HPLC ANALYSIS OF VITAMIN B₁, B₂, B₃, B₆, B₉, B₁₂ AND VITAMIN C IN VARIOUS FOOD MATRICES

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ABSTRACT

Various methods had been described for the analysis of vitamins in food matrices, with more and more of these including the use of HPLC to measure the levels of these micronutrients in foodstuffs. The renewed interest in rapid and accurate quantification of micronutrients in foodstuffs is due to more stringent requirements by food regulatory agencies around the world. Legislation now demands that the nutrition information displayed on food labels be backed up by reliable results obtained using validated analyses. Three challenges are common in terms of quantifying vitamins in food matrices: 1) extraction techniques that are sufficiently effective to liberate the various forms of the vitamin from each unique matrix, 2) ensuring that labile forms of the vitamin are protected against degeneration by light and/or air (oxygen) for a sufficiently long period to afford accurate quantification and 3) obtaining an analytical method with sufficient sensitivity, selectivity, accuracy and precision, with cost and time also being considerations. The chapter dealt with these aspects concerning vitamin B₁, B₂, B₃, B₆, B₉, B₁₂ and vitamin C. Extraction procedures were described, as well as typical HPLC methods and recent improvements in this field.
INTRODUCTION

The family of water-soluble vitamins (WSVs) comprises nine vitamins, namely thiamine (vitamin B₁), riboflavin (vitamin B₂), niacin / nicotinic acid / nicotinamide (vitamin B₃), pantothenic acid (vitamin B₅), pyridoxine / pyridoxal (vitamin B₆), biotin (vitamin B₇ or B₈ (as D-(p)-biotin)), folic acid (vitamin B₉), cyanocobalamin (vitamin B₁₂) and L-ascorbic acid / L-dehydroascorbic acid (vitamin C) (Heudi, 2012; Nohr & Biesalski, 2009).

Like fat-soluble vitamins, WSVs are essential components in the human diet since they play a vital role in optimal health owing to their involvement in fundamental functions of the body, such as growth and metabolism (Groff et al., 1995; Kim, 2011). Their importance in preventing deficiency diseases is well-established, with mounting evidence of the prophylactic role of many of these vitamins protecting against other forms of disease, such as elevated maternal folate intake resulting in reduced incidence of neural tube defects (NTDs) (Berry et al., 2010; Quinlivan & Gregory, 1997) and vitamin B₁₂ and folate supplementation normalizing blood homocysteine concentrations, thus minimizing a risk factor for heart disease (Groff et al., 1995) and other chronic diseases, such as cancer and age-related dementia (Iyer & Tomar, 2009; Kim, 2007; Russell, 2012). As a result of the crucial role that vitamins play in optimum nutrition, disease-prevention and as therapeutic aids, many dietary supplements (also known as nutrition or food supplements) are available (Heudi, 2012), while mandatory fortification of specified foodstuffs are enforced by around 75 governments around the globe (Samaniego-Vaesken et al., 2013). Hence, there is a global thrust to develop accurate and precise analytical techniques for compliance monitoring and also for establishing reliable information for nutrient databases (Blake, 2007) and food labels (Russell, 2012).

The WSVs are a group of biomolecules with considerable variation as concerns their molecular weight, structure, chemical properties and biological activity (Groff et al., 1995;
Russell, 2012). In addition, in biological samples they can occur as a number of closely related compounds, or vitamers (examples are pyridoxine, pyridoxal or pyridoxamin), while these can be present either in free or phosphorylated form (such as riboflavin-5’-adenosyldiphosphate (FAD)) or non-covalently, but strongly, bound to polysaccharides and proteins (Ball, 2006; Heudi, 2012). The diversity in chemical reactivity or sensitivity to degradation, as well as the necessity to release the bound forms of the various WSV species highlight the challenges in terms of quantitative liberation of the analytes via effective sample extraction, particularly for methods aimed at simultaneous quantification of various WSVs in non-fortified food matrices (Ball, 2006; Heudi, 2012). Generally, extraction processes include acid or alkaline hydrolysis of the sample (Ball, 2006), heat denaturation of proteins, often by autoclaving (Van Wyk & Britz, 2010; 2012), deproteinization with trichloroacetic acid or similar agent (Ball, 2006) and digestion with appropriate enzymes (Lim et al., 1998; Pfeiffer et al., 2010).

While the extraction process is of critical importance, it is also vital to apply an analytical method that is accurate, precise, selective and sensitive, as well as rapid. Hence, while the time-consuming microbiological assays were the gold standard for many years (Blake, 2007), they have largely been replaced by other methods that produce results more swiftly (Ball, 2006; Heudi, 2012). Due to the hydrophobic, non-volatile character of the WSVs, they are most suitable for reversed phase high performance liquid chromatography (RP-HPLC) methods (Russell, 2012), which feature prominently among the afore-mentioned rapid methods (Ball, 2006; Heudi, 2012). The first generation of the LC methods used UV detection most frequently, followed by fluorescence and electrochemical detection methods (Russell, 2012). More recent developments are the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Ball, 2006; Heudi, 2012; Chandra-Hioe et al., 2011; Freisleben et al., 2003) and even ultraperformance liquid chromatography-tandem mass
spectrometry (UPLC-MS/MS) coupled with isotope dilution MS. An electron-spray ionisation (ESI) interface facilitates the exploitation of the high selectivity of MS detection with the improved sensitivity when operating in multiple reactions monitoring (MRM) mode.

The application of isotope dilution mass spectrometry (IDMS) facilitates the simultaneous determination of various WSVs (Heudi, 2012), including different vitamers (folates), with enhanced quantitative accuracy (Chandra-Hioe et al., 2011), while UPLC effects very high resolution separations and fast separations (Edelmann et al., 2012; Chandra-Hioe et al., 2011; Heudi, 2012) due to the high pressure (up to 1 000 atm) and small stationary phase particles (<2 μm) (Russell, 2012). However, the cost and complexity of operating the equipment based on MS detection (Iyer & Tomar, 2009; Pfeiffer et al., 2010), with still higher costs applying to stable isotope labelling (SIL) and greater complexity to IDMS (Heudi, 2012), put it beyond the reach of the average routine analytical or research laboratory.

The aim of this chapter is to focus on the extraction of selected water-soluble vitamins in food matrices, followed by quantification using HPLC methods. Since several recent reviews have been published (Heudi, 2012; Kumar et al., 2010; Plonka et al., 2012; Russell, 2012), including an excellent treatise on method validation (Russell, 2012), in most cases the narrative will concentrate on the period after 2010. The exceptions will be folate and vitamin B₁₂ where a more comprehensive description includes the work performed in our own laboratories, these two vitamins being a key element in one of our research focus areas (Van Wyk, 2002; Van Wyk & Britz, 2010; Van Wyk et al., 2011; Van Wyk & Britz, 2012). At the end of the chapter, a summary of a selection of the most recent methods is presented in Table 1, with the information arranged in alphabetical order based on food matrix. Since food analytical methods are often based on methods originally developed for non-food matrices, some of these methods are also included at the end of the table to provide a complete picture in terms of the available methodology.
In addition, a brief generic description, structures, nutritional and physiological importance is also covered for each vitamin. As much as this includes a description of typical deficiency diseases in each case, it is important to note that in most modern societies, single-nutrient deficiencies are rare. Generally, a diet sub-optimal in one vitamin would be sub-optimal in others. In addition, the metabolism of some vitamins are interconnected, such as the interrelationship between vitamins B₉ and B₁₂ in terms of megaloblastic anaemia, while vitamins B₆, B₉ and B₁₂ are involved in homocysteine synthetic and degradative reactions (Rivlin, 2007), preventing hyperhomocysteinemia (Shaik & Gan, 2013). Moreover, vitamin B₆ plays a role in the synthesis of niacin from tryptophan, thus forming the basis of vitamin B₆ deficiency potentially causing secondary niacin deficiency (Rivlin, 2007).

**VITAMIN B₁ (THIAMINE)**

**Generic description and structures – Vitamin B₁**

Vitamin B₁ in the form of its pyrophosphate acts as a coenzyme for reactions involving the transfer of activated aldehyde groups. Other forms of this vitamin are thiamine itself, thiamine monophosphate, thiamine triphosphate (Russell, 2012) and protein-bound thiamine (Bucholz et al., 2012). A deficiency of this vitamin results in the development of beri-beri. The latter deficiency disease is characterized by neurological and cardiac aberrations resulting in amnesia, hence the suggestion that it can be used to treat Alzheimer’s disease. A range of other symptoms include muscle weakness, lack of coordination and, in severe cases, death (Akyilmaz et al., 2006; Harper, 2006).

The structures of various forms of vitamin B₁ are depicted in Figure 1. More thiamine is found in plant products while thiamine pyrophosphate is predominant in muscle foods where pork is the exception (Russell, 2012). Reactions in which thiamine vitamers participate
are best described by the main enzymes which use it as a coenzyme. These are pyruvate dehydrogenase, transketolase and α-ketoglutarate dehydrogenase (Belitz & Grosch, 1999).

Figure 1. Vitamin B₁ structure (thiamine).

Nutritional/Physiological importance and Dietary sources – Vitamin B₁

Thiamine, together with its counterpart riboflavin (see later), are two major water-soluble vitamins used in fortification of food products (Boyaci et al., 2012). As a coenzyme, it plays a role in the mechanism for the decarboxylation of α-keto acids. Through mediation or participation in these reactions, the deficiency symptoms are generated when thiamine is not available. In developed countries there are marginal deficiencies of this vitamin (Nohr & Biesalski, 2010), but severe deficiencies, resulting in brain damage are still common among certain population groups such as children and the aged (Harper, 2006).

The main dietary sources of thiamine are meat (Lombardi-Boccia, 2005), the pericarp and germ of cereals (Belitz & Grosch, 1999), baked products, legumes, nuts yeast and organ-rich meats (Russell, 2012), milk, oats, rye, sunflower, lentils, broccoli and potatoes (Nohr & Biesalski, 2010).
**Assay methodology for Vitamin B₁ (vitamins B₂, B₃ and B₆) – General**

Due to the similarities among the assay techniques, the following discussion grouped vitamins B₁ to B₆ together. Microbiological assays exist for all of these vitamins, but the extraction and assay procedures are tedious and time-consuming. Other methods that are used are: HPLC, capillary electrophoresis (thiamine); HPLC, direct fluorometric, electrophoretic and biosensor-based methods (riboflavin); HPLC and colorimetric methods (niacin); and HPLC (pyridoxine) (Da Silva et al., 2013; Heudi et al., 2005; Gao et al., 2008; Hidiroglou et al., 2008; Sikorska, 2007). A recent paper describes the simultaneous quantification of thiamine, nicotinic acid, niacin, pyridoxine and even vitamin C in a yeast supplement, beer and dietary supplements using capillary electrophoresis with diode array detection (CE-DAD) (Mazina & Gorbatskova, 2010).

There are very few papers between 2010 and now dealing with single-vitamin analytical techniques. The majority of recent analytical work conducted with respect to the B-group vitamins has been related to multi-vitamin analysis. Examples of this is seen in the following articles *viz.* Aslan et al. (2013), De Arruda et al. (2012), Hucker et al. (2012), Jin et al. (2012), León-Ruiz et al. (2013), Rudenko & Kartsova (2010), San José et al. (2012), (Table 1), Hälvin et al. (2013), Zand et al. (2012) amongst others. However, there are established methods and ongoing research into single-vitamin assays (Gratacos-Cubarsi et al. 2011, Yoshida et al. 2012).

There are standard methods for the analysis of the vitamins as a group and for B₁ in particular such as those of the American Association of Cereal Chemists International e.g. thiochrome method, fluorometric method, HPLC and microbiological methods. Biosensor-based methods (Akyilmaz et al., 2006) and a method using adsorptive stripping voltammetry (Tyszczuk-Rotko, 2012) are also available. Microbiological methods are still the standard against which to reference (Chen et al., 2009).
However, most of the methodology being used is based on HPLC modifications of established methods e.g. thiochrome. Table 1 summarizes a number of HPLC-based methods based on the matrix involved, most of which again is based on multi-vitamin analysis with a few exceptions. In terms of the methods summarized for vitamins B₁ to B₆ in the table, the literature (with a few exceptions) was only polled back to 2010. The work by Russell (2012) can be considered a baseline upon which this writing is built. It elegantly captures the state of the analytical field with an observation that the thiochrome-based method is well-matured to the extent very little new comes out in terms of its basic principles and further development. One area where development does take place is in terms of hardware e.g. the move from HPLC to UHPLC with its quicker run times and better resolution (Di Stefano et al. 2012).

