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

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Abstract

Water-soluble vitamins are essential dietary components with a multitude of important functions that require quantification from food sources to characterise the nutritional status of food. In this study, we have developed a hydrophilic interaction chromatography (HILIC) based method coupled to single-quadrupole mass spectrometry (MS) for the analysis of selected water-soluble vitamins. Due to their involvement in energy release from macronutrients, the quantification of thiamine (B_1), riboflavin (B_2), nicotinamide (B_3) and pyridoxine (B_6) offers significant value in food analysis.

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

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

Key Words

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

Abbreviations

HILIC, hydrophilic interaction chromatography; MS, mass spectrometry; MRM, Multiple Reaction Monitoring; RP, reversed-phase; LOD, limit of detection; LOQ, limit of quantification; DTT, DL-dithiothreitol; SIM, single ion monitoring; ES-API, Electrospray – Atmospheric pressure ionization; CV, coefficient of variation; SD, standard deviation; B_1, thiamine; B_2, riboflavin; B_3, nicotinamide; B_6, pyridoxine.

1. Introduction

Water-soluble vitamins and their derivatives are important dietary components essential for health due to their wide range of functions within central metabolism. The B group vitamins, for example, function to release energy from macronutrients, play crucial roles in redox reactions and act as enzyme co-factors [1]. The accurate quantification of water-soluble vitamins in foodstuffs is of significant value and importance. However, simultaneous determination and quantification of these vitamins from various food matrices represents a substantial challenge. In part, this is due to the diverse structural forms and different chemical compounds that make up these vitamins, many of which occur side by side in various foods. Naturally occurring vitamins often occur covalently bound to enzymes as cofactors, or as phosphorylated forms bound non-covalently to proteins and carbohydrates [2]. Thiamine (B_1) occurs in plant-derived foods chiefly in its free form but is present in animal foods as mono-, di-, or triphosphates and their esters, protein complexes, and as thiamine disulfides and their pyrophosphoric acid esters [1, 3]. This renders precise quantification of vitamin levels in food a difficult process, as conventional methods require the parent compound in its free form to compare against pure standards. Another limitation is related to the extraction process employed to release bound vitamins, as B group vitamins can be labile and degrade in the presence of light, heat and pH conditions [2, 4-6]. Due to the reasons presented above highlights the difficultly to develop one method to analyse all vitamins in a given sample and the reason several methodologies exist for each vitamin [7-10].

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

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

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

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

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.

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

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

2.4 Calibration, Precision and Quality Control Measures

Calibration curves for targeted vitamins were made for multi-vitamin standards by plotting the multi-vitamin concentration against peak area. The calibration curve for vitamins B_1, B_2 and B_6 consisted of 7 points ranging from 5-400 ng/mL, whereas B_3 calibration curve comprised of 6 points from 10 ng/mL to 400 ng/mL. Calibration curves were made fresh each day of analysis and an average taken from multiple injections on separate days.

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

2.5 LOD and LOQ

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

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

Where: $\sigma =$ the standard deviation of the y-intercept

$S =$ Calibration curve slope

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

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

2.6 Food Sample Extraction

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

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

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

2.7 Recovery and Precision

Sample recovery was performed on the lyophilised sample by spiking 5 g soup sample in quintuplicate with either 0.5 mL mobile phase B (n=5), or 0.5 mL of a multi-vitamin standard at a concentration of 2.5 µg/mL (n=5) prior to placement in the lyophiliser (VirTis SP Scientific, Sentry 2.0), followed by the extraction procedure. Alternatively, recovery was investigated in the non-lyophilised, spiking 5 g with a multi-vitamin standard (0.5 ml) comprising of B_1, B_2, B_3 and B_6 at a concentration of 10 µg/mL, with the exception of B_3 at a concentration of 20 µg/ml. The multi-vitamin standard concentrations (2.5-20 µg/mL) used for sample recovery were diluted throughout both extraction protocols (lyophilised and non-lyophilised), as outlined in above section (2.6) with meta-phosphoric acid/DTT solution and acetonitrile. This ensured the multi-vitamin standard spiking concentrations fell inside the investigated linear range, by the final stage of extraction. Percentage recovery was calculated based on the quantity of each analyte recovered according to the calibration curve, as a percentage of the known spiked quantity the soup contained. However, when calculating percentage recovery in the non-lyophilised sample, average percentage moisture of the soup (92%) was taken into consideration.

