

1 type of extraction was trialled initially but was ineffective to extract all vitamins of interest at
2 the same time. To simplify, we incorporated a strongly acidic environment, in order to
3 hydrolyse covalent bonds and release the free vitamins, with a reducing agent to generate
4 the free vitamins in their native form [2, 46]. We have previously used a similar extraction
5 procedure for vitamin C [1], that contains meta-phosphoric acid as a reducing agent and
6 stabiliser.

7 A typical LC-MS chromatogram of the vitamins extracted from food is shown in Figure 3. It
8 can be seen that the vitamin peaks are clearly identified with no contamination that would
9 influence quantitation, facilitated through SIM mode utilisation. However, food matrices are
10 complex and other extracted components may co-elute in the MS source, potentially
11 interfering with the ionization of the analytes [47, 48]. Additionally, a chromatogram of
12 extracted vitamins from food, overlaid with a spiked food sample is shown in Figure 4. To
13 verify the vitamin peaks, we analysed the fragmentation pattern of each vitamin against a
14 pure standard, which agrees with published work [2].

15 Next, we looked at the reproducibility of the methodology (Table 4). Intra-day and inter-day
16 precision were very good, and all vitamins displayed variation <7% for intra-day and variation
17 <10% for inter-day. Thiamine was the only vitamin where inter-day precision was slightly
18 higher at 11%. The precision values of the presented method can be evaluated against other
19 published methodologies, although these values will be dependent on the food matrices
20 investigated. Gratacós-Cubarsí *et al.* (2010) [22] reported an extraction method for thiamine
21 from dry-cured sausages, and demonstrated good intra-day repeatability of <7.0% and inter-
22 day variability of <7.8%. Nurit *et al.* (2015) [12] method showed intra-day precision values of
23 between 3.2-9.3% and inter-day CV's ranging from 6.2-13.8% across thiamine, riboflavin,
24 nicotinamide and pyridoxine extracted from different wheat flour products. The
25 reproducibility of their extraction methods are similar to the assessed reproducibility
26 reported here.

27

28 3.2.2 Recovery Studies

29 The recovery of each vitamin using our methodology was assessed, by spiking soup with a
30 known concentration of vitamin mix (Table 5). The percentage recovery of riboflavin and
31 pyridoxine were desirable, between 96-118%, but nicotinamide and thiamine experienced
32 lower recovery (30-50%). Recovery of nicotinamide is known to be problematic as recoveries
33 in the region of 29-32% have been reported [2], and recovery appears to be dependent on
34 the food matrix investigated. Nicotinamide in foods will coexist with nicotinic acid and are
35 collectively referred to as niacin. For example, based on niacin profiles of vegetables, onions
36 comprise of 72% nicotinamide and 28% nicotinic acid, whereas potatoes contain 13%
37 nicotinamide and 87% nicotinic acid [49]. Further, upon acid extraction some of the
38 nicotinamide would have been converted to the acid form [50]. However, the conversion of
39 forms has not been accounted for in this paper, as nicotinic acid was not detected on the MS.

1 To assess any influence of the food matrix, we performed recovery experiments both from a
2 soup sample or from a blank sample and results indicated minimal differences in recovery.
3 For example, riboflavin recovery from soup was 96%, while from blank 84% (data not shown),
4 indicating there was limited interference from the food matrix in our experiments and
5 therefore, matrix effect was not investigated further. Alternatively, we also investigated %
6 recovery when the soup samples were not lyophilised prior to extraction, as part of a separate
7 preliminary investigation, data included in Table 4. Omission of the lyophilisation step
8 resulted in an increase in nicotinamide recovery to 76%. Freeze-drying has been shown in
9 previous studies to cause loss of nicotinamide forms in plant tissue, similar to the findings in
10 this report and potentially contributed to the observed low recovery of nicotinamide [51].
11 However, removal of the lyophilisation step, simultaneously resulted in thiamine falling below
12 the detection limit in soup samples (non-spiked). Therefore, lyophilisation was required as a
13 concentration step to analyse all vitamins.