In general, based on the observations in Table 1 regarding the B-group vitamins, the following position emerges in terms of analytical regimes.

After reducing the sample (milling, homogenization, cutting, shearing), an acid treatment at elevated temperature (sometimes autoclaving) is usually employed to free the vitamers from the sample (Boyaci et al., 2012; Ersoy & Özeren, 2009). This may include enzyme treatment e.g. taka-diastase (Khair-un-Nisa et al., 2010) or Clara-diastase (Bui & Small, 2009) and other steps such as sonication (Santos et al., 2012) toward releasing vitamers bound to protein. Other enzymes that may be employed include papain and α-amylase (Chandra-Hioe et al., 2011; San José et al., 2012). To assist with this the sample may also be de-proteinized by trichloroacetic acid treatment or by using other precipitating agents (Bucholtz, at al., 2012). In some cases liquid-liquid separation of vitamins may be employed (Giorgi et al., 2012). In most cases buffers are employed as part of the suspending agent. Where dry samples are required after preparation, drying is conducted using a stream of nitrogen (Gratacos-Cubarşi et al., 2011).
In the case of vitamins B₁ and B₂, derivitization to a stable thiochrome is performed using alkaline ferricyanide (Boyaci et al., 2012) or cyanogen bromide (Bucholtz et al., 2012). The latter is not common due to the toxicity of cyanogen bromide.

All other standard HPLC procedures, including micro-filtration, are employed in the general analytical scheme with permutations based on the nature of the required output e.g. different types of micro-filters with different porosities.

General hardware required for the run included branded equipment, the most important of which is the column and column conditions employed. This ranges from the most common i.e. reversed phase C18 HPLC columns at ambient or at 30°C (Bui & Small, 2009; San José et al., 2012) to UHPLC columns with higher resolution and shorter run-times (Mihalevski et al., 2013) to a hydrophilic interaction chromatography (HILIC) column at ambient temperature (Gratacos-Cubarši et al., 2011).

In terms of elution, the generic methods are isocratic and gradient elution including both concentration differences as well as, in one case, flow rate differences.

Detection was mostly UV (single fixed wavelength or DAD at a specific set of wavelengths) or fluorescence at appropriate excitation and emission wavelengths pertinent to each study reported. In some cases, a mass spectrometer was also employed.

For any analytical technique, one of the more important steps that dictate the outcomes and quality of results is sample preparation, in particular extraction of the analytes. In this case the analyte(s) is a vitamin (with possibly more than one vitamer) with its own degree of stability and sensitivity to different reagents. Thiamine itself is sensitive to pH (stable at pH 2.0 to 4.00 and highly unstable at alkaline pH), unstable in solution, easily oxidized and can be degraded by UV light (Russell, 2012; Santos et al., 2012).

A more detailed summary of extractions and analytical work is given in the next section per vitamin and is common to a range of vitamins where multi-vitamin analysis is conducted.
**HPLC assay methodology for Vitamin B₁**

For cereal grains extraction of the vitamin may be conducted by placing a sample in cold 4% trichloroacetic acid to be milled or homogenized before filtering, derivatizing (in this case using cyanogen bromide) after which the pH is adjusted to 10 (the thiochrome formed fluoresces strongly). Further treatment may involve cooling, centrifugation and SPE (Bucholtz et al., 2012).

A more environmentally friendly derivatizing agent is alkaline ferricyanide. In this case the trichloroacetic acid mixture is centrifuged after which the supernatant is treated further (Hucker et al., 2012). At this point the sample can be injected directly onto a column to determine riboflavin while a second supernatant stream is further treated with alkaline potassium ferricyanide, degassed, vortexed and neutralized and then filtered prior to injection onto the column. Fluorescence detection is done at excitation/emission wavelengths of 360/245 nm for thiamine.

For fruit and vegetable matrices the fruit is peeled and extracted in methanol, centrifuged and filtered. The sample can then be clarified at this point followed by SPE using different solvents to extract at least three different vitamin fractions eventually allowing for analysis of ascorbic acid, thiamine, riboflavin, nicotinamide, pantothenic acid, pyridoxine and folic acid using DAD at appropriate wavelengths (Plonka et al., 2012).

For complex cereal foods a sample may be mixed in 0.05M H₂SO₄ with sodium acetate, papain, diastase and amylase and incubated at 37°C to release vitamins from other cereal components. Both thiamine and riboflavin may be determined using UV detection at 268 nm (San José et al., 2012). However, the detection method of choice is fluorescence.
VITAMIN B₂ (RIBOFLAVIN)

Generic description and structures – Vitamin B₂

Various forms of vitamin B₂ occur, namely riboflavin, FMN (flavine mononucleotide), FAD (flavine adenine dinucleotide) and lumiflavin. The structure of riboflavin is depicted in Figure 2. This vitamin is quite stable to heat processing but is extremely sensitive to UV light, thus necessitating protection from light when the existing pool in a matrix is to be kept stable. This is further related to light barriers in packaging e.g. dark bottles, opaque materials and light-tight seals. The effect of light in the 420–560 nm range cleaves ribitol from the parent structure leading to the formation of lumiflavin (Belitz & Grosch, 1999). This is also related to analytical challenges associated with losses during sample preparation (Ciulu et al., 2011).

Figure 2. Vitamin B₂ (riboflavin) structure.

Based on its role in the generic reactions involving decarboxylation, hydroxylation and reduction of oxygen to hydrogen peroxide amongst others, it plays a role in a wide range of
biochemical reactions and pathways. This is done via FAD and FMN. These include the electron transport chain, Krebs cycle, oxidation of amino acids and the replenishment of reduced glutathione which has a protective effect against oxidation in cells (Russell, 2012; Nohr & Biesalski, 2010).

**Nutritional/Physiological importance and Dietary sources – Vitamin B2**

Riboflavin (7,8-dimethyl-10-(1′-D-ribityl)isoalloxazine) is the counterpart to thiamine used in the fortification of food products (Boyaci et al. 2012). It is associated with FMN and FAD. These coenzymes are involved in reactions requiring electron carriers e.g. hydroxylation and decarboxylation, and have a wide variety of roles to play in general metabolism (Nohr & Biesalski, 2010). Vitamin B2 is not associated with any specific deficiency symptom since it is a very stable pool in the body (Belitz & Grosch, 1999; Nohr & Biesalski, 2010). The main dietary source is muscle products as well as liver and other organs, milk and eggs (Russell, 2012), wheat germ, yeast, soya beans and mackerel (Nohr & Biesalski, 2010).

**Assay methodology for Vitamin B2 – General**

Apart from microbiological assay and HPLC methods, analytical techniques for vitamin B2 include fluorescence, chemiluminescence, capillary electrophoresis and spectro-electrochemical and electrochemical methods. Electrochemical methods had been found to show sensitivity and selectivity for the determination of riboflavin in multivitamin preparations and foods like milk, cereal and beer (Safavi et al., 2010).

**HPLC assay methodology for Vitamin B2**

There is a great deal of overlap between the extraction and determination of thiamine and riboflavin as is noted in the previous section on thiamine. The standard extraction techniques
together with the added value of enzymatic and derivatizing techniques apply toward detection by fluorescence due to the thiochrome formed. However, based on advances in technology regarding analytical hardware, there is a trend toward mass spectroscopy as an added technique toward identifying and quantifying analytes, including riboflavin. However, due to the costs and challenges of mass spectrometry and the sensitivity and selectivity of fluorescence detection, methods based on thiochrome generation are still favoured (Hålcin et al., 2013).

The simultaneous determination of thiamine and riboflavin in malt may be used as an example of a typical experimental regime (Hucker et al., 2012) as was previously described. A further method for the determination of riboflavin and thiamine is described by Santos et al. (2012) where green, leafy vegetables are freeze-dried, milled, extracted with 10 mM ammonium acetate in methanol containing an antioxidant (butylated hydroxytoluene) and then sonicated. After centrifuging at 14 000 g for 15 minutes and filtering through a 0.45 µm nylon filter, it was concentrated in a nitrogen stream prior to injection and detection using DAD in the 200 to 680 nm range. This allows determination of a number of other water-soluble vitamins as well.

The methods available all have different permutations which involve enzymic digestion, use of opaque flasks, trichloroacetic acid protein precipitation and inactivation of interfering enzymes by acid treatment (Englberger et al., 2010). Choices may be made based on cost, speed and sensitivity for each specific user and application. Co-enzymes need to be extracted in the pH 5.0–7.0 range due to instability outside of the range. This may be done using an acetonitrile/phosphate buffer mixture (Russell, 2012).
VITAMIN B₃ (NIACIN)

Generic description and structures – Vitamin B₃

The structures of two forms of vitamin B₃ are depicted in Figure 3. There are more vitamers than that represented graphically below (Mihhalevski et al., 2013). This vitamin is quite stable to different processing conditions. Its active forms (NAD and NADP) are involved in oxidation reduction reactions, acting as electron carriers. Similar to riboflavin and its coenzyme forms, niacin is indirectly involved in a wide range of central biochemical reactions.

![Nicotinic acid](image1.png)  ![Nicotinamide](image2.png)

**Figure 3.** Vitamin B₃ structures.

Nutritional/Physiological importance and Dietary sources – Vitamin B₃

Niacin (nicotinic acid amide) is better known in the form of nicotinamide adenine dinucleotide (NAD) or its phosphorylated form i.e. nicotinamide adenine dinucleotide phosphate (NADP). A lowered NAD or NADP level is an indicator of deficiency, the symptoms of which are pellagra (skin, digestive and nervous disorders) (Belitz & Grosch, 1999). The main dietary source is muscle products as well as plant products, being more bio-available in meat and in milk (Nohr & Biesalski, 2010).
HPLC assay methodology for Vitamin B₃

There is a further deal of overlap between the extraction and determination of the B vitamins, including thiamine, riboflavin and niacin. However, the many different vitamers of this vitamin do provide challenges when conducting quantitative determinations in different food matrices. The different vitamers are those mentioned previously viz. nicotinamide, nicotinic acid, nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. Further vitamers are nicotinic acid dinucleotide, nicotinic acid dinucleotide phosphate, N-ribosyl nicotinamide and N-ribosyl nicotinic acid (Mihhalevski et al., 2013).

These vitamers (as with other vitamins) are usually integrated into the food matrix, including binding to protein or other structural components, in some cases being unavailable to extraction procedures and quantitation (Russell, 2012; Mihhalevski et al., 2013; Plonka et al., 2012). This, together with poorly defined vitamers in terms of what exactly is being extracted, makes for some uncertainty in terms of niacin analysis.

Santos et al. (2012) have developed a method for the simultaneous determination of water- and fat-soluble vitamins in leafy vegetables using LC-MS/MS and LC-DAD, the former for analyzing the water-soluble components. Sample extraction from green, leafy vegetables included protection from light and by maintaining samples on ice. In this instance samples were extracted into 10 mM ammonium acetate/methanol 50/50 v/v containing butylated hydroxytoluene as an antioxidant (Santos et al., 2012). Hippuric acid was used as a standard. The samples were shaken and then sonicated for 15 minutes with the temperature not going above 25°C. The centrifuged sample (14 000 g for 12 minutes) was then passed through a 0.45 μm nylon filter, concentrated to remove methanol in a nitrogen stream and injected onto an HPLC-MS/MS system to determine water-soluble vitamins. The detection system was a DAD and MS via an electrospray interface. The column was an ACE-100 C18 (100 X 2.1 mm i.d., 3 μm particle size). Separation of vitamins in a single run was achieved.
using a complex gradient with 3 mobile phases viz. A: 10 mM ammonium acetate (pH 4.5), B: 0.1% acetic acid and C: 0.3% acetic acid. The method was validated and then used to study vitamins (including nicotinamide) in the vegetables as a function of storage time.