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

2.8 Data Analysis

All statistical analysis completed to determine precision and reproducibility of method was calculated based on mean and standard deviation of replicates. The standard deviation was further divided by the mean of the replicates, to give the percentage coefficient of variation (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. B_6), 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 vitamers.

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

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

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

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

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

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

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

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

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

3.2.2 Recovery Studies

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

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

3.2.3 Quantification of Vitamins in a Soup-Based Food Matrix

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

4. Conclusions

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

for quantification of targeted vitamins (B_1, B_2, B_3 and B_6) that were isolated from a commercially available soup utilising an acid extraction. The simple extraction method resulted in comparable performance to that of other more complex methods which require further processing steps and techniques. In terms of quantification, employing a single-quad LC-MS system provided good sensitivity, with low detection limits varying from 2.4-9.0 ng/mL and quantification limits between 8-30 ng/mL. Linearity ranged from 5-400 ng/mL and was successfully used to determine vitamin content in this work. Four targeted B vitamins were reliably quantified within a total run time of 19 mins, with a method applicable for a single-quad MS. Overall, the method developed has shown to be applicable to procedures that require high throughput and good sensitivity, with the opportunity to monitor nutritional content in foods.

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Declaration of interest

No competing interests.

References

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

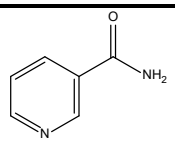
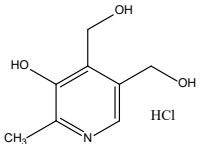
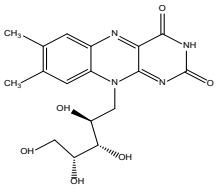
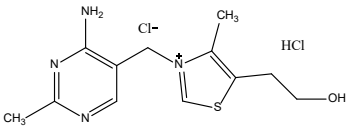
[17] J.-H. Suh, D.-H. Yang, B.-K. Lee, H.-Y. Eom, U.-Y. Kim, J.-H. Kim, H.-Y. Lee, S.-B. Han, Simultaneous determination of B group vitamins in supplemented food products by high performance liquid