14

15 3.2.3 Quantification of Vitamins in a Soup-Based Food Matrix

16 Following extraction and analysis by our method, the following quantities were found to be
17 present in our soup sample; thiamine 67 ± 7 ng/g, riboflavin 423 ± 39 ng/g, nicotinamide 856
18 ± 77 ng/g and pyridoxine 133 ± 11 ng/g. Santos *et al.* (2012) [21] published a paper
19 determining B vitamin content in vegetables where they observed a higher abundance of
20 nicotinamide, when compared to riboflavin and thiamine. Food labelling or nutritional
21 databases contain no vitamin content information for commercially available soup, and
22 popular nutritional databases including Nutritics™ (Nutritics, 5.098 research edition, Dublin
23 Ireland) or MyFitnessPal™ (MyFitnessPal, Inc) also have no firm estimates. However, the
24 vitamin content presented can be compared to McCance & Widdowson's (2002) [52] food
25 composition tables for vegetable soup; thiamine 900 ng/g; riboflavin 200 ng/g; and
26 pyridoxine 100 ng/g. The content of nicotinamide cannot be compared, as the value
27 published was based on total niacin content. The quantity of pyridoxine was comparable,
28 whereas the detected riboflavin was greater than reported, while thiamine content was
29 underrepresented in the current study, potentially due to the vegetable composition of the
30 studied soup. The analytical methods utilised for vitamin determination by McCance &
31 Widdowson's (2002) [52], comprised of HPLC and microbiological assays with the latter
32 technique lacking efficiency and accuracy. It has also been previously suggested, that
33 microbial assays as a quantification tool for water-soluble vitamins may overestimate true
34 vitamin content [53].

35

36 4. Conclusions

37 We have developed a relatively simple yet sensitive method to extract and quantify several B
38 vitamins from a food matrix. HILIC chromatography coupled with MS detection was applied

1 for quantification of targeted vitamins (B₁, B₂, B₃ and B₆) that were isolated from a
2 commercially available soup utilising an acid extraction. The simple extraction method
3 resulted in comparable performance to that of other more complex methods which require
4 further processing steps and techniques. In terms of quantification, employing a single-quad
5 LC-MS system provided good sensitivity, with low detection limits varying from 2.4-9.0 ng/mL
6 and quantification limits between 8-30 ng/mL. Linearity ranged from 5-400 ng/mL and was
7 successfully used to determine vitamin content in this work. Four targeted B vitamins were
8 reliably quantified within a total run time of 19 mins, with a method applicable for a single-
9 quad MS. Overall, the method developed has shown to be applicable to procedures that
10 require high throughput and good sensitivity, with the opportunity to monitor nutritional
11 content in foods.

12 **Funding**

13 This research did not receive any specific grant from funding agencies in the public,
14 commercial, or not-for-profit sectors.

15 **Declaration of interest**

16 No competing interests.

17

1 References

2

3 [1] S. Langer, J.K. Lodge, Determination of selected water-soluble vitamins using hydrophilic
4 chromatography: A comparison of photodiode array, fluorescence, and coulometric detection, and
5 validation in a breakfast cereal matrix, *Journal of Chromatography B*, 960 (2014) 73-81.

6 [2] A. Gentili, F. Caretti, G. D'Ascenzo, S. Marchese, D. Perret, D. Di Corcia, L.M. Rocca, Simultaneous
7 determination of water-soluble vitamins in selected food matrices by liquid
8 chromatography/electrospray ionization tandem mass spectrometry, *Rapid Communications in Mass
9 Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute
10 Research in Mass Spectrometry*, 22 (2008) 2029-2043.

11 [3] G.F. Combs, *The vitamins : fundamental aspects in nutrition and health*, 4th ed.. ed., Amsterdam :
12 Elsevier/Academic Press, Amsterdam, 2012.

13 [4] M.A. Sheraz, S.H. Kazi, S. Ahmed, Z. Anwar, I. Ahmad, Photo, thermal and chemical degradation
14 of riboflavin, *Beilstein Journal of Organic Chemistry*, 10 (2014) 1999-2012.

15 [5] A.L. Voelker, J. Miller, C.A. Running, L.S. Taylor, L.J. Mauer, Chemical stability and reaction
16 kinetics of two thiamine salts (thiamine mononitrate and thiamine chloride hydrochloride) in
17 solution, *Food research international*, 112 (2018) 443-456.

18 [6] B. Saidi, J.J. Warthesen, Influence of pH and light on the kinetics of vitamin B6 degradation,
19 *Journal of Agricultural and Food Chemistry*, 31 (1983) 876-880.

20 [7] Y. Gao, F. Guo, S. Gokavi, A. Chow, Q. Sheng, M. Guo, Quantification of water-soluble vitamins in
21 milk-based infant formulae using biosensor-based assays, *Food Chemistry*, 110 (2008) 769-776.

22 [8] A. Lebidzińska, M.L. Marszał, J. Kuta, P. Szefer, Reversed-phase high-performance liquid
23 chromatography method with coulometric electrochemical and ultraviolet detection for the
24 quantification of vitamins B1 (thiamine), B6 (pyridoxamine, pyridoxal and pyridoxine) and B12 in
25 animal and plant foods, *Journal of Chromatography A*, 1173 (2007) 71-80.

26 [9] V. León-Ruiz, S. Vera, A.V. González-Porto, M.P. San Andrés, Analysis of water-soluble vitamins in
27 honey by isocratic RP-HPLC, *Food analytical methods*, 6 (2013) 488-496.

28 [10] A. Loporati, D. Catellani, M. Suman, R. Andreoli, P. Manini, W.M.A. Niessen, Application of a
29 liquid chromatography tandem mass spectrometry method to the analysis of water-soluble vitamins
30 in Italian pasta, *Analytica Chimica Acta*, 531 (2005) 87-95.