The above method did not specify SPE even though it is usually standard practice to do so. In addition, the use of enzymes or other protein precipitating agents were also not used for this particular sample matrix. The latter, together with heating, would be suited to other matrices such as cereals.

León-Ruiz et al. (2013) conducted studies on honey using a simpler system and included the determination of nicotinic acid and nicotinamide. The vitamins all occur in solution in honey and are thus more easily extracted via dilution of honey in water followed by filtration through a 0.45 µm nylon filter. Isocratic elution was conducted using a mobile phase containing 0.01 % sulphuric acid and 2% methanol (v/v) at pH 3.5 and a column temperature of 25°C. This gave both high sensitivity and low limits of detection. A µBondapak C18 column (150 X 3.9 mm, 10 µm) was used.

**VITAMIN B₆ (PYRIDOXINE)**

**Generic description and structures – Vitamin B₆**

The structures of various forms of vitamin B₆ are depicted in Figure 4. While these include pyridoxine, pyridoxal, pyridoxamine, vitamers that are not depicted are pyridoxine phosphate, pyridoxal phosphate, pyridoxamine phosphate and 5’-O-β-D-glucopyranosyl pyridoxine (Mihhalevski et al., 2013). As much as it is considered relatively stable as a group of vitamers, pyridoxine itself can be affected in highly processed foods. As with other groups of vitamers, different forms are either free or bound to different elements of food matrices. Due to this, hydrolysis or SPE is necessary prior to HPLC analysis (Zand et al., 2012).
Figure 4. Vitamin B₆ structures.

Nutritional/Physiological importance and Dietary sources – Vitamin B₆

Pyridoxine (vitamin B₆) is the precursor for pyridoxal phosphate. The latter is a prosthetic group for a group of enzymes. The latter includes amino acid decarboxylases, racemases, dehydrases, transferases and synthases (Belitz & Grosch, 1999; Zand et al., 2012). Ingestion is usually in the form of pyridoxal or pyridoxamine. The most stable form is pyridoxal.

This vitamin is found in most food products and also, due to its stability, is often used for fortifying food products (Russell, 2012). It is also much more bio-available in animal than in plant sources. A deficiency of this vitamin (or its vitamers) is protein metabolism-related, e.g. hemoglobin synthesis. It also plays a role in homocysteine synthetic and degradative reactions (Rivlin, 2007)

HPLC assay methodology for Vitamin B₆

Zand et al. (2012) developed a UHPLC-DAD method for determining riboflavin and pyridoxine together with a parallel LC-MS analysis using the same solvents. This was considered advantageous in terms of one method being used to validate another.

A simple sample preparation process included different baby food samples which were homogenized and diluted with 1% acetic acid and incubated at 70°C for 40 minutes, cooled
and made to volume with acetic acid. After centrifuging at 8 000 g for 10 minutes at 5°C, the supernatants were passed through a 0.45 µm filter prior to injection. The system consisted of a UHPLC system and rapid resolution high definition C18 column (2.1 X 50 mm, 1.8 µm particle size, RRHD Eclipse Plus). The flow rate was 0.5 mL/ minute with A = water:acetic acid (99:1) and B = acetonitrile:acetic acid (99:1). The gradient started at A:B = 90:10 to reach 10:90 after 3 minutes (20 µL injection volume). Pyridoxine was measured at 280 nm.

The LC-MS version was by the same authors using a C18 Ultrasphere Behcam (4.6 mm X 25 cm, 5 µm particle size) column coupled to a mass spectrometer with electrospray ionization interface. Conditions were the same as for the UHPLC system. This allows then for validation of the UHPLC method and for the determination of other water-soluble species in the samples.

Other multivitamin methods include that by Santos et al. (2012) and Jin et al. (2012) which include the determination of B₆ vitamers.

**Improvements and recent developments: Vitamin B₆ methods**

One of the recent developments was in terms of microbiological assay (MA), where a colorimetric microbial viability assay based on the reduction of a tetrazolium salt was used to determine vitamin B₆ in a wide range of foods and beverages with satisfactory accuracy and within 24 hours, as opposed to the 2 days required for the conventional MA (Tsukatani et al., 2011).

With respect to HPLC methods, Hálvin et al. (2013) developed an analytical system using nutritional yeast as a food model to conduct LC-MS/TOF to determine B complex vitamins since they considered that HPLC methods employing UV or fluorescence detection did not always have enough sensitivity and selectivity if proper clean-up parameters are not
observed. In this instance, isotope-labeled standards were employed to compensate for losses during sample preparation and for matrix effects during MS and MS/MS analysis.

Finely ground yeast was weighed into flasks to which were added labeled vitamin standards and 0.05 M ammonium formate buffer to volume. This was centrifuged (14 000 rpm, 5 minutes at room temperature) and passed through a 0.2 µm PTFE filter prior to injection. All analyses were conducted using an ACQUITY UPLC® with a time of flight mass spectrometer. An ACQUITY UPLC HSS C18 (2.1 X 150 mm, 1.8 µm) column was used with two eluents viz. A: water + 0.1% formic acid and B: acetonitrile + 0.1% formic acid. The gradient was run at 0–3 minutes100% A, 3–8.5 minutes (A:B = 80:20), 8.5–10 minutes (A:B = 5:95) and 10–15 minutes (100% A). The flow rate was 0.25 mL/minutes, temperature of 25°C and a sample injection volume of 5 µL.

This method was proven to work without pre-concentration for determination of the B group vitamins, including that of pyridoxine. Challenges were encountered, including the need to prepare additional labeled vitamers viz. pantheine, N-ribosyl nicotinamide and N-ribosyl nicotinic acid. A second challenge was the selection of enzyme preparations to minimize the inclusion of interfering agents in these preparations with respect to MS-TOF.

VITAMIN B₉ (FOLATE)

Generic description and structures – Vitamin B₉

Pteroyl-l-glutamic acid (PGA or PteGlu) is the synthetic form of the vitamin that is most commonly known as folic acid, while “folate” is the collective name for a wide range of PGA derivatives, some of which are depicted in Figure 5. As the most stable vitamer, folic acid is the preparation of choice for inclusion in fortified food products and in dietary supplements (Kim, 2011; Pfeiffer et al., 2010; Shane, 2010). The folates that occur in nature are the 7,8-
dihydro- and 5,6,7,8-tetrahydro-reduced forms of PGA, resulting in Dihydrofolate (DHF) and tetrahydrofolate (THF). Substitution at the N-5 and/or N-10-position with a one-carbon adduct generates 5-methyl-THF, 5-formyl-THF, 5-formino-THF, 10-formyl-THF, 5,10-methylene-THF and 5,10-methenyl-THF (Fig. 5). Although only the monoglumate form is metabolically active, approximately 80% of all native folates are polyglutamates, with 5–8 conjugated glutamate units most abundant (Bailey, 2006; McGuire, 2006; Russell, 2012; Vahteristo & Finglas, 2000), while a chain length of 2–14 conjugated glutamates has been reported (Garrat et al., 2005). In vivo, monoglutamates, notably 5-CH₃-THF, is the transport form of folate, while the storage form in tissues like the liver (the main storage organ), is polyglutamates (Alegría et al., 2008; Shane, 2010).
Figure 5. Vitamin B<sub>9</sub> (folate) structures.
**Nutritional/Physiological importance – Vitamin B₉**

The nutritional importance of folate lies in its vital role in one-carbon metabolism. In the body, various THF derivatives serve as co-factors which transfer one-carbon units in numerous biosynthetic, catabolic and interconversion reactions. These include amino acid synthesis (synthesis of serine, glycine, re-methylation of homocysteine to methionine), degradation of histidine and purine and pyrimidine synthesis (Groff *et al.*, 1995; Bailey, 2007; McGuire, 2006). The latter is critical for DNA synthesis and repair, for all cell replication, including normal foetal development (Groff *et al.*, 1995; Iyer & Tomar, 2009; McGuire, 2005; Stover, 2010). Persistent low dietary folate intake results in deficiency with bone marrow cells becoming megaloblastic and manifestation of anaemia. The formation of the large immature erythrocytes which are characteristic of megaloblastic anaemia is the consequence of impaired DNA synthesis (Groff *et al.*, 1995; MacGuire, 2006). Folate administration as therapeutic treatment against megaloblastic anaemia has been practiced for almost seven decades (Bailey, 2007; Ye *et al.*, 2008). More recently, it was discovered that increased periconceptual folate consumption significantly reduces the incidence of birth defects (Hobbs *et al.*, 2010; Oakley, 2009), including Down syndrome, orofacial clefts and congenital heart defects (Hobbs *et al.*, 2010; Russell, 2012; Wallis *et al.*, 2010) and, in particular, neural tube defects (NTDs), with anencephaly and spina bifida the most common and severe (Hobbs *et al.*, 2010; Oakley, 2009). This precipitated mandatory folate fortification on a global scale. The USA was the first to introduce such programme, targeting grain flour and other cereal products (Berry *et al.*, 2010), with other countries, including Canada, Mexico, Hungary (Gregory, 2004; Oakley, 2009), Australia (Chandra-Hioe *et al.*, 2011) and South Africa (Anonymous, 2003) following suit. Countries where mandatory fortification programmes have been implemented reported that the intended effect of reducing NTDs had been achieved (Berry *et al.*, 2010; De Wals *et al.*, 2007; Gregory, 2004).
An association was indicated between methylenetetrahydrofolate reductase (MTHFR) polymorphisms and the incidence of NTDs (Bailey et al., 2002; Trembath et al., 1999) and the risk for Down Syndrome (Wu et al., 2013), especially if coupled with reduced folate and/or cobalamin status (Brouns et al., 2008). Current research on the role of folate in health promotion centres on the finding that similar single-nucleotide polymorphisms in folate transport or metabolism genes have been implicated as risk factors for many age-related chronic diseases associated with debility and mortality. These include cardiovascular diseases, neurodegenerative disorders (such as stroke, Parkinson’s and Alzheimer’s disease), macular degeneration and various cancers (Bailey et al., 2002; Caudill, 2004; Shaik & Gan, 2013; Ye et al., 2008). By interacting with the proteins encoded by the variant genes, folate can reduce disease risk (Ye et al., 2008). Protection against cardiovascular disease (Iyer & Tomar, 2009; Stover, 2010), as well as a range of vascular, neurological, ocular, renal and pulmonary abnormalities is also linked to its role in preventing hyperhomocysteinemia (i.e. elevated levels of homocysteine in the serum) (Caudill, 2004; Bottiglieri & Reynolds, 2010; Russell, 2012; Shaik & Gan, 2013). While McGuire (2006) described the causal relationship between folate deficiency and DNA damage in eukaryotes (including humans) leading to chromosomal abnormalities associated with oncogenesis of leukemias, lymphomas and tumors, epidemiological studies produced compelling evidence indicating that increased folate intakes may reduce the risk of colorectal cancer (Iyer & Tomar, 2009; Kim, 2007). Further research is required to confirm that folate plays a prophylactic or preventative role in osteoporosis, psychiatric illness, depression and senile dementia (Russell, 2012).

Another aspect of the increasing interest in folate in terms of health promotion is the recent discovery that it may play an important role as an antioxidant in vivo, both by preventing the adverse effect of reactive oxygen species (ROS), as well as by inhibiting lipid peroxidation (Merola et al., 2013). There is a tantalizing possibility that this is linked to one
of the consequences of folate and vitamin B₁₂ deficiency, hyperhomocysteinemia. It was suggested that hyperhomocysteinemia may be accompanied by increased ROS, elicited by homocysteine. When folate (vitamin B₉), pyridoxine (vitamin B₆), betaine and vitamin B₁₂ were combined as supplements in a protein restricted diet administered to homocystinuric patients (a genetic disease characterised by hyperhomocysteinemia), the treatment resulted in a significant reduction of indicators of oxidative stress, such as malondialdehyde (Vanzin et al., 2011). The antioxidant potency of folate, pyridoxine (and thiamine) was also reported by Gliszczynska-Swiglo, 2006).