chromatography-diode array detection, Bulletin of the Korean Chemical Society, 32 (2011) 2648-2656.
[18] A. Zafra-Gómez, A. Garballo, J.C. Morales, L.E. García-Ayuso, Simultaneous determination of eight water-soluble vitamins in supplemented foods by liquid chromatography, Journal of agricultural and food chemistry, 54 (2006) 4531-4536.
[19] S. Vidović, B. Stojanović, J. Veljković, L. Pražić-Arsić, G. Roglić, D. Manojlović, Simultaneous determination of some water-soluble vitamins and preservatives in multivitamin syrup by validated stability-indicating high-performance liquid chromatography method, Journal of chromatography A, 1202 (2008) 155-162.
[20] M. Ciulu, S. Solinas, I. Floris, A. Panzanelli, M.I. Pilo, P.C. Piu, N. Spano, G. Sanna, RP-HPLC determination of water-soluble vitamins in honey, Talanta, 83 (2011) 924-929.
[21] J. Santos, J.A. Mendiola, M.B.P.P. Oliveira, E. Ibáñez, M. Herrero, Sequential determination of fat- and water-soluble vitamins in green leafy vegetables during storage, Journal of Chromatography A, 1261 (2012) 179-188.
[22] M. Gratacós-Cubarsí, C. Sárraga, M. Clariana, J.G. Regueiro, M. Castellari, Analysis of vitamin B1 in dry-cured sausages by hydrophilic interaction liquid chromatography (HILIC) and diode array detection, Meat science, 87 (2011) 234-238.
[23] A.E. Karatapanis, Y.C. Fiamegos, C.D. Stalikas, HILIC separation and quantitation of water-soluble vitamins using diol column, Journal of separation science, 32 (2009) 909-917.
[24] B. Hucker, L. Wakeling, F. Vriesekoop, The quantitative analysis of thiamin and riboflavin and their respective vitamers in fermented alcoholic beverages, Journal of agricultural and food chemistry, 59 (2011) 12278-12285.
[25] C.A. Mondragón-Portocarrero, L. Vázquez-Odériz, M. Romero-Rodriguez, Development and validation of an HPLC method for the determination of thiamine and riboflavin in green leafy vegetables using clara-diastase, Journal of food science., 76 (2011) C639-642.
[26] M.L. Marszałł, A. Lebiedzińska, W. Czarnowski, P. Szefer, High-performance liquid chromatography method for the simultaneous determination of thiamine hydrochloride, pyridoxine hydrochloride and cyanocobalamin in pharmaceutical formulations using coulometric electrochemical and ultraviolet detection, Journal of Chromatography A, 1094 (2005) 91-98.
[27] K.E. Vigneau-Callahan, A.I. Shestopalov, P.E. Milbury, W.R. Matson, B.S. Kristal, Characterization of diet-dependent metabolic serotypes: analytical and biological variability issues in rats, The Journal of nutrition, 131 (2001) 924S-932S.
[28] Z. Gazdik, O. Zitka, J. Petrlova, V. Adam, J. Zehnalek, A. Horna, V. Reznicek, M. Beklova, R. Kizek, Determination of vitamin C (ascorbic acid) using high performance liquid chromatography coupled with electrochemical detection, Sensors, 8 (2008) 7097-7112.
[29] N.A. Cellar, S.C. McClure, L.M. Salvati, T.M. Reddy, A new sample preparation and separation combination for precise, accurate, rapid, and simultaneous determination of vitamins B1, B2, B3, B5, B6, B7, and B9 in infant formula and related nutritionals by LC-MS/MS, Analytica Chimica Acta, 934 (2016) 180-185.
[30] R.J. Goldschmidt, W.R. Wolf, Simultaneous determination of water-soluble vitamins in SRM 1849 Infant/Adult Nutritional Formula powder by liquid chromatography–isotope dilution mass spectrometry, Analytical and bioanalytical chemistry, 397 (2010) 471-481.
[31] A. Kakitani, T. Inoue, K. Matsumoto, J. Watanabe, Y. Nagatomi, N. Mochizuki, Simultaneous determination of water-soluble vitamins in beverages and dietary supplements by LC-MS/MS, Food Additives & Contaminants: Part A, 31 (2014) 1939-1948.
[32] K. Hälvin, T. Paalme, I. Nisamedtinov, Comparison of different extraction methods for simultaneous determination of B complex vitamins in nutritional yeast using LC/MS-TOF and stable isotope dilution assay, Analytical and Bioanalytical Chemistry, 405 (2013) 1213-1222.
[33] A. Sentkowska, K. Pyrzyńska, Zwitterionic hydrophilic interaction liquid chromatography coupled to mass spectrometry for analysis of beetroot juice and antioxidant interactions between its bioactive compounds, LWT, 93 (2018) 641-648.

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

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

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

Vitamin/s	Vitamin structure*	Retention Time Window (min) – SIM Positive Mode	Precursor Ion $[M+H]^+$	Fragment ion
Nicotinamide (B_3)		0.00-0.80	122.1	80.1
Pyridoxine Hydrochloride (B_6)		0.80-1.50	170.1	152.1
Riboflavin (B_2)		1.50-3.00	377.1	122.0
Thiamine Hydrochloride (B_1)		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