31 [11] C.J. Blake, Analytical procedures for water-soluble vitamins in foods and dietary supplements: a
32 review, *Anal Bioanal Chem*, 389 (2007) 63-76.

33 [12] E. Nurit, B. Lyan, A. Piquet, G. Branlard, E. Pujos-Guillot, Development of a LC-MS/MS method
34 for the simultaneous screening of seven water-soluble vitamins in processing semi-coarse wheat
35 flour products, *Analytical and Bioanalytical Chemistry*, 407 (2015) 3471-3479.

36 [13] R. Engel, É. Stefanovits-Bányai, L. Abrankó, LC simultaneous determination of the free forms of
37 B group vitamins and vitamin C in various fortified food products, *Chromatographia*, 71 (2010) 1069-
38 1074.

39 [14] O. Heudi, T. Kiliç, P. Fontannaz, Separation of water-soluble vitamins by reversed-phase high
40 performance liquid chromatography with ultra-violet detection: Application to polyvitaminated
41 premixes, *Journal of Chromatography A*, 1070 (2005) 49-56.

42 [15] A.E. Karatapanis, Y.C. Fiamegos, C.D. Stalikas, Study of the behavior of water-soluble vitamins in
43 HILIC on a diol column, *Chromatographia*, 71 (2010) 751-759.

44 [16] B. Klejdus, J. Petrlová, D. Potěšil, V. Adam, R. Mikelová, J. Vacek, R. Kizek, V. Kubáň,
45 Simultaneous determination of water- and fat-soluble vitamins in pharmaceutical preparations by
46 high-performance liquid chromatography coupled with diode array detection, *Analytica Chimica
47 Acta*, 520 (2004) 57-67.

48 [17] J.-H. Suh, D.-H. Yang, B.-K. Lee, H.-Y. Eom, U.-Y. Kim, J.-H. Kim, H.-Y. Lee, S.-B. Han, Simultaneous
49 determination of B group vitamins in supplemented food products by high performance liquid

1 chromatography-diode array detection, *Bulletin of the Korean Chemical Society*, 32 (2011) 2648-
2 2656.

3 [18] A. Zafrá-Gómez, A. Garballo, J.C. Morales, L.E. García-Ayuso, Simultaneous determination of
4 eight water-soluble vitamins in supplemented foods by liquid chromatography, *Journal of*
5 *agricultural and food chemistry*, 54 (2006) 4531-4536.

6 [19] S. Vidović, B. Stojanović, J. Veljković, L. Pražić-Arsić, G. Roglić, D. Manojlović, Simultaneous
7 determination of some water-soluble vitamins and preservatives in multivitamin syrup by validated
8 stability-indicating high-performance liquid chromatography method, *Journal of chromatography A*,
9 1202 (2008) 155-162.

10 [20] M. Ciulu, S. Solinas, I. Floris, A. Panzanelli, M.I. Pilo, P.C. Piu, N. Spano, G. Sanna, RP-HPLC
11 determination of water-soluble vitamins in honey, *Talanta*, 83 (2011) 924-929.

12 [21] J. Santos, J.A. Mendiola, M.B.P.P. Oliveira, E. Ibáñez, M. Herrero, Sequential determination of
13 fat- and water-soluble vitamins in green leafy vegetables during storage, *Journal of Chromatography*
14 *A*, 1261 (2012) 179-188.

15 [22] M. Gratacós-Cubarsí, C. Sárraga, M. Clariana, J.G. Regueiro, M. Castellari, Analysis of vitamin B1
16 in dry-cured sausages by hydrophilic interaction liquid chromatography (HILIC) and diode array
17 detection, *Meat science*, 87 (2011) 234-238.

18 [23] A.E. Karatapanis, Y.C. Fiamegos, C.D. Stalikas, HILIC separation and quantitation of water-soluble
19 vitamins using diol column, *Journal of separation science*, 32 (2009) 909-917.

20 [24] B. Hucker, L. Wakeling, F. Vriesekoop, The quantitative analysis of thiamin and riboflavin and
21 their respective vitamers in fermented alcoholic beverages, *Journal of agricultural and food*
22 *chemistry*, 59 (2011) 12278-12285.

23 [25] C.A. Mondragón-Portocarrero, L. Vázquez-Odériz, M. Romero-Rodriguez, Development and
24 validation of an HPLC method for the determination of thiamine and riboflavin in green leafy
25 vegetables using clara-diestase, *Journal of food science.*, 76 (2011) C639-642.

26 [26] M.L. Marszał, A. Lebiezińska, W. Czarnowski, P. Szefer, High-performance liquid
27 chromatography method for the simultaneous determination of thiamine hydrochloride, pyridoxine
28 hydrochloride and cyanocobalamin in pharmaceutical formulations using coulometric
29 electrochemical and ultraviolet detection, *Journal of Chromatography A*, 1094 (2005) 91-98.