**Dietary sources – Vitamin B₉**

Good food sources of folates include fortified cereals (Russell, 2012), spinach, broccoli, peas, (Vahteristo et al., 1997b), liver and kidneys (Kim, 2011; Vahteristo et al., 1996a), egg yolk, yoghurt (Vahteristo et al., 1997a), lima beans, mushrooms (Groff et al., 1995) and orange juice (Ye et al., 2008).

In cows’ milk, 90–95% occurs as 5-CH₃-THF (Ye et al., 2008), while 5-CHO-THF is the dominant folate in fermented milk products (Van Wyk & Britz, 2012). In fruit (bananas, oranges and strawberries) and vegetables (spinach, lettuce, cabbage, peas and beetroot) 5-CH₃-THF is predominant (Jastrebova et al, 2003; Vahteristo et al., 1997b), while THF dominates in liver tissue (Garratt et al., 2005; Vahteristo et al., 1996a) and in fresh fish (Vahteristo et al., 1997a). The major folate forms in cereals are 5-CHO-THF, 10-CHO-THF and 5-CH₃-THF, with 5-CHO-THF dominating in bread, while 5-CH₃-THF dominates in rice (De Brouwer et al., 2008) and also in baker’s yeast, followed by THF (Patring et al., 2009). However, the bioavailability of native dietary folates average only 50% (Gregory, 2004), with polyglutamates being 70–80% bioavailable compared to monoglutamate forms (Bailey, 1988).
Digestibility is also affected by the matrix (Bailey, 1988). While protein in general binds folate (De Souza & Eitenmiller, 1990; Strålsjö et al., 2002), folate binding protein strongly and specifically binds folate in biological fluids like milk (Ball, 2006; Indyk, 2010; Shane, 2010; Witthöft et al., 1999), but heating at 100°C for 5 minutes denatures the binding proteins (Lim et al., 1998), liberating the folates. Most native folates occur as folylpolyglutamates that are unable to traverse the cell membrane (Shane, 1982; 2010) and are likely to be retained intracellularly. However, digestive processes (in vivo) (Iyer & Tomar, 2009), or heating at 100°C for 6 minutes (in vitro) releases intracellular folates (Shane, 1982; 2010).

Assay methodology for Vitamin B<sub>9</sub> – General

The increasing significance of folates in health and disease, including the global wave of mandatory folate fortification programmes, prompted by its proven role in protecting against NTDs, has also focused attention on the need for accurate folate assay methods in order to establish a credible database of folate levels in dietary sources high in native folates, as well as in foods fortified with folates (Arcot & Shrestha, 2005; Rader et al., 1998). Methods to quantify folates in biological samples in general and in food, in particular, are complicated due to the numerous forms of native folates, their instability, the complexity of food matrices and the relatively low concentration of the analytes (Arcot & Shrestha, 2005).

Folate analytical methods were developed over the last 50 years, with the microbiological assay (MA) being the first. It was also the standard reference method for determining folates with biological activity for decades and still finds wide application in routine folate assays in food (Ball, 2006; Pfeiffer et al., 2010; Kim, 2011). The preferred organism when applying the MA to food samples is Lactobacillus rhamnosus (ATCC 7469) (or the chloramphenicol-resistant strain (ATCC 27773; NCIB 10463)), rather than
Enterococcus hirae (ATCC 8043) since the latter does not respond to many folate forms, a notable exclusion being 5-CH₃-THF, one of the most abundant folate forms in foods (Kariluoto et al., 2001; Rader et al., 1998). The principle of the turbidimetric MA is the specific growth-dependence of L. rhamnosus on folate. Hence, the growth response is directly proportional to the amount of folate in the medium. While the MA does not require sophisticated instrumentation, is relatively economical and has high sensitivity (quantitation of folate concentrations as low as 0.12 ng/mL is possible), its precision is inferior to other methods, it is labour intensive, even with semi-automated procedures and results are only available after 2–5 d. Moreover, it measures only total folates and shows a lower growth response when PGA is used as the calibrator, compared to 5-CH₃-THF, resulting in an underestimation of the folate concentration to some extent (Pfeiffer et al., 2010; Rader et al., 1998). Until recently, the MA was the only method given official status by the AOAC (Iyer & Tomar, 2009), but two new methods were recently awarded “AOAC First Action Official Method℠” status. AOAC 2011.05 is entitled “An Optical Biosensor Assay for the Determination of Folate in Milk and Nutritional Dairy Products” and is based on a surface plasmon resonance (SPR) optical biosensor. AOAC 2011.06 (“Total Folates in Various Foods by Trienzyme Extraction and UPLC-MS/MS Quantitation) uses UPLC-MS/MS to measure total folates of seven folate forms (Sullivan, 2012).

Protein-binding assays to determine folates were developed during the 1970–1980s as more rapid and simpler alternatives to the MA. These methods were developed for clinical laboratories and started out as immunoassays using folate-specific antibodies as the folate binder. In modern protein-binding assays, primarily folate binding protein (FBP) is used, either in competitive binding assays with radiolabelled tracer folate (radioassays) or automated protein-binding assays (using chemiluminescence as detection) or enzyme immunoassays (typically using fluorescence detection). Two major limitations impede the
suitability of protein-binding assays to food samples, namely the questionable accuracy when mixtures of folates are present and the limited dynamic range (typically extending to only 20 ng/mL serum folate), often coupled with inaccurate dilution linearity (Pfeiffer et al., 2010). However, the accuracy of an enzyme linked immunosorbent assay (ELISA) was established when comparing folate levels in fortified wheat flours analysed using MA > HPLC > ELISA (Alaburda et al., 2008). Satisfactory results were obtained when using an optical biosensor assay utilising folate binding protein to measure folates in milk and milk-based paediatric formulae (Indyk, 2010), meat homogenate, skim milk, breakfast cereals, broccoli, egg yolk and other food matrices (Indyk & Woollard, 2013).

HPLC methods for the determination of folates in foodstuffs are numerous (Doherty & Beecher, 2003; Hefni et al., 2010; Lebiedzińska et al., 2008; Ndag et al., 2001; Puthesseri et al., 2013; Samaniego-Vaesken et al., 2013; Vahteristo et al., 1997a and b; Van Wyk & Britz, 2012; Wawire et al., 2012; Yazynina, et al., 2008). The water-soluble nature as well as variations in hydrophobicity and ionic properties of the folate vitamers enable their separation by ion exchange or reversed-phase liquid chromatography (Gregory, 1984). UV (as variable wavelength or diode array), electrochemical (in the oxidative mode) and fluorescence detection methods are the most common (Gregory et al., 1984; Hefni et al., 2010; Lebiedzińska et al., 2008; Vahteristo et al., 1997a and b; Van Wyk & Britz, 2012; Wawire et al., 2012) and are equally sensitive (Ball, 2006). The latter applies to the reduced forms of folate which shows native fluorescence that is pH dependent, with pH 2.2–2.3 the best compromise relative to the individual optima (Ball, 2006; Gounelle et al., 1989; Russell, 2012; Van Wyk & Britz, 2012). Inter-laboratory studies confirmed that fluorescence detection is superior to UV absorbance detection, due to the greater sensitivity displayed by fluorescence detection (Russell, 2012). The poor inter-laboratory agreement in terms of the results for folate vitamers in foodstuffs, other than 5-CH₃-THF (Finglas et al., 1999), was
attributed to the relative instability of the other reduced folate vitamers (Vahteristo et al., 1996b), while Puwastien et al. (2005) attributed it to lack of uniformity/standardisation in terms of methods of folate extraction and detection, as well as the paucity of reliable reference materials. However, intra- and inter-laboratory studies confirm the superior precision of LC methods, compared to the MA: coefficients of variation (CV) of up to 35%, with a mean value of 17.5% reported for inter-laboratory assays (MA) (Finglas et al., 1999; MacLean et al., 2010; Puwastien et al., 2005), while the corresponding mean for HPLC assays was 14.5%. For intra-laboratory studies, the CVs were 8.9% (MA) and 5.6% (HPLC) (Finglas et al., 1999).

Assays performed using LC systems where detection is achieved using mass spectrometry (LC-MS and LC-MS/MS) (Patring, et al., 2009; de Brouwer et al., 2008) are adjudged the most selective, specific and sensitive (Pfeiffer et al., 2010). Quantitative accuracy can be further improved by using isotope dilution MS with (13C5) 5-CH3-THF used as the Internal Standard (IS) in an UPLC-MS/MS system (Chandra-Hioe et al., 2011).

**HPLC assay methodology for Vitamin B9**

In general, extraction procedures are aimed at liberating protein-bound folates and deconjugating folylpolyglutamates. Hence, the procedure involves suspension in a suitable buffer and then a heat treatment to break up particles, denature proteins and gelatinize starch (Ball, 2006; Lim et al., 1998). Microwave heating was found to increase the measurable folates, compared to conventional heating. This is ascribed to the rapidity of the microwave heating, preventing folate losses due to the activity of endogenous enzymes (Puthesseri et al., 2013). Heating is followed by followed by enzymatic deconjugation (Van Wyk & Britz, 2012). The enzyme preparations used to achieve deconjugation contain folate conjugase, the common name for pteroylpolyglutamate hydrolase or folylpoly-γ-glutamyl carboxypeptidase
(EC 3.4.22.12) (Rader et al., 1998). Various deconjugase preparations are available, each named for the source that it originated from, encompassing a variety of mammalian tissues or fluids, including human and porcine jejunal tissue and pancreatic fluid (Ball, 2006), chicken pancreas, hog kidneys and rat and human plasma, as well as vegetable extracts (cabbage) (Eitenmiller & Landen, 1999). Most conjugase preparations yield monoglutamates, but chicken pancreas conjugase produces diglutamyl folates. Since HPLC methods predominantly require complete deconjugation, it is essential to use either hog kidney, human or rat plasma conjugase to produce monoglutamates (Strålsjö et al., 2002; Ye et al., 2008).

Several researchers employed the trienzyme extraction method and reported that it enhanced the yield of measureable folate significantly (Bui & Small, 2007; Johnston et al., 2002a; 2002b; Kim, 2011; Lim et al., 1998; Pfeiffer et al., 2010; Rader et al., 1998; Shrestha et al., 2000). However, this method also has its detractors whom reported that the reactions with the α-amylase and protease are time-consuming, that the analyst often faces the dilemma of which pH to select due to the substantial differences in pH optima for the three different enzymes (Ball, 2006), that the method sometimes has no effect on folate levels (Ndaw et al., 2001; Strålsjö et al., 2002) and may result in folate losses, compared to the single-enzyme methods (Yazynina et al., 2008). Di-enzyme methods have been used to good effect. For example, α-amylase in a starchy matrix (Chandra-Hioe et al., 2011; Hefni et al., 2010; Vahteristo et al., 1997b) and protease in high-protein foods and dairy products (De Souza & Eitenmiller, 1990) achieved enhanced liberation of the folates bound to polysaccharides and proteins, respectively, followed by reaction with a suitable folate deconjugase (Chandra-Hioe et al., 2011; De Souza & Eitenmiller, 1990). Moreover, combining CP conjugase, which possesses significant amylolytic (Pedersen, 1988) and proteolytic activity (Strålsjö et al., 2002), and HK conjugase, complete folate extraction and conjugation without long incubation times was attained (Vahteristo et al., 1996; Van Wyk &
Britz, 2012). However, the commercial supply of CP conjugase has been erratic in recent times and although Soongsongkiet et al. (2010) reported satisfactory results with a lyophilised crude CP extract prepared by them, in our laboratory we were unable to source raw material that had been produced under consistently controlled conditions to ensure a preparation of consistent quality. As a result, we are presently doing a comparative investigation to ascertain the effectiveness of various dienzyme and trienzyme extraction and deconjugation regimes (results unpublished).

In solution, all folates are susceptible to oxidative degradation, which is exacerbated by oxygen, heat, metal ions, such as cupric and ferric ions (Russell, 2012) and especially light (Garrat et al., 2005; Kim, 2011). Moreover, the stability of the various folate forms varies (Garrat et al., 2005), dependent on their composition, particularly the state of reduction and substitution at the N-5 or N-10 position. Reduced forms are more labile, with THF the least stable and substitution at the N-5 position increasing stability (Russel, 2012). The order of stability is: 5-CHO-THF > 5-CH₃-THF > 10-CHO-THF > THF (Wittöft et al., 1999). Hence, the extraction is always conducted in the presence of at least ascorbic acid as antioxidant, but often with mercapto-ethanol as well, and under subdued light (Pfeiffer et al., 2010; Van Wyk & Britz, 2012).