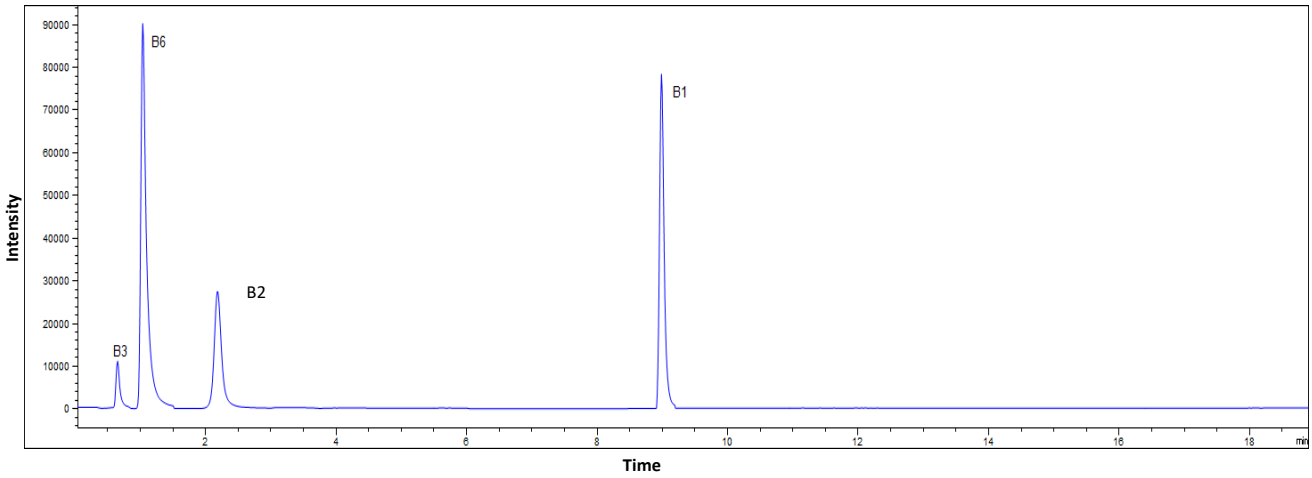


Figure 1: Representative chromatogram of a multi-vitamin mix (standard) at 400 ng/mL, comprising of nicotinamide (B_3), pyridoxine (B_6), riboflavin (B_2) and thiamine (B_1). MS operated in positive single ion monitoring mode (SIM) mode.