30 [27] K.E. Vigneau-Callahan, A.I. Shestopalov, P.E. Milbury, W.R. Matson, B.S. Kristal, Characterization
31 of diet-dependent metabolic serotypes: analytical and biological variability issues in rats, *The Journal*
32 *of nutrition*, 131 (2001) 924S-932S.

33 [28] Z. Gazdik, O. Zitka, J. Petrlova, V. Adam, J. Zehnalek, A. Horna, V. Reznicek, M. Beklova, R. Kizek,
34 Determination of vitamin C (ascorbic acid) using high performance liquid chromatography coupled
35 with electrochemical detection, *Sensors*, 8 (2008) 7097-7112.

36 [29] N.A. Cellar, S.C. McClure, L.M. Salvati, T.M. Reddy, A new sample preparation and separation
37 combination for precise, accurate, rapid, and simultaneous determination of vitamins B1, B2, B3, B5,
38 B6, B7, and B9 in infant formula and related nutritional by LC-MS/MS, *Analytica Chimica Acta*, 934
39 (2016) 180-185.

40 [30] R.J. Goldschmidt, W.R. Wolf, Simultaneous determination of water-soluble vitamins in SRM
41 1849 Infant/Adult Nutritional Formula powder by liquid chromatography–isotope dilution mass
42 spectrometry, *Analytical and bioanalytical chemistry*, 397 (2010) 471-481.

43 [31] A. Kakitani, T. Inoue, K. Matsumoto, J. Watanabe, Y. Nagatomi, N. Mochizuki, Simultaneous
44 determination of water-soluble vitamins in beverages and dietary supplements by LC-MS/MS, *Food*
45 *Additives & Contaminants: Part A*, 31 (2014) 1939-1948.

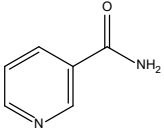
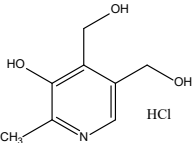
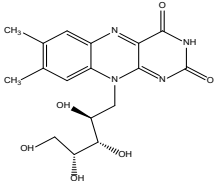
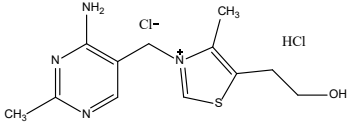
46 [32] K. Hälvin, T. Paalme, I. Nisamedtinov, Comparison of different extraction methods for
47 simultaneous determination of B complex vitamins in nutritional yeast using LC/MS-TOF and stable
48 isotope dilution assay, *Analytical and Bioanalytical Chemistry*, 405 (2013) 1213-1222.

49 [33] A. Sentkowska, K. Pyrzyńska, Zwitterionic hydrophilic interaction liquid chromatography coupled
50 to mass spectrometry for analysis of beetroot juice and antioxidant interactions between its
51 bioactive compounds, *LWT*, 93 (2018) 641-648.

