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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

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ABSTRACT

Water-soluble vitamins are an important class of compounds that require quantification from food sources to monitor nutritional value. In this study we have analysed six water-soluble B vitamins ([thiamine (B_1), riboflavin (B_2), nicotinic acid (B_3, NAc), nicotinamide (B_3, NAm), pyridoxal (B_6), folic acid (B_9)], and ascorbic acid (vit C) with hydrophilic interaction liquid chromatography (HILIC), and compared UV, fluorescent (FLD) and coulometric detection to optimise a method to quantitate the vitamins from food sources.

Employing UV/diode array (DAD) and fluorimetric detection, six B vitamins were detected in a single run using gradient elution from 100% to 60% solvent B [10 mM ammonium acetate, pH 5.0, in acetonitrile and water 95:5 (v:v)] over 18 min. UV detection was performed at 268 nm for B_1, 260 nm for both B_3 species and 284 nm for B_9. FLD was employed for B_2 at excitation wavelength of 268 nm, emission of 513 nm, and 284 nm/317 nm for B_6. Coulometric detection can be used to detect B_6 and B_9, and vit C, and was performed isocratically at 75% and 85% of solvent B, respectively. B_6 was analysed at a potential of 720 mV, while B_9 was analysed at 600 mV, and vit C at 30 mV. Retention times (0.96 to 11.81 min), intra-day repeatability (CV 1.6 to 3.6), inter-day variability (CV 1.8 to 11.1), and linearity (R 0.9877 to 0.9995) remained good under these conditions with limits of detection varying from 6.6 to 164.6 ng mL^{-1}, limits of quantification between 16.8 and 548.7 ng mL^{-1}. The method was successfully applied for quantification of six B vitamins from a fortified food product and is, to our knowledge, the first to simultaneously determine multiple water-soluble vitamins extracted from a food matrix using HILIC.

1. Introduction

It has long been recognised that water-soluble vitamins and their biological derivatives are crucial co-factors of many enzymes involved in vital metabolic processes [1] and represent important dietary components essential for health. They therefore represent an important class of molecules that require quantification from food matrices. However, the simultaneous determination and accurate quantification of water-soluble vitamins from food, represents a substantial challenge. Vitamins that are added to fortified food occur in their free forms, while endogenous vitamins from natural sources are present largely in numerous covalently bound forms e.g. phosphorylated [2], that renders precise quantification of vitamin levels in food a difficult process. For example, thiamine (vitamin B_1) occurs in plant-derived foods chiefly in its free form but is present in animal foods as mono-, di-, or triphophates and their esters, protein complexes, and as thiamine disulfides and their pyrophosphoric acid esters [1].

Traditional methods of vitamin analysis were typically conceived for the analysis of individual vitamins only, and are not deemed appropriate any longer as they are laborious and often not selective or reproducible [3–8]. For vitamins B_1, B_2 and B_3, the AOAC recommends fluorescence methods [9–11], while for vitamins B_5, B_6, B_9 and B_{12} microbiological assays are suggested [12,13]. Recent

Abbreviations: B_1, thiamin; B_2, riboflavin; B_6, pyridoxal; B_9, folic acid; CV, coefficient of variation; DAD, diode array detector; DTT, DL-dithiothreitol; FLD, fluorescence detector; HILIC, hydrophilic interaction liquid chromatography; LU, luminescence units (arbitrary units); mAU, milli absorbance unit; NAc, nicotinic acid; NAm, nicotinamide; RPLC, reversed-phase liquid chromatography; RT, room temperature; SD, standard deviation; WSV, water-soluble vitamins; vit C, ascorbic acid.

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technological developments prompted a move towards more efficient and accurate chromatographic methods such as high-performance liquid chromatography (HPLC), ultrahigh-pressure liquid chromatography (UHPLC), and liquid chromatography/mass spectrometry (LC/MS). HPLC in the reversed-phase mode (RPLC) has become the method of choice for many researchers, and in the last ca. 10 years a number of HPLC methods for the simultaneous analysis of water-soluble vitamins have been described. Modern HPLC systems most commonly utilise UV/vis detection [2–7,14–33], but also fluorescence [5,8,34], electrochemical/coulometric [21,23,35,36], and mass spectrometry (MS) [22,33,37–39] techniques were applied in the simultaneous analysis of water-soluble vitamins.

In the present report, chromatographic separation of some of the main food-based water-soluble vitamins was achieved by use of hydrophilic-interaction liquid chromatography (HILIC). Previous application of HILIC stationary phases has been restricted to analysis of pure standards of B vitamins [24,25,29–31,33,39] or to determination of merely single vitamins (ascorbic acid and cognates [28,32,40]; thiamine [41]) in food extracts, but to our knowledge no reports are present that describe the simultaneous analysis of more than two water-soluble vitamins on a HILIC stationary phase that were extracted from a food matrix. Furthermore, the present report aims to compare different detection methods (UV, fluorescence, coulometric) for the simultaneous determination of thiamine (B_1), riboflavin (B_2), nicotinic acid (B_3, NAc), nicotinamide (B_3, NAm), pyridoxal (B_6), folic acid (B_9), and ascorbic acid (C), and to apply the method in an example of a fortified food product.