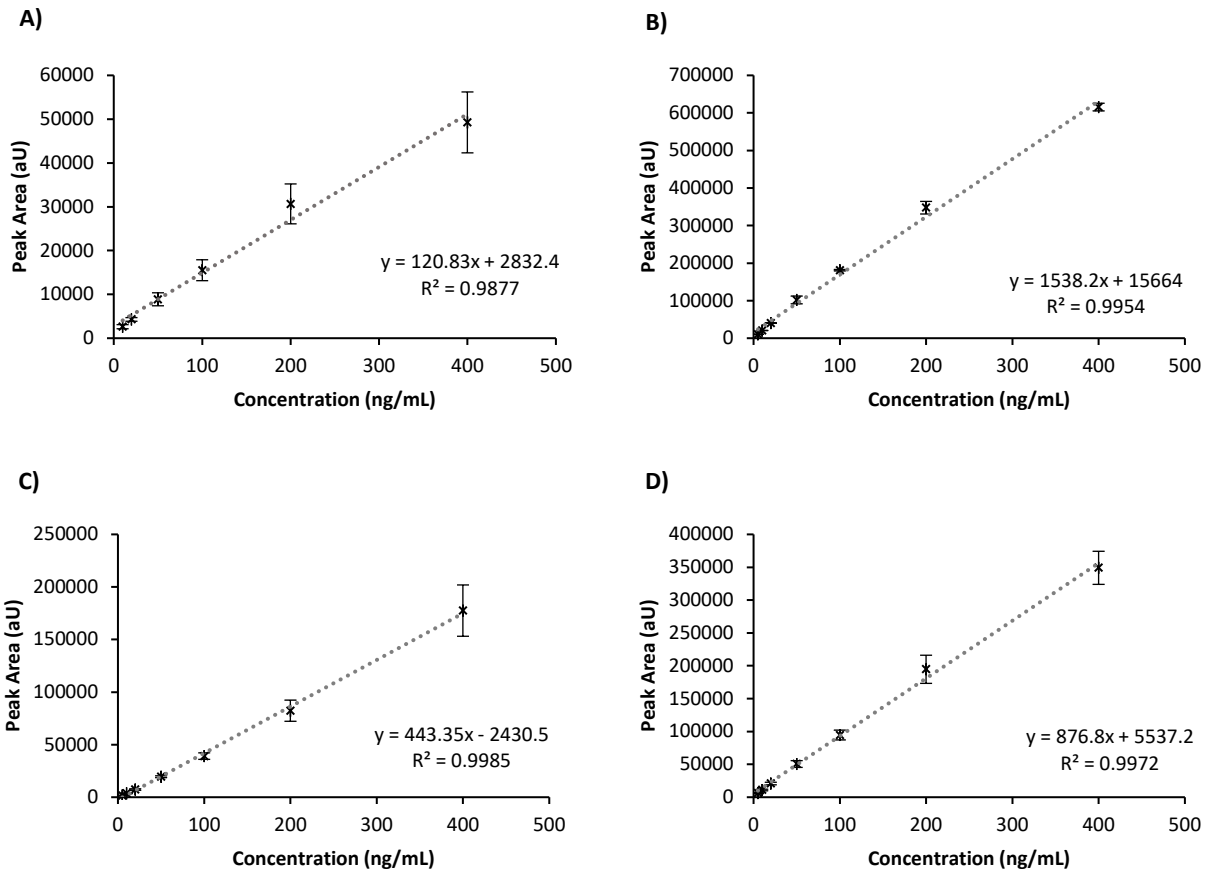


Figure 2: Calibration curves of selected water-soluble vitamins; A) nicotinamide, B) pyridoxine, C) riboflavin and D) thiamine. The calibration curves display the mean slopes and intercepts of multiple injections including the associated error for each data point expressed by a 95% confidence interval.

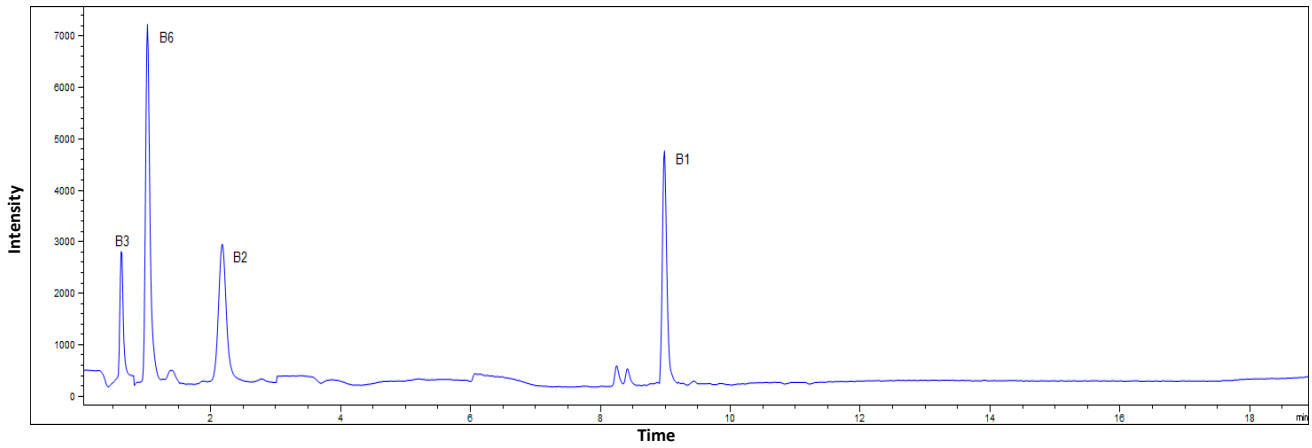


Figure 3: Representative chromatogram of targeted vitamins; nicotinamide (B_3), pyridoxine (B_6), riboflavin (B_2) and thiamine (B_1) in a commercial vegetable soup sample. MS operated in positive single ion monitoring mode (SIM) mode.