- 1 [34] H.G. Gika, G.A. Theodoridis, U. Vrhovsek, F. Mattivi, Quantitative profiling of polar primary
2 metabolites using hydrophilic interaction ultrahigh performance liquid chromatography-tandem
3 mass spectrometry, *J Chromatogr A*, 1259 (2012) 121-127.
- 4 [35] P. Chen, W.R. Wolf, LC/UV/MS-MRM for the simultaneous determination of water-soluble
5 vitamins in multi-vitamin dietary supplements, *Anal Bioanal Chem*, 387 (2007) 2441-2448.
- 6 [36] Z. Fatima, X. Jin, Y. Zou, H.Y. Kaw, M. Quinto, D. Li, Recent trends in analytical methods for
7 water-soluble vitamins, *Journal of Chromatography A*, 1606 (2019) 360245.
- 8 [37] N.S. Chatterjee, K.A. Kumar, K. Ajeeshkumar, K. Kumari, K. Vishnu, R. Anandan, S. Mathew, C.
9 Ravishankar, Screening Natural Content of Water-Soluble B Vitamins in Fish: Enzymatic Extraction,
10 HILIC Separation, and Tandem Mass Spectrometric Determination, *Journal of AOAC International*,
11 100 (2017) 579-585.
- 12 [38] I.H.T. Guideline, Validation of analytical procedures: text and methodology Q2 (R1), In
13 International conference on harmonization, Geneva, Switzerland DOI (2005) 1-17.
- 14 [39] J. Bernal, A.M. Ares, J. Pól, S.K. Wiedmer, Hydrophilic interaction liquid chromatography in food
15 analysis, *Journal of Chromatography A*, 1218 (2011) 7438-7452.
- 16 [40] A.J. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids
17 and other polar compounds, *Journal of Chromatography A*, 499 (1990) 177-196.
- 18 [41] F. Raposo, Evaluation of analytical calibration based on least-squares linear regression for
19 instrumental techniques: A tutorial review, *TrAC Trends in Analytical Chemistry*, 77 (2016) 167-185.
- 20 [42] N. Zand, B.Z. Chowdhry, F.S. Pullen, M.J. Snowden, J. Tetteh, Simultaneous determination of
21 riboflavin and pyridoxine by UHPLC/LC-MS in UK commercial infant meal food products, *Food
22 chemistry*, 135 (2012) 2743-2749.
- 23 [43] Z. Chen, B. Chen, S. Yao, High-performance liquid chromatography/electrospray ionization-mass
24 spectrometry for simultaneous determination of taurine and 10 water-soluble vitamins in
25 multivitamin tablets, *Analytica Chimica Acta*, 569 (2006) 169-175.
- 26 [44] S. Ndaw, M. Bergaentzlé, D. Aoudé-Werner, C. Hasselmann, Extraction procedures for the liquid
27 chromatographic determination of thiamin, riboflavin and vitamin B6 in foodstuffs, *Food Chemistry*,
28 71 (2000) 129-138.
- 29 [45] M.J. Esteve, R. Farré, A. Frígola, J.M. García-Cantabella, Determination of vitamin B6
30 (pyridoxamine, pyridoxal and pyridoxine) in pork meat and pork meat products by liquid
31 chromatography, *Journal of Chromatography A*, 795 (1998) 383-387.
- 32 [46] I. Odriozola-Serrano, T. Hernández-Jover, O. Martín-Belloso, Comparative evaluation of UV-
33 HPLC methods and reducing agents to determine vitamin C in fruits, *Food Chemistry*, 105 (2007)
34 1151-1158.
- 35 [47] W. Zhou, S. Yang, P.G. Wang, Matrix effects and application of matrix effect factor, *Future
36 Science*, 2017.
- 37 [48] B. Matuszewski, M. Constanzer, C. Chavez-Eng, Strategies for the assessment of matrix effect in
38 quantitative bioanalytical methods based on HPLC- MS/MS, *Analytical chemistry*, 75 (2003) 3019-
39 3030.
- 40 [49] J. Çatak, M. Yaman, Research Article Determination of Nicotinic Acid and Nicotinamide Forms of
41 Vitamin B3 (Niacin) in Fruits and Vegetables by HPLC Using Postcolumn Derivatization System, DOI
42 (2019).
- 43 [50] K.J. Scott, P.M. Finglas, NIACIN | Properties and Determination, in: B. Caballero (Ed.)
44 *Encyclopedia of Food Sciences and Nutrition (Second Edition)*, Academic Press, Oxford, 2003, pp.
45 4116-4119.
- 46 [51] Z. Zhao, X. Hu, C.W. Ross, Comparison of tissue preparation methods for assay of nicotinamide
47 coenzymes, *Plant physiology*, 84 (1987) 987-988.
- 48 [52] R.A. McCance, McCance and Widdowson's the composition of foods, 6th summary ed /
49 compiled by Food Standards Agency and Institute of Food Research.. ed., Cambridge : Royal Society
50 of Chemistry, Cambridge, 2002.

- 1 [53] D. Hampel, D.K. Dror, L.H. Allen, Micronutrients in Human Milk: Analytical Methods, *Adv Nutr*, 9
- 2 (2018) 313S-331S.
- 3

Table 1: Signal parameters set on the LC-MS for targeted water-soluble vitamins. Fragmentation (10), Dwell (590msec)

Vitamin/s	Vitamin structure*	Retention Window SIM Mode	Time (min) – Positive	Precursor Ion [M+H] ⁺	Fragment ion
Nicotinamide (B₃)		0.00-0.80		122.1	80.1
Pyridoxine Hydrochloride (B₆)		0.80-1.50		170.1	152.1
Riboflavin (B₂)		1.50-3.00		377.1	122.0
Thiamine Hydrochloride (B₁)		8.70-9.2		265.1	243

*Vitamin structures based on standards purchased from Sigma-Aldrich (Gillingham, Dorset).

Table 2: Standard curve characteristics of selected water-soluble vitamins

Vitamin/s	RT (min)	±SD	Slope	Slope CV (%)	Intercept	r^2	Linear range* (ng/mL)	Retention factor (k')	Selectivity factor (α)	LOD (ng/mL)	LOQ (ng/mL)
Nicotinamide	0.651	0.003	120.858	20.8	2824	0.985	10 - 400	1.01	2.22	9.0	30.1
Pyridoxine	1.050	0.014	1494.665	4.1	34165	0.983	5 - 400	2.25	2.65	5.4	18
Riboflavin	2.253	0.044	443.186	19.4	-2383	0.994	5 - 400	5.97	4.52	2.4	8
Thiamine	9.042	0.047	876.331	11.1	5672	0.994	5 - 400	26.98	4.52	6.5	21.7

MS operated in positive single ion monitoring (SIM) mode, consisting of an average of 6-7 concentrations (6 concentrations of nicotinamide and 7 concentrations of pyridoxine, riboflavin, thiamine) injected in quadruplicate, or duplicate across multiple days.