Sample clean-up is often considered essential to ensure reliability of the results. Affinity chromatography with folate-binding protein is used effectively to this end, as well as reversed-phase (phenyl) or strong-ion exchange (SAX) solid phase extraction (Ball, 2006; Jastrebova et al., 2003; Johansson et al., 2008; Kariluoto et al., 2001 Kim, 2011; Vahteristo & Finglas, 2000; Van Wyk & Britz, 2012).

The following is a typical extraction, purification and HPLC assay protocol that is used in our laboratories as reported in Van Wyk & Britz (2012) with some modifications, as applied to various dairy products. Ten mL phosphate extraction buffer was added to 2 mL or
2 g sample, followed by homogenisation under a constant flow of nitrogen. The homogenate was flushed with nitrogen and heated in a microwave oven at 75% power (610 kW) for 1 minute. The tube was then placed in a boiling waterbath at 100°C for 10 minutes to release protein-bound folates. After cooling and centrifugation, decanting the supernatant into clean centrifuge tubes, pH of the extract was then adjusted to 7.0 using NaOH. 25 mg α-amylase was added per sample and dispersed, flushed with nitrogen, capped and incubated at 37°C for 4 h, followed by addition and dispersion of 25 mg protease and incubation at 37°C for 4 h. The extracts were then transferred to a boiling waterbath (100°C) for 5 minutes to inactivate the enzymes, followed by cooling and then the addition of 40 mg lyophilised Human plasma (HP) conjugase, and incubation as before. Extracts were again heated at 100°C for 5 minutes to inactivate the enzymes, followed by rapid cooling and centrifugation.

Deconjugation efficiency in each sample type was tested by adding 110.4 nmol of PteGlu₃ to the sample extract prior to the addition of the enzymes. Control samples, where 110.4 nmol of PteGlu₃ was added after inactivation of the deconjugase enzymes, were also assayed. In the sample treated with the HP deconjugase, the absence of the PteGlu₃, having been replaced by a PteGlu peak, testified to complete deconjugation of the Pte-triglutamate.

The folate extracts were effectively purified using quaternary amine-based strong anion exchange solid phase extraction (SPE) columns (Strata SAX/3 mL/500 mg) (Phenomenex, Torrance, California).

Oxidative degeneration of the labile reduced folates induced by light and oxygen was minimised by expedient handling of all samples and standards under subdued light (gold fluorescent), the use of opaque containers or amber glassware, inclusion of antioxidants into solutions and flushing with nitrogen gas.

An Agilent 1100 quaternary HPLC system (Agilent Technologies, Waldbron, Germany), with a scanning fluorescence detector with excitation and emission wavelengths
set at 290 and 356 nm was used. Folic acid and PteGlu\textsubscript{3} were detected with the diode array UV detector at a wavelength range of 270–300 nm.

Two analytical columns (3 µm Luna-C\textsubscript{18} column (Phenomenex, Torrance, California)), followed by a 3.5 µm Zorbax SB-C\textsubscript{18} column (Agilent Technologies, Waldbronn, Germany) were used. The column temperature was maintained at 30 ± 1°C during separation of the vitamers by means of gradient elution with acetonitrile and a 30 mM potassium phosphate buffer (pH 2.2) at a flow rate of 0.45 mL/min. The gradient was started at 5% acetonitrile and then linearly increased to 13% over 18 min, increased to 25% acetonitrile over the next 7 min, followed by recycling to initial conditions before the following injection.

The limits of detection (LOD) and limits of quantitation (LOQ) for the different folate vitamers differ and so do the percentage recovery (quantitative validity or accuracy), but these, as well as the folate levels determined in the matrices were in agreement with other authors (Van Wyk & Britz, 2012).

It has become the norm to use Standard Certified Reference Materials (SRMs) as a quality control tool to confirm the accuracy of the assay, while Certified Reference Materials are used to validate the method (Johansson et al., 2008; Phillips et al., 2006; 2011; Rader et al., 1998; Samaniego-Vaesken et al., 2013). Examples are SRM 1849, a milk-based infant/adult nutritional powder with reference values defined in terms of folic acid (Anon., 2012) and CRM 421 (spray-dried milk powder). During the study in our laboratories described above, CRM 421 was not available, having been discontinued by the supplier, the Institute for Reference Materials and Measurements (Geel, Belgium). A substitute was obtained by combining a food grade folic acid fortificant (DSM Nutritional Products, Isando, SA) with fat free milk powder (Elite, Clover, Roodepoort, SA) to yield a folate concentration of 100 mg per 100 g. The results of our study agreed with the theoretical value, as well as
with the results obtained when a commercial accredited laboratory analysed a duplicate sample (Van Wyk & Britz, 2012).

In addition, the method was validated against results of an MA. Congruent with several other researchers (Alaburda et al., 2008; Doherty & Beecher, 2003; Edelmann et al., 2012; Puthusseri et al., 2013), we found that the HPLC results were in agreement (p < 0.05) with the results of the MA (Van Wyk & Britz, 2012).

**Improvements and recent developments – Vitamin B₉ methods**

A UPLC-DAD/Fluorescence method was employed to assay folates in oats. Contrary to HPLC methods applied to cereals, the UPLC method showed a high correlation with the MA. The method enabled the determination of seven different folate monoglutamates in the samples, with satisfactory precision and accuracy and substantially faster separation (Edelmann et al., 2012).

Garra et al. (2005) assayed the folate content in several food matrices, namely vegetables (including spinach, cauliflower and peas), wheat and liver tissue. They overcame the problems posed by the customary intricate and laborious extraction and deconjugation methods by applying a simple extraction technique based on snap-freezing tissue in liquid nitrogen, followed by homogenization in an ice cold 95% methanol / phosphate buffer (75 mM K₂PO₄, 0.4 M ascorbic acid, 0.8% MCE, pH 6.0), followed by centrifugation and filtration. They used an internal standard mixture which included polyglutamates. Assays were performed using LC-MS/MS (liquid chromatography/negative ion electrospray ionization tandem mass spectrometry). Apart from the fact that they could not distinguish between 5-formyl-tetrahydrofolyl- and 10-formyl-tetrahydrofolyl-polyglutamates, they obtained full quantitative analysis for 16 folates, including folylpolyglutamates where the chain contained less than eight residues. Semi-quantitative analysis was possible for folates
with up to eight conjugated glutamates and qualitative results were obtained for folylpolyglumates with up to 14 conjugated glutamates.

Pawlosky et al. (2003) reported that acceptable precision and accuracy was demonstrated when doing a comparative study between folates determined in various food matrices and CRMs using HPLC-Fluorescence vs a stable isotope LC-MS method with ESI. However, there are clear advantages in using LC-MS methods, when directly compared to HPLC-Fluorescence methods. Not only did the stable isotope LC-MS method allow quantification of 5-CHO-THF, but it also yielded folate values that were 67% higher than the HPLC-Fluorescence method, confirming the greater sensitivity of the former (Freisleben et al., 2003). A further demonstration of the superior selectivity of MS detection is the use of 5 kDa cut-off ultrafiltration that was sufficient in terms of sample purification, in contrast to the affinity binding or SPE purification methods that are required for LC-UV/fluorescence detection (Russell, 2012). Further LC-MS methods are listed in Table 1 (Chandra-Hioe et al., 2011; De Brouwer et al., 2008; Delchier et al., 2013; Young et al., 2011). However, as mentioned before, the cost and complexity of the MS-based methods limit their wide-spread application, even though they are superior in terms of sensitivity, selectivity and accuracy (Russell, 2012).

**VITAMIN B₁₂ (COBALAMIN)**

**Generic description and structures – Vitamin B₁₂**

The term vitamin B₁₂ is routinely used as a generic descriptor for all cobalamins that have biochemical functions in the human (Eitenmiller et al., 2008), including their role in antipernicious anaemia activity (Ball, 2006).
The structures of various vitamin B₁₂ (cobalamin) derivatives are depicted in Figure 6. Described as an octahedral cobalt complex, the structure consists of an equatorial chiral ligand called corrin (the tetapyrrole macrocycle), a lower axial ligand and various upper axial ligands. The lower ligand, comprising a rare α-ribofuranoside of 5,6-dimethylbenzimidazole, is attached to a corrin ring side chain, forming a “nucleotide loop” and coordinates to the cobalt via the N-3 atom of the imidazole. The upper ligand varies from cyanide to 5’-deoxyadenosine (Fig. 6) (Chandra & Brown, 2008; Randaccio et al., 2006).

**Nutritional/Physiological importance – Vitamin B₁₂**

Cyanocobalamin (CNCbl) is the most stable form of the vitamin and hence the form used in dietary supplements and food fortification (Ball, 2006; Kim, 2011). OHCbl is also used in this role (Eitenmiller et al., 2008). CNCbl is not biologically active and needs to be enzymatically modified (Wong, 1989) to methylcobalamin (MeCbl) or 5’-Deoxyadenosylcobalamin (co-enzyme B₁₂, AdoCbl) which are important cofactors for methionine synthase (MeCbl) and 1.-methylmalonyl-CoA mutase as well as leucine aminomutase (AdoCbl) (Randaccio et al., 2006; Russell, 2012). Eitenmiller et al. (2008) tabulated the complete set of vitamin B₁₂-dependent enzymes.

The remethylation of methionine by 5-CH₃-THF:homocysteine methyl transferase (EC 2.1.1.13) occurs via the transfer of the methyl group to the cobalamin co-enzyme and then to homocysteine, forming methionine and regenerated THF (Bailey, 2006; Ball, 2006), illustrating the close relationship between folate and vitamin B₁₂ deficiencies. As with folate, vitamin B₁₂ deficiency results in impaired DNA synthesis, resulting in megaloblastic anaemia which is clinically indistinguishable from that due to folate deficiency. The vitamin B₁₂-induced anaemia is known as pernicious or Addisonian anaemia (Ball, 2006).
Other important roles of vitamin B₁₂ in normal metabolism include synthesis of DNA, proteins, phospholipids, creatine and neurotransmitters. Apart from the haematological effects, vitamin B₁₂ deficiency also results in neurological changes, caused by the inability to synthesise the lipid component of myelin. This leads to general demyelination of nerve tissue. The first symptoms are peripheral neuropathy with the feet and fingers are first to be affected, then the spinal cord, culminating in brain damage if not treated (Parker-Williams, 2013; Russell, 2012). Hence, the concern that high folate intakes may mask the haematological effects of vitamin B₁₂ deficiency, thus allowing the progression of neuropsychiatric damage, lies in the fact that advanced neurological damage cannot be reversed (Ball, 2006; Groff et al., 1995; Russell, 2012). Cerebellar ataxia (Morita et al., 2003), triggering seizures in epileptics (Thiel & Fowkes, 2004) and reversible involuntary movements in adults (Celik et al., 2003) are further conditions ascribed to vitamin B₁₂ deficiency.
Vitamin B<sub>12</sub> is also associated with the same chronic diseases as folate, due to the coincidences in their biological functions (Russell, 2012). Hence, subnormal vitamin B<sub>12</sub> levels have been linked to reversible dementia (Tucker <i>et al.</i>, 1996), while low folate and vitamin B<sub>12</sub> levels have been implicated in psychiatric diseases, such as bipolar disorder (Ozbek <i>et al.</i>, 2008). Optimum vitamin B<sub>12</sub> intake may also play an ameliorating role in depression, senile dementia (Eitenmiller <i>et al.</i>, 2008), Alzheimer’s disease, cardiovascular disease, breast cancer, hearing loss, osteoporosis, NTDs (Green & Miller, 2007) and multiple sclerosis (Kocer <i>et al.</i>, 2009). Furthermore, not only were low vitamin B<sub>12</sub> levels recorded in HIV-infected patients (Hepburn <i>et al.</i>, 2004), but adequate vitamin B<sub>12</sub> may slow the onset of Aids in HIV-positive individuals (Green & Miller, 2007).