2. Materials and methods

2.1. Chemicals and consumables

Ammonium acetate and ammonium formate were of HPLC-grade and acquired from Fluka (Sigma Aldrich, Gillingham, UK). HPLC-grade acetonitrile was from Fisher Scientific (Loughborough, UK). Nicotinic acid, nicotinamide, (−)-riboflavin, pyridoxal hydrochloride, thiamine hydrochloride, folic acid, L-ascorbic acid, DL-dithiothreitol (DTT) and papain were supplied by Sigma (Sigma-Aldrich, Gillingham, UK). Acetic acid, formic acid, hydrochloric acid and clara-diastase were obtained from Fluka. 35% ammonia solution was from Fisher Scientific. Water was purified to 18.2 MΩ using a Milli-Q Integral three water purification system (Merck-Millipore, Watford, UK). All chemicals were of analytical grade. 0.2 μm Nylon filter membranes were from Chromacol (Fisher Science).

2.2. Standard preparation

For individual vitamin stock solutions, nicotinic acid, nicotinamide, pridoxal hydrochloride and thiamine were prepared at a concentration of 2 mg mL^{-1} in deionised water including 0.12% acetic acid; riboflavin and folic acid were dissolved at a concentration of 0.5 mg mL^{-1} in a 0.28 M ammonia solution and the pH was subsequently adjusted to 7.0 with acetic acid. Ascorbic acid was prepared in water with 3% meta-phosphoric acid and 200 mg L^{-1} DTT. Stocks were prepared in subdued light and stored for 3 days in the dark at 4 °C; working standards and multi-component standards were prepared daily by diluting stocks in 10 mM ammonium acetate pH 5.0 in 95:5 (v:v) acetonitrile:water (solvent B).

2.3. Chromatographic conditions

Chromatographic separation was carried out in a Dionex UltiMate 3000 RS liquid chromatography system (Thermo Fisher, Hemel Hempstead, UK), equipped with a 4-channel pump, autosampler with a Rheodyne 20 μL loop, column oven, diode array detector (DAD), fluorescence detector (FLD), maintained at 35 °C, and an electrochemical coulometric CoulArray model 5600A detector (ESA, Chelmsford, MA). The CoulArray detector was equipped with 16 channels, set at even increments of 30 or 60 mV from 0 to 450 or 900 mV, and was maintained at 35 °C. Software packages Chromeleon v.7.1.1 and CoulArray DataStation 3.10 were used for data analysis.

Depending on the method of analysis, three different chromatographic methods were employed. The same column and mobile phases (albeit under varying gradient conditions) were used throughout. The column was a ZORBAX HILIC Plus silica column (Agilent, Wokingham, UK), 100 × 4.6 mm, 3.5 μm. Mobile phase A was 10 mM ammonium acetate (pH 5.0) in water:acetonitrile (95:5, v:v), mobile phase B was 10 mM ammonium acetate (pH 5.0) in acetonitrile:water (95:5, v:v).

Method 1 was used for the analysis of thiamine, riboflavin, nicotinic acid, nicotinamide, folic acid and pyridoxal; the detection was performed with DAD and FLD. The flow rate was 0.8 mL min^{-1}, injection volume 5 μL, column temperature 30 °C, FLD detector temperature 35 °C. The gradient consisted of 100% B for 4.5 min, decreased to 60% B over 6 min and went back up to 100% B over 6.5 min and concluded at 18 min with 100% B. The Diode Array detection wavelengths were set to 260 nm (for nicotinic acid and nicotinamide), 268 nm (thiamine) and 284 nm (folic acid). The FLD was set to wavelengths of excitation of 284 nm/emission 317 nm (sensitivity 7) for pyridoxal and excitation 268 nm/emission 513 nm (sensitivity 4) for riboflavin. Prior to use in Method 1 the column was equilibrated in 60% mobile phase B for 2 h. Method 2 used coulometric detection for the analysis of pyridoxal and folic acid (5 min duration). Chromatography conditions used were 75% solvent B over 5 min, at a flow rate of 0.5 mL min^{-1} and 5 μL injection. The optimal potentials used were 720 mV for pyridoxal and 600 mV for folic acid. Method 3 also used coulometric detection for the analysis of ascorbic acid (5 min duration). Chromatography conditions used were 85% solvent B, over 5 min, at a flow rate of 0.5 mL min^{-1} and an injection volume of 5 μL. Optimal potential was 30 mV. Equilibration time was 3–4 h at starting mobile phase conditions for Methods 2 and 3.

2.4. Calibration

Calibration curves were constructed for multi-component vitamin standard solutions by plotting concentration against peak area. A $(1/x)^n$ weighting was used. The calibration curves values (Table 1) for DAD and FLD were the average of injection of five to six different concentrations in quadruplicates on four separate days, while the CoulArray values represent the average of six different concentrations injected in duplicate on four separate days. A partial validation study was undertaken according to published guidelines [42,43].

2.5. Food sample preparation

As a sample fortified food, cornflakes (Kellogg's, Manchester) were used. Sample treatment for DAD and FLD analysis was adapted from a previously reported method [8]. Samples were extracted at room temperature (RT) or underwent mineral acid hydrolysis followed by enzymatic treatment in order to liberate complexed vitamers. Cornflakes were ground in a mortar. 1.0 g of cornflake meal were added to 4 mL of 0.1 M HCl and vortexed for 1 min. Samples were either spiked with 0.5 mL of a multi-component vitamin solution (that contained 80 μg each of B_1, B_2, NAc, NAm, B_6 and B_9), or water. Samples were either extracted at room temperature (RT), or incubated in a water bath at 100 °C for 30 min. After cooling to room temperature and adjustment to pH 4.5 with 0.15 mL of 2.5 M sodium acetate buffer, either 20 mg (48 U) papain and 12.5 mg