*Linearity was based on investigated range to determine vitamins naturally occurring in the food matrix investigated in this study.

Table 3: Standard injection precision data

Vitamin/s	Multi-vitamin standard quantity (ng/mL)	Average quantity based off calibration curve (ng/mL)*	CV (%)
Nicotinamide	50	47	0.9
Pyridoxine	50	47	3.4
Riboflavin	50	48	1.3
Thiamine	50	47	3.3

*Average based off multiple injections of multi-vitamin standard in a run (n= 4 pyridoxine, riboflavin, thiamine and n= 3 nicotinamide)

Table 4: Extraction precision data

Vitamin/s	CV (%) (intra-day)	CV (%) (inter-day)
Nicotinamide	6.56	8.96
Pyridoxine	4.46	8.07
Riboflavin	1.56	9.19
Thiamine	5.89	10.97

For intra-day precision the average was taken by quintuplicate analysis of one extracted soup sample. Inter-day precision was determined by the average of extracting and injecting 5 soup samples.

Table 5: Recovery from matrix, including comparison between lyophilised and non-lyophilised soup samples

Vitamin/s	Recovery from vegetable soup lyophilised sample			Recovery from vegetable soup Non-lyophilised sample		
	Spiked with*	Mean % Recovery	CV (%)	Spiked with**	Mean % Recovery	CV (%)
Nicotinamide	2.5 µg/mL	30	9.8	20 µg/mL	76	3.3
Pyridoxine	2.5 µg/mL	118	1.35	10 µg/mL	94	7.1
Riboflavin	2.5 µg/mL	96	4.55	10 µg/mL	104	6.9
Thiamine***	2.5 µg/mL	50	3.11	10 µg/mL	-	-

*5 replicates spiked with 0.5 mL of a multi vitamin standard at 2.5 µg/mL, therefore 1.25 µg was added to a 5 g soup sample.

**3 replicates spiked with 0.5 mL of a multi vitamin standard.

***Mean % recovery of thiamine in the non-lyophilised sample was unable to be calculated, as a result of thiamine failing below the detection limit in the non-spiked sample. Therefore, the native quantity in the soup could not be determined and accounted for without the lyophilisation step.

Figures with captions

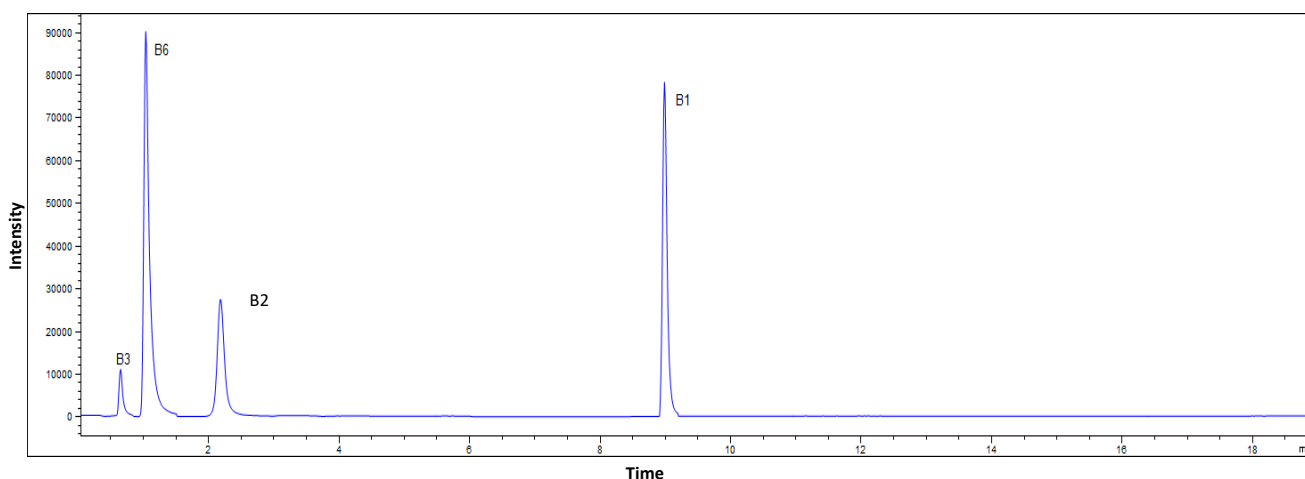


Figure 1: Representative chromatogram of a multi-vitamin mix (standard) at 400 ng/mL, comprising of nicotinamide (B₃), pyridoxine (B₆), riboflavin (B₂) and thiamine (B₁). MS operated in positive single ion monitoring mode (SIM) mode.