**Dietary sources – Vitamin B<sub>12</sub>**

The vitamers that predominate in foodstuffs include AdoCbl in animal liver (Metzler, 1977), with hydroxocobalamin (OHCbl), AdoCbl and MeCbl dominant in animal tissue in general (Alegría <i>et al.</i>, 2008; Kumar <i>et al.</i>, 2010) and hydroxocobalamin (OHCbl) or aquacobalamin (H<sub>2</sub>Ocbl) are the forms most prevalent in milk (Ball, 2006; Kumar <i>et al.</i>, 2010). The native vitamin B<sub>12</sub> vitamers present in food are normally bound to proteins in the matrix, while certain foods contain specific vitamin B<sub>12</sub>-binding protein reducing the bioavailability. In contrast to folate, milk does not contain vitamin B<sub>12</sub> binding protein, while raw egg yolk and egg white do. Heating denatures the protein and releases the vitamin B<sub>12</sub> (Eitenmiller <i>et al.</i>, 2008; Russell, 2012).

All native vitamin B<sub>12</sub> in the human diet is originally synthesised by microorganisms, either in the rumen and intestinal tract of animals or in soil and water, which is then assimilated by animals (Ball, 2006; Eitenmiller <i>et al.</i>, 2008; Kim, 2011; Nohr & Biesalsky, 2009). Commercial production of vitamin B<sub>12</sub> also exploits biosynthetic processes, since
chemical synthesis is extremely complex (Murooka et al., 2005). The micro-organisms most widely used in these fermentations include *Pseudomonas denitrificans* (Mazumder et al., 1987), methanogens (*Methanosarcina barkeri, Methanobacterium* spp.) and *Bacillus* spp. (Mazumder et al., 1987; Yang et al., 2004) and *Propionibacterium* spp. (Gardner & Champagne, 2005), the preferred organisms for vitamin B₁₂ production (Murooka et al., 2005).

The most important dietary sources of vitamin B₁₂ are meat and animal organ meats, particularly liver (Alegría et al., 2008; Van Heerden et al., 2007; Schönfeldt & Gibson, 2008). Ruminant meat accounts for two-thirds of the daily intake of vitamin B₁₂ in the human diet (Alegría et al., 2008). Shellfish, dairy products, eggs and fortified cereal products are also dietary sources (Russell, 2012).

**Assay methodology for Vitamin B₁₂ – General**

Vitamin B₁₂ assay techniques include microbiological assays (MA), HPLC analyses, radioisotope dilution assay (RIDA), electrothermal atomic absorption spectrometry, capillary electrophoresis, biomolecular interaction analysis (BIA), chemiluminescence techniques (Alegría et al., 2008; Ball, 2006; Cheung et al., 2009; Gao et al. 2008; Kim, 2011; Markopoulou et al., 2002), fluorescence resonance energy transfer (FRET) combined with flow-injection analysis (FIA) (Xu et al., 2008) as well the more novel approach of a dipstick-based immunochemiluminescence biosensor used for energy drinks (Selvakumar & Thakur, 2011; 2012). Of the abovementioned methods, only the MA, HPLC, RIDA and chemiluminescence methods have been used successfully to quantify vitamin B₁₂ in foods (Eitenmiller et al., 2008; Kumar et al., 2010; Selvakumar & Thakur, 2012).

Until recently the MA was the only official AOAC method and is still widely used to determine vitamin B₁₂ in food (Ball, 2006; Eitenmiller et al., 2008; Kim, 2011), but is very
protracted (Alegría et al., 2008). The organism most frequently used for vitamin B12 determination in foods is *Lactobacillus delbrueckii* ssp. *Lactis* (ATCC 7830). The growth response (measured titrimetrically) (Van Wyk & Britz, 2010) is similar for most forms of cobalamin (CN-, NO-, OH-, SO3Cbl). However, AdoCbl produces a greater and MeCbl a lesser response, but exposure of the samples to fluorescent light accomplishes the complete conversion of MeCbl and AdoCbl to OHCbl (Kim, 2011).

Alternatives to the MA were recently adopted as official methods by the AOAC. An SPR optical biosensor protein-binding assay for vitamin B12 in milk products (Official method 2011.16) and three HPLC methods to determine vitamin B12 in food products were adopted. The HPLC methods include “Determination of Vitamin B12 in food products by Liquid Chromatography/UV detection with Immunoaffinity extraction: Single laboratory validation” (Campos-Giménez et al., 2008) (Official method 2011.08), “Determination of vitamin B12 in baby food (milk formulas) using HPLC after purification on an immunoaffinity column, cleanup and LC-UV quantitation” (Official method 2011.09) and “Determination of vitamin B12 by HPLC” (Official method 2011.10) (Sullivan, 2012). Several researchers employed HPLC methods to determine vitamin B12 in multivitamin and mineral tablets. These matrices are relatively simple, compared to foodstuffs, making extraction and resolution of the vitamin simple in comparison (Markopoulou et al., 2002). Although vitamin B12 levels in foods are often too low for detection using HPLC methods (Ball, 2006), reversed-phase HPLC methods with immunoaffinity extraction (Heudi et al., 2006) and UV detection (Heudi et al., 2006; Van Wyk & Britz, 2010; Van Wyk et al., 2011), and coulometric electrochemical detection (Lebiedzińska et al., 2007) have been used effectively to determine vitamin B12 in dairy products (Van Wyk & Britz, 2010), petfood and various infant formulae (Heudi et al., 2006), fruit juice and various seafood (Lebiedzińska et al., 2007).
HPLC assay methodology for Vitamin B₁₂

Extraction of cobalamins from tissues or fluids is a prerequisite for all assays. The ideal process achieves full recovery of the cobalamins, while preventing degradation to noncobalamin corrinoids (Gimsing & Beck, 1986). Using phosphate-buffers containing sodium metabisulphate or acetate-buffers containing KCN, combined with a heat treatment, liberates the vitamin B₁₂ from the matrix, converts all forms of vitamin B₁₂ to the more stable sulfitocobalamin or cyanocobalamin, respectively, as well as achieves protein denaturation (Alegría et al., 2008; Ball, 2006; Kim, 2011; Russell, 2012; Van Wyk, 2002). In high-protein foods, like meat and dairy products, heat-induced or protease-catalysed protein denaturation is essential, since both vitamin B₁₂ and folate are protein-bound in these products (Alegría et al., 2008; Deeth & Tamime, 1981; Kumar et al., 2010).

Cobalamins are stable to thermal processing, with cyanocobalamin the most stable. However, strong acid and alkaline conditions, oxidizing agents and intense visible light inactivate the vitamin (Ball, 2006; Kim, 2011). Hence, during extraction and subsequent sample handling, it is advisable to avoid any of the above. Light exposure is minimized by working under subdued light and using amber glassware (Van Wyk & Britz, 2010).

Purification of extracted cobalamins will achieve removal of compounds that may interfere with the resolution of cobalamins, as is present in most biological samples. Purification of the extracted cobalamins should ideally remove all these interfering substances, in such a manner that the extracted cobalamins react similar to standards during the analysis. Removal of these substances is based on the amphoteric nature of cobalamins, i.e. they possess both polar and non-polar groups. Hence, in aqueous solutions, cobalamins are absorbed to nonpolar materials such as silanized silica gel, Amberlite XAD-2, or alkyl-bonded phase columns such as C₈ and C₁₈ reverse phase columns, while water or weak
organic solvents wash off less hydrophobic compounds. When followed by application of higher concentrations of organic solvents, yet not concentrated enough to elute compounds that are more hydrophobic than cobalamins, complete elution of purified cobalamins is achieved (Gimsing & Beck, 1986; Blanche et al., 1990; Dalbacke & Dahlquist, 1991). Immunoaffinity, ion exchange chromatography (Eitenmiller et al., 2008), or SPE methods can also be used to purify and/or concentrate CNCbl in extracts (Van Wyk & Britz, 2010).

RP-LC on C18 columns with gradient elution of the analyte(s) is the most common approach. The mobile phase consists of water or buffers and methanol or acetonitrile. Since all vitamin B12 absorb UV light, detection based on absorbance in the UV–Visual range with absorbance maxima from 264–551 nm, with the absorbance maxima of CNCbl 278, 361 and 551 nm (Eitenmiller et al., 2008). CNCbl is most often detected at 361 (Russell, 2012; Van Wyk & Britz, 2010) or 550 nm (Russell, 2012). UV photodiode array detectors are preferred to single lamp instruments.

Some of the most recent methods are described in Table 1 (Campos-Giménez et al., 2008; Guggisberg et al., 2012; Heudi et al., 2006; Lebiedzińska et al., 2007), including multivitamin (Luo et al., 2006) and human serum assays (Shaik & Gan, 2013). Details of the method used in our laboratories (Van Wyk & Britz, 2010, with modifications) follow.

Samples were extracted using a 0.31 M KCN-acetate buffer at pH 4.5. The sample and buffer were combined in a 4:10 ratio, autoclaved at 121°C for 25 min, cooled and filtered into amber vials. Work with OHCbl standards showed that all OHCbl is converted to CNCbl during this extraction, so it is reasonable that all other cobalamins are converted to CNCbl (Van Wyk, 2002).

The HPLC system described in the previous section was used. Separation was achieved using a Phenomenex Luna (150 X 4.6 mm, 5 μm) column, with the column temperature at 30°C and the flow rate at 0.05 mL/min. Gradient elution was used: A = Acetonitrile; and B =
10 mM phosphate buffer (pH 2.2). Starting with 5% A: 95% B initial conditions, the gradient is increased to 15%A:85%B (0–10 min), then 40%A:60%B (10–13 min), 15%A:85%B (13–15 min), 10%A:90%B (15–20 min) and then 5%A:95%B (20–25 min), with 5 min post run time at initial conditions.

Detection was achieved using a diode array detector at 360.4 nm (sample) and 100 nm (reference). The LOD and LOQ were 0.002 and 0.005 µg/mL sample and all measures of sensitivity, selectivity, precision and accuracy were satisfactory. However, sensitivity was enhanced even more by using SPE columns to purify (Chromabond SB/3 mL/500 mg, Machery-Nagel) and concentrate (Chromabond C18ec/6 mL/1000 mg column, Machery-Nagel) the extracts. Moreover, the HPLC results were validated against the microbiological assay (Van Wyk & Britz, 2012).

**Improvements and recent developments – Vitamin B₁₂ methods**

HPLC analysis of vitamin B₁₂ in foodstuffs had recently received attention from the AOAC, resulting in the adoption of three official methods (Sullivan, 2012), as described in a previous section. LC-MS methods are not much in evidence, but those that have been reported (Luo *et al.*, 2006 – also see Table 1), indicate their potential in terms application of soft ionization techniques suitable to thermolabile and nonvolatile compounds to improve the selectivity and sensitivity of vitamin B₁₂ analyses (Eitenmiller *et al.*, 2008).

**VITAMIN C (ASCORBIC ACID)**

**Generic description and structures – Vitamin C**

Vitamin C, also referred to as l-ascorbic acid belongs to a group of vitamins called water-soluble vitamins. Its systematic name is 5(R)-5-[(1S)-1,2-Dihydroxyethyl]-3,4-dihydroxy-
2(5H)-furanone. It occurs naturally in food as L-ascorbic acid (AA) and as its oxidised product, dehydro- L-ascorbic acid (DHAA) (Figure 7) (Eitenmiller, 2008; Lee, 2004). Humans must obtain vitamin C through their diet. Some other mammals have the ability to produce their own vitamin C supply by converting it from glucose using the enzyme L-gulono-γ-lactone oxidase (Basu, 1996). L-ascorbic acid has a stereoisomer, isoascorbic acid (IAA), which can only be synthetically produced. IAA has its oxidised product called dehydroisoascorbic acid (DHIAA).

![Vitamin C structures](image)

**Figure 7.** Vitamin C structures.

**Nutritional/Physiological importance and Dietary sources**

Vitamin C is present in fresh fruit, vegetables, juices and organ meats such as liver (Combs, 1992; Jurczuk, 2005). Since vitamin C is highly unstable it can be used as an indicator of the quality of foodstuffs and fruit beverages during manufacturing and storage. It can be degraded by enzymes, oxygen, heat, light and metal ions (Bhagavan *et al.*, 2001).