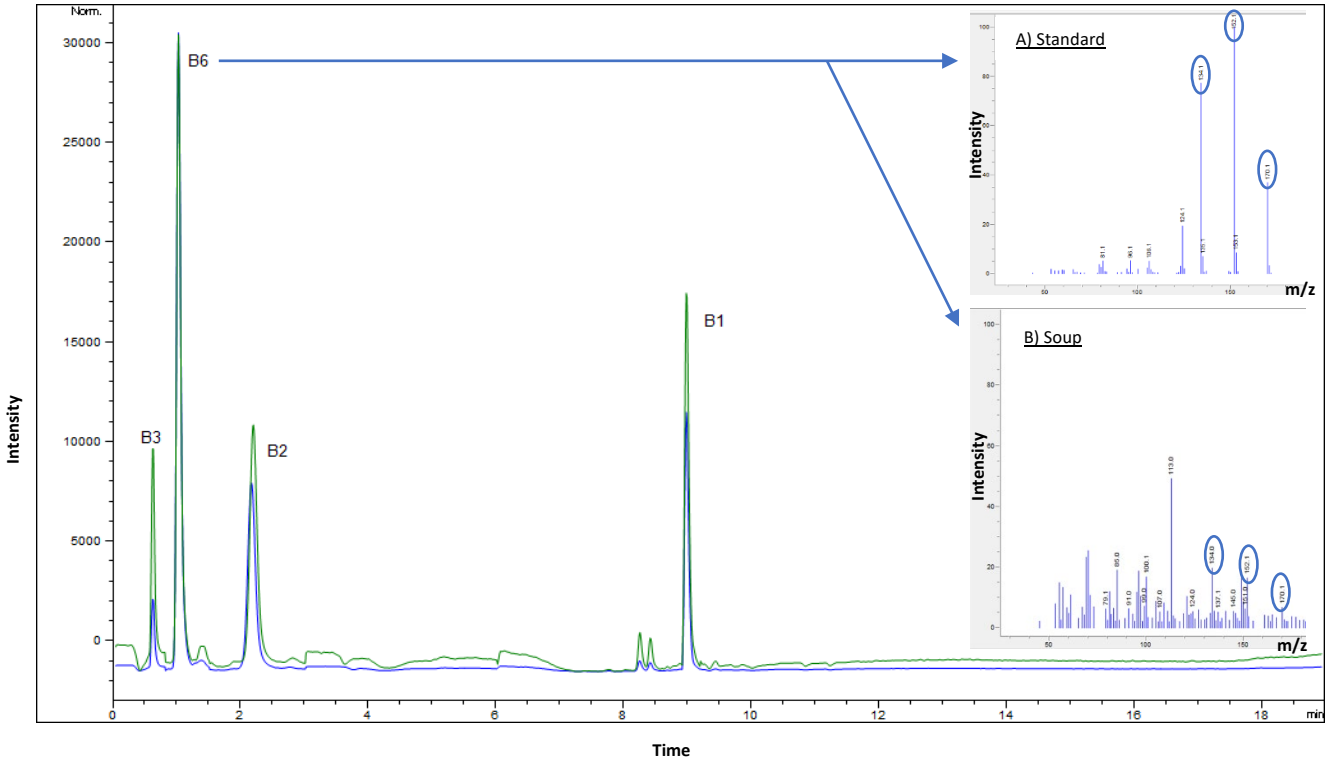


Figure 3: Representative chromatogram of targeted vitamins; nicotinamide (B_3), pyridoxine (B_6), riboflavin (B_2) and thiamine (B_1) in commercial vegetable soup (blue), overlaid with a spiked soup sample (green). MS operated in positive single ion monitoring mode (SIM) mode. The fragmentation pattern of a B_6 standard (A) against B_6 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