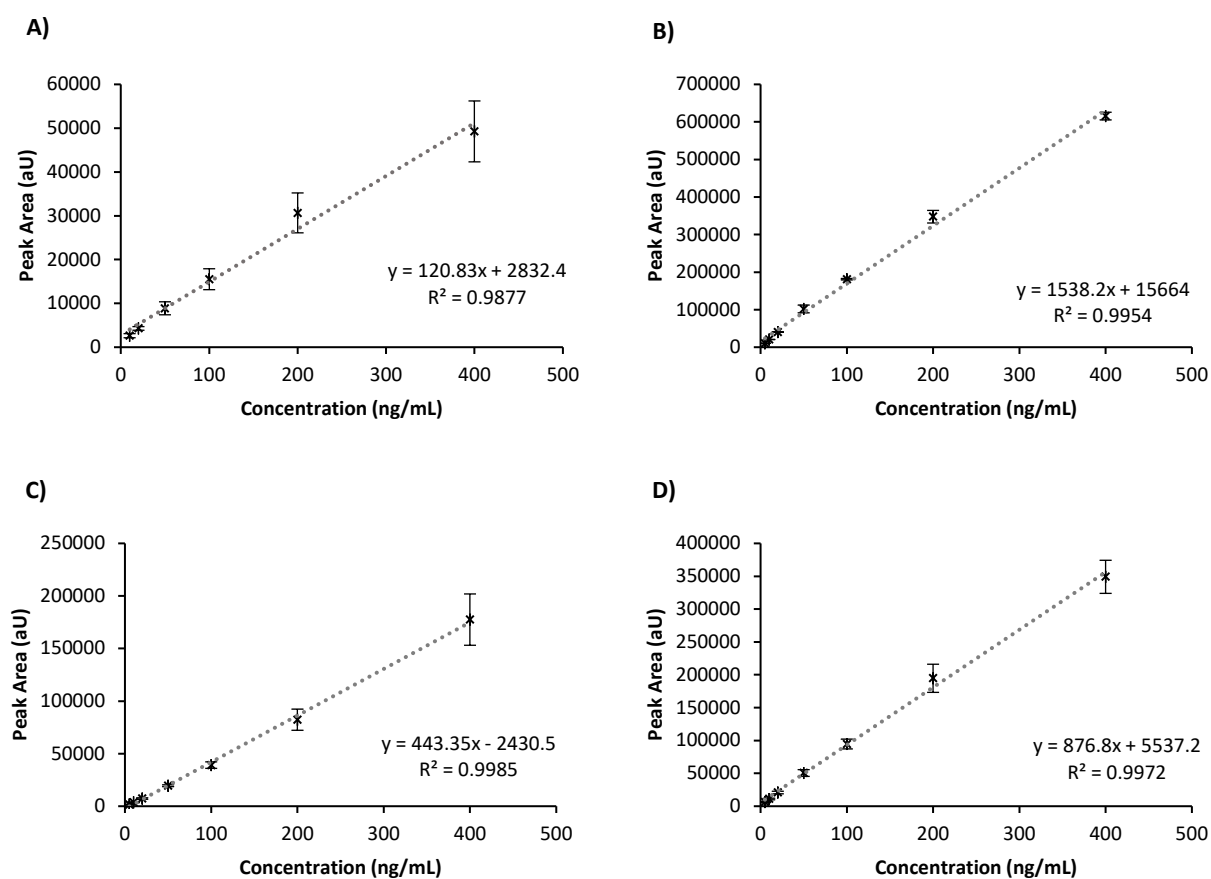


Figure 2: Calibration curves of selected water-soluble vitamins; A) nicotinamide, B) pyridoxine, C) riboflavin and D) thiamine. The calibration curves display the mean slopes and intercepts of multiple injections including the associated error for each data point expressed by a 95% confidence interval.