Vitamin C is well known for its antioxidant properties as it assists the body in contesting viral infection, bacterial infections and toxicity. It is required for the synthesis of collagen, an important structural component of tendons, bones, teeth, blood vessels and muscles. Vitamin C enhances iron adsorption and regenerates other antioxidants such as
vitamin E (Brody, 1994). Vitamin C also plays a role in wound healing (Fox, 1989). A deficiency in vitamin C causes bruising, bleeding, dry skin and depression (Olson, 1999).

These are all symptoms of a deficiency-induced potentially fatal skin disease called scurvy. The symptoms are all related to diminished levels of collagen in bones, blood vessels and connective tissue. Over-consumption of vitamin C may cause a vitamin B₁₂ deficiency (Herbert & Jacob, 1974). It also acts as an antioxidant and protects carbohydrates, fats, proteins and nucleic acids (DNA and RNA) from damage induced by free radicals and other reactive species. (Bhagavan et. al, 2001; Cathcart et al., 1991).

**Assay methodology for vitamin C – General**

Many analytical methods have been reported for the determination of vitamin C (l-ascorbic acid) such as spectrophotometric, cyclic voltammetry (Güçlü, et al., 2005; Pisoschi, et al., 2008) as well as enzymatic methods (Akyilmaz, 1999). Before the development of high performance liquid chromatography (HPLC) as a method of analysis, one of the most commonly used methods was titration, which is based on the reduction of 2,6-dichlorophenolindophenol by ascorbic acid (Anon., 1999). It has since been modified (Matei et al., 2004) and is used in the determination of vitamin C not only in fruit, vegetables and other food products but also in pharmaceutical products (Hernandez et al., 2006). The volumetric techniques have limited use as they can only be applied to samples not containing other reducing agents (O’Connell, 2001).

With the development of HPLC, many HPLC based methods proved to be selective and specific to vitamin C determination (Iwase & Ono, 1998; Kall & Anderson, 1999; Oliviera & Watson, 2000). These methods also allow simultaneous determination of l-ascorbic (AA) and dehydro-ascorbic acid (DHAA) (Nisperos-Carriedo et al., 1992), and separation of ascorbic acid and its stereoisomer, isoascorbic acid (IAA) (Kall & Anderson, 1999). Russell (2012)
covers HPLC methods employed by various researchers and selected methods were summarised in Table 1.

**HPLC assay methodology for Vitamin C**

The extraction of vitamin C in various matrices requires procedures that limit or prevent its degradation as it is labile. Most extraction methods tend to use metaphosphoric acid as an extraction solution as it precipitates protein from the samples and thus preventing its interference with the results and degradation from enzymes and metal induced oxidation (Davey *et al.*, 2000; Eitenmiller, 2008). Based on a specific matrix, different concentrations of metaphosphoric acid combined with acetic acid, sulfuric acid, ethylenediaminetetra-acetic acid (EDTA), acetone or ethanol can be used at acidic pH to prevent factors that contribute to instability of vitamin C (Ball, 2006; Kall & Andersen, 1999; Nyyssönen, *et al.*, 2000). The recommended AOAC 967.22 method for extraction of vitamin C in most food matrices uses the metaphosphoric acid/glacial acetic acid extractant (Anon., 1993). More extraction protocols based on matrix type are listed in Table 1. The stability of vitamin C can further be increased by using low actinic glassware or bubbling inert gas through the extraction process.

Amongst the most reported HPLC methods, reversed-phase is the most commonly used by means of C_{18} stationary phase column with 5 µm particle size and polar mobile phase with diode array detector (DAD) or ultraviolet (UV) absorbance detection as indicated in Table 1. The advantage with diode array detection is that it combines absorbance detection and peak purity. The methods report absorbance wavelengths that range between 210 nm and 265 nm (Shafqat *et al.*, 2012; Tarrago-Trani *et al.*, 2012; Valls *et al.*, 2002). Electrochemical monitoring and fluorescence detection can also be utilised after chemical derivatisation to a fluorescent compound (Vanderslice & Higgs, 1984; Bognár & Daoood, 2000).
Iwase (2000) and Rizzolo (2002) reported HPLC methods with electrochemical detection as more selective and sensitive for vitamin C analysis in foodstuffs and biological fluids. Refractive Index detection has been reported by Doner (1981) to be non-selective, with low sensitivity and not suitable for accurate measurement of small amounts of L-dehydroascorbic acid and other oxidation products. The vitamer and its oxidised products are commonly eluted isocratically with varied flow rates, or with gradient elution to improve resolution of the peaks. Mass spectrometer (MS) detection of Vitamin C has been reported but is limited to multivitamin assays (Russell, 2012). However, Garrido Frenich et al. (2005) reported the HPLC analysis of vitamin C in tomatoes, mango and kiwi fruit with MS detection.
### Table 1

Water-soluble Vitamin Estimation by HPLC

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Analyte</th>
<th>Extraction</th>
<th>Column</th>
<th>Elution</th>
<th>Detection</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bread, sourdough</td>
<td>Thiamine</td>
<td>Incubation of freeze-dried samples with agitation at 37°C for 18 hours; centrifuge 14 00 rpm; filter 0.2 µm</td>
<td>Acquity UPLC HSS C-18 1.8 µm (2.1 X 150 mm)</td>
<td>A: Water + 0.1% formic acid and B: Acetonitrile + 0.1% formic acid gradient at 2.5 mL/min and 5 µL injection (0-3 min 100% A, 3-6.5 min 80% A and 20% B, 8.5-10 min 5% A and 95% B, 10-15 min 100% A)</td>
<td>LCT Premier™ XE ESI TOF MS (Waters)</td>
<td>Mihhalevski et al. (2013)</td>
</tr>
</tbody>
</table>
| Bread, white, wholemeal and multigrain | Folic Acid (FA) and 5-Methyl-THF (SMTHF) | Phosphate buffer (0.1 M, pH 6.1) with 2% (w/v) Na-ascorbate and 0.1% (w/v) mercapto-propanol. 