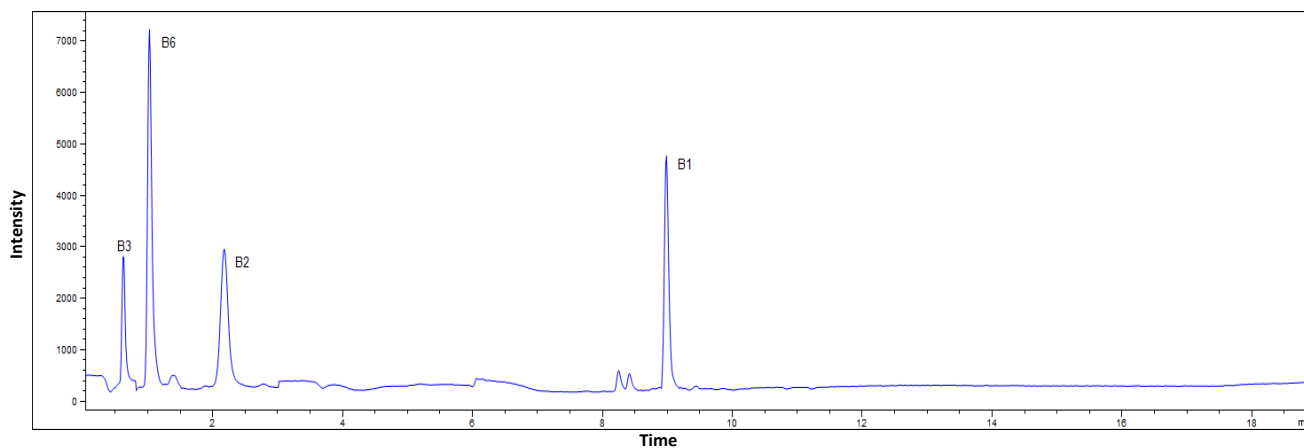


Figure 3: Representative chromatogram of targeted vitamins; nicotinamide (B₃), pyridoxine (B₆), riboflavin (B₂) and thiamine (B₁) in a commercial vegetable soup sample. MS operated in positive single ion monitoring mode (SIM) mode.

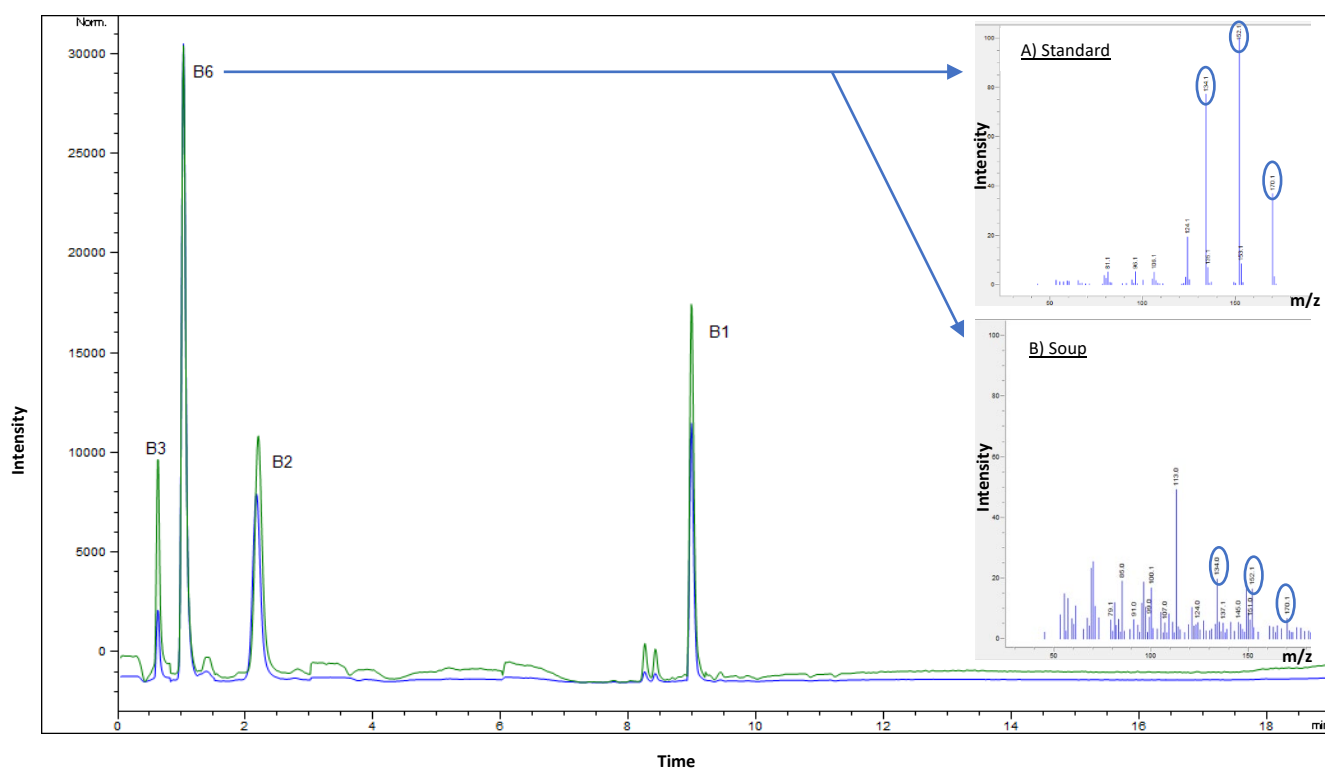


Figure 3: Representative chromatogram of targeted vitamins; nicotinamide (B₃), pyridoxine (B₆), riboflavin (B₂) and thiamine (B₁) in commercial vegetable soup (blue), overlaid with a spiked soup sample (green). MS operated in positive single ion monitoring mode (SIM) mode. The fragmentation pattern of a B₆ standard (A) against B₆ fragmentation pattern in soup (B) is shown as an example. Circled are the fragments of interest with MS operated in positive scan mode, and fragmentation energy set to 150.

Colour required for figure 4