\[ ^{13}C_6 \text{ FA and } ^{13}C_5 \text{ SMTHF added to extraction buffer. Homogenised samples treated with } \alpha\text{-amylase to hydrolyse starch, followed by deconjugation with rat serum SPE and ultrafiltration used for sample cleanup and concentration} \] | Acquity UPLC HSS C-18 1.8 µm (2.1 X 150 mm) | A: 0.1% Formic acid in Milli-Q water and B: acetonitrile. 200 µL/min and 20 µL injection (0-0.2 min 99.5% A, 0.2-2.2 min 65% A and 35% B, 2.2-3.0 min 30% A and 70% B, 3.0-3.2 min 30% A and 70% B, recycled to initial conditions. Total run time 6 min | Thermo TSQ Quantum Access tandem quadrupole MS (Thermo-Scientific) operated in positive ion ESI mode | Chandra-Hloe et al. (2011) |
<table>
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<tr>
<th>Matrix</th>
<th>Analyte</th>
<th>Extraction</th>
<th>Column</th>
<th>Elution</th>
<th>Detection</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereal grains 2 (fortified)</td>
<td>Thiamine and its</td>
<td>4 g samples were milled and extracted with cold 4% aqueous trichloroacetic</td>
<td>C-18 connected column to NH₂</td>
<td>Gradient elution gave reversed elution order due to the two columns</td>
<td>Fluorescence detection</td>
<td>Bucholtz et al. (2012)</td>
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<td>phosphate esters</td>
<td>phosphate esters</td>
<td>acid with stirring. This centrifuged at 1500 g for 5 min and supernatant</td>
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<td>linked (according to Poel et al., 2009)</td>
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<td>filtered through fine glass wool (3 X). To 2 mL a 1 mL volume of 3%</td>
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<td>aqueous cyanogen bromide was added and the pH adjusted to 10 with 1 M NaOH.</td>
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<td>For thiochrome reaction, it was left in dark for 25 min on ice, pH</td>
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<td>adjusted to 7, centrifuged at 16000 g for 2 min and subjected to</td>
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<td>solid phase extraction (SPE).</td>
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<tr>
<td>Corn extrudates</td>
<td>Thiamine</td>
<td>50 mL 0.1 N H₂SO₄ added to 1 g extrudate, boiled for 30 min with vortexing,</td>
<td>μ-Bondapak column (3.9 X 300</td>
<td>Injection volume = 20 μL. Flow rate = 1 mL/min. Temperature = 30°C.</td>
<td>Fluorescence with excitation</td>
<td>Boyaci et al. (2012)</td>
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<tr>
<td>(fortified)</td>
<td>Riboflavin</td>
<td>cooled, takadiastase in 2.5 M Na acetate added, incubated (4 h in 37°C</td>
<td>mm, 10 μm)</td>
<td>Isocratic elution with methanol: water = 30:70 v/v</td>
<td>wavelength = 365 nm and</td>
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<td>waterbath). Diluted to 100 mL with water, filtered through Whatman 541</td>
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<td>emission wavelength = 435 nm</td>
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<td>paper. Riboflavin: Filtered through 0.2 μm nylon filter before HPLC.</td>
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<td>for Thiamine and 450 nm and</td>
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<td>Thiamine: 5 mL filtrate plus 1 mL of 0.18 M potassium ferricyanide in</td>
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<td>510 nm for riboflavin</td>
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<td>3.5 M NaOH. Filtered through 0.2 μm nylon filter before HPLC</td>
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<td>Matrix</td>
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<td>Extraction</td>
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<td>Elution</td>
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<tr>
<td>Complex cereal foods</td>
<td>Thiamine, Riboflavin</td>
<td>15 g cereal in 90 mL 0.1 N H2SO4, adjusted to pH 4.5 with 2.5 M sodium acetate which was added papain, diastase and α-amylase, incubated 37°C overnight, made to 100 mL with water, filtered through Whatman 40 paper and through 0.45 μm polyvinylidene fluoride membrane</td>
<td>Purospher® STAR RP-18e (250 X 4 mm, 5 μm particle size)</td>
<td>Eluent: 12.5 mM sodium acetate in methanol / water (25/75) + 2.4 mM sodium heptanesulphonate. Flow rate = 0.9 mL/min</td>
<td>UV-Vis at 268 nm</td>
<td>San José et al. (2012)</td>
</tr>
</tbody>
</table>
| Cooked meals, with cereals, vegetables and legumes. Milk and fruit | Folates (five vitamins) | Samples homogenised and freeze-dried. To 0.5-1 g 10 mL HEPES-CHES buffer, pH 7.85, containing 2% Na-ascorbate and 0.01 M 2-mercaptoethanol. Placed in waterbath for 10 min at 100°C, cooled. Trienzyme extraction with a protease, α-amylase and human plasma (various pH and times, all at 37°C), followed by heating for 10 min at 100°C, cooling and centrifugation. Purification achieved on SAX SPE cartridges. Final eluate reduced to dryness in vacuum centrifuge and resuspended in 100 μL 0.1% formic acid | C18 RP column (Zorbax Eclipse, 150 X 2.1 mm, 5 μm particle size) | A: Aqueous formic acid (0.1%), B: Acetonitrile
Elution:
0-4 min: Isocratic at 7% B
4-16 min: Linear gradient: 30% B
16-10 min: Linear gradient: 7% B
Flow rate: 0.2 mL/min | Quantification achieved using selected monitoring (SR) MS. System: HPLC coupled to ion trap MS via an ESI interface. MS operated in positive ion mode. Quantification of folates using 13C-labelled I.S. | Vishnumohan et al. (2013) |
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Analyte</th>
<th>Extraction</th>
<th>Column</th>
<th>Elution</th>
<th>Detection</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td>Dates</td>
<td>Vitamin B₁</td>
<td>Samples homogenised, 25 mL buffer (Na-hexane sulphonate + KH₂PO₄ + DI water + trimethylamine) added, heated in shaking waterbath for 40 min at 70°C, cooled, filtered and diluted</td>
<td>Waters Symmetry C₁₈ (250 X 4.6 mm, 5 µm particle size)</td>
<td>A: 940 mL DI water + 5 mL triethylyamine + H₃PO₄ to pH 3.0; B: Methanol isocratic elution with buffer:methanol at 96:4. Flow rate: 1 mL/min</td>
<td>UV, using two channels simultaneously at 210 nm, a bandwidth of 5 nm and another wavelength of 280 nm</td>
<td>Asiam et al. (2013)</td>
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<td>Vitamin B₂</td>
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<td>Vitamin B₃</td>
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<td>Vitamin B₆</td>
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<td>Vitamin B₂</td>
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<td>Vitamin B₁₂</td>
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<td>Dried bee pollen</td>
<td>Thiamine</td>
<td>Simultaneous extraction of 5 g pollen with 50 mL 0.1 M HCl in boiling bath for 30 min. pH adjusted to 4.6 with 2.5 M sodium acetate followed by adding 0.5 g fungal diastase, incubating 2 h at 42°C in water bath, cooling, diluted to 100 mL with water, homogenizing and filtering through paper and 0.45 µm prior to injection</td>
<td>C₁₈ reversed-phase column (250 mm X 4.6 mm, 5 µm particle size) with pre-column (5 µm/10 mm X 4.6 mm)</td>
<td>Thiamine and riboflavin: Flow rate = 1 mL/min, injection volume = 20 µL. Mobile phase: 10 mM phosphate buffer (pH 7.2) and dimethylformamide (85:15). Niacin vitamers: Flow rate = 1.5 mL/min, injection volume = 20 µL. Mobile phase: phosphate / copper sulphate / hydrogen peroxide buffer complex with UV treatment in the loop. Vitamin B₆ vitamers: Flow rate = 0.6 mL/min, injection volume = 20 µL. Mobile phase: 39 mM phosphate buffer (pH 2.5) and ion pair (PIC 7) and acetonitrile (96:4)</td>
<td>Fluorescence detection (excitation/emission) for thiamine (368/440 nm), riboflavin (450/530 nm), niacin and niacinamide (332/380 nm) and B₆ vitamers (296/390 nm)</td>
<td>De Arruda et al. (2013)</td>
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<tr>
<td></td>
<td>Riboflavin</td>
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<td>Nicotinic acid</td>
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<td></td>
<td>Nicotinamide</td>
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<td>Dry-cured sausages</td>
<td>Thiamine</td>
<td>Frozen samples milled with bowl cutter, 1 g; incubated for 30 min at 100°C in 9 mL water and this treated with 2.5 M Na-acetate and 10% taka-diatase overnight at 37°C, centrifuged at 4°C at 4 500 g for 5 min, supernatant diluted with water, pH adjusted to 6.0 with Na-acetate. Loaded onto pre-treated Owasis® WCX cartridge, thiamine washed off sequentially, evaporated to dryness under nitrogen at 25°C, re-dissolved in mobile phase A and filtered through 0.45 µm PTFE filter</td>
<td>Luna™ HILIC (150 mm X 3.0 mm i.d., 3 µm particle size) column at ambient temperature</td>
<td>Gradient elution at 0.5 mL/min using (A): acetonitrile:50mM ammonium acetate pH 5.8 (90:10 v/v) and (B): acetonitrile:10mM ammonium acetate pH 5.8 (50:50 v/v) by varying B from 0 to 10% in 8 min. Injection volume = 40 µL</td>
<td>UV detection at 270 nm and spectral trace from 220 nm to 420 nm</td>
<td>Gratacos-Cubarši et al. (2011)</td>
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<td>Feed, pre-mixes and supplements</td>
<td>Thiamine</td>
<td>Extraction with 0.01 M HCL at 40°C for 10 min, centrifuged at 8 000 rpm for 10 min followed by SPE using various adsorberts followed by filtration through a microfilter</td>
<td>Supelco C18 column (250 X 4.6 mm); 5 µm particle size</td>
<td>Gradient elution at 1 mL/min using (A) 11mM phosphate pH 2.5 buffer containing 4% acetonitrile and (B) acetonitrile (permutations available in original article).</td>
<td>UV at 260 nm and 280 nm</td>
<td>Rudenko &amp; Kartsova (2010)</td>
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<td>Riboflavin</td>
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<td>Nicotinic acid</td>
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<td>nicotinamide</td>
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<td>Fennel</td>
<td>Ascorbic acid</td>
<td>Homogenise with 0.2% potassium metabisulphite. Dilute with 20 mM phosphate buffer. Keep in the dark for 5 min and filter (0.2 µm). Flush with nitrogen and protect from light</td>
<td>Precolumn: Aqua C18 (Phenomenex), Analytical: Aqua C18 (250 X 4.6 mm, 5 µm, Phenomenex)</td>
<td>Isocratic: 20 mM phosphate buffer, pH 2.14 at 1 mL/min</td>
<td>UV absorbance: 240 nm</td>
<td>Galgano et al. (2002)</td>
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<td>Fish</td>
<td>Thiamine, Riboflavin, Niacin, Pyridoxine</td>
<td>Weighed into 0.1 M HCl and autoclaved. pH adjusted to 6.5 and 4.5, made to volume with water and filtered through paper. Any turbidity removed by centrifugation at 6,000 rpm for 10 min and then passed through 0.45 μm filter.</td>
<td>C18 Omnisphere 5 (250 X 4.6 mm)</td>
<td>Flow rate = 1 mL/min. Injection volume = 20 μL. Isocratic elution with phosphate buffer: methanol = 100:36. Run time = 22 min</td>
<td>UV at 254 nm</td>
<td>Ersoy &amp; Özeren (2009)</td>
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<td>Fish</td>
<td>Thiamine, Riboflavin, Niacin, Pyridoxine</td>
<td>30g fish was extracted with 60 mL extraction solution (10 mL acetic acid and 50 mL acetonitrile in 1 L water), boiled in an ultrasonic bath at 70°C for 30 min and cooled. This was filtered through Whatman paper and a 0.45 μm filter.</td>
<td>Lichospher 60 RP-select B (5 μm) LiChroCART 250-4 HPLC cartridge</td>
<td>Flow rate = 1 mL/min. Injection volume = 20 μL. Isocratic elution with buffer containing phosphate, hexanesulfonic acid and trimethylamine at pH 2.4–2.5</td>
<td>UV detection at as follows: 0–6.5 min. at 264 nm for niacin, 6.5–20 min at 6.4–20 min for riboflavin and pyridoxine and 20–35 min for thiamine</td>
<td>Erkan et al. (2010)</td>
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<td>Food Supplement (athlete)</td>
<td>Total vitamin C as ascorbic acid</td>
<td>Mix sample with 0.2% phosphoric acid containing 20µM L-methionine at 5°C; filter (0.45 μm). Protect from light.</td>
<td>Vydac 201-HS C18, 10 µm, 250 X 4.6 mm</td>
<td>Isocratic: 0.2% phosphoric acid, pH 2.1, 0.4 mL/min. 5μl injection volume.</td>
<td>Electrochemistry: glassy carbon working electrode, +400 mV vs. Ag/AgCl reference electrode</td>
<td>Iwase (2000)</td>
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<tr>
<td>Food products and multivitamin-</td>
<td>Vitamin B₁₂</td>
<td>50 mM Na-acetate buffer, pH 4.0 + 1% NaCN + α-amylase + pepsin; digest for 3 h at 37°C; cool, adjust pH to 4.8; Heat for 35 min at 100°C; Cool, dilute, centrifuge and filter (0.45 μm membrane). Ginsenoside used as I.S.</td>
<td>Xierra MS C18 column (150 mm X 3.9 mm), 5 μm</td>
<td>Gradient, water/acetonitrile. Flow rate 1 mL/min</td>
<td>ESI-MS, positive ion mode</td>
<td>Luo et al. (2006)</td>
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<td>mineral tablets</td>
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<td>Fruit juices (apple), fortified</td>
<td>Pyridoxamine</td>
<td>Variable based on matrix and analytes, in general: acid digestion and enzymatic hydrolysis (papain and diastase), followed by enzyme inactivation and filtration into amber glass. For cyanocobalamin extraction, the acid digestion was replaced with autoclaving after addition of a NaCN-containing acetate buffer at pH 4.0</td>
<td>Supelco C18 column (250 X 4.6 mm); 5 μm particle size</td>
<td>Isocratic elution with methanol-phosphate buffer (10:90) and 0.018 M trimethylamine at pH 3.55. Flow rate 1 mL/min</td>
<td>Electrochemical (EC) detector Coulochem II with dual analytical cell and guard cell connected in-line before the injection port. Detector response set to give full scale at 1 μA and 50 μA current output received from analytical cell. Peak area of the EC signal at the porous graphite electrode used for quantitative analysis</td>
<td>Lebiedzińska et al. (2007)</td>
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<tr>
<td>fruit juices, salmon, oysters,</td>
<td>Pyridoxal</td>
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<td>scallop</td>
<td>Pyrodoxin</td>
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<td>Thiamine</td>
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<td>Cyanocobalamin</td>
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<tr>
<td>Fruit drinks, fortified fruit drinks, juices and cereal products</td>
<td>Folic acid (vitamin B9)</td>
<td>Phosphate buffer (pH 6.8) + sample; homogenized; 10 min at 100°C, cooling, pH adjusted to 4.9; tri-enzyme treatment (Hog Kidney conjugase); heating 5 min at 100°C to inactivate enzyme; cooling, centrifugation, filtration (Whatman no. 1), storage at -20°C; filtration through 0.22 μm pore size prior to HPLC. Protection against light; subdued light</td>
<td>LC 18 column (250 mm X 4.6 mm), 5 μm, Supelco, Inc.</td>
<td>40 mM sodium phosphate dibasic heptahydrate and 8% acetic anhydride (v/v), adjusted to pH 5.5 with 85% phosphoric acid. Column temperature: 25°C Flow rate 0.9 mL/min</td>
<td>Electrochemical detector Coulochem II with dual analytical cell and guard cell connected in-line before the injection port. Detector response set to give full scale at 1 μA and 50 μA current output received from analytical cell. For obtaining optimum detection, electrode potential set at 0.85 V (guard cell), 0.20 V (electrode E1) and 0.80 V (electrode E2)</td>
<td>Lebiedzińska et al. (2008)</td>
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<td>Fruit (fresh tomatoes)</td>
<td>Ascorbic acid (AA), pentanophenone (internal standard)</td>
<td>Homogenize with 6% HPCN (H2O and MeOH), mix aliquot of slurry with MeOH, add 0.05% pentanophenone (internal standard), centrifuge, dilute with MeOH containing 0.0015 M pyrogallol</td>
<td>Precolumn: Optimal ODS-H C18 (20 X 4.6 mm, 5 μm, Capital HPLC). Analytical: Optimal ODS-H C18 (250 X 4.6 mm, 5 μm, Capital HPLC) at 25°C.</td>
<td>MeOH/H2O/MeCN (60% 40% 4) containing 0.5 mM tridecyl ammonium formate, adjusted to pH 4.25</td>
<td>UV absorbance: 247 nm</td>
<td>Russell (1986)</td>
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<td>Fruit, dog rose</td>
<td>Ascorbic acid</td>
<td>Freeze in liquid nitrogen and homogenise in 5% MPA, centrifuge at 4°C. Dilute with water and filter (0.45 μm)</td>
<td>Precolumn: Inertsil ODS-3 C18 (10 X 3 mm, 5 μm). Analytical: Inertsil ODS-3 C18 (150 X 3mm, 5 μm; GL Sciences)</td>
<td>Isocratic: Methanol + 0.25% MPA at 1 mL/min. Injection volume: 20 μl</td>
<td>UV absorbance: 245 nm</td>
<td>Nojavan et al. (2008)</td>
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<td>Fruit and vegetables</td>
<td>Ascorbic acid</td>
<td>Homogenise sample in methanol + 3% MPA containing 8% acetic acid (1 + 5, v/v), filter, make to volume with 0.1% acetic acid. Protect from light during extraction Injection volume = 10 µL</td>
<td>Analytical column: Symmetry C18 (75 X 4.6 mm, 3.5 µm, Waters) + Atlantis dC18 (150 X 2.0 mm, 5 µm, Waters) in series; 30°C</td>
<td>Isocratic: 0.005% acetic acid in methanol + 0.05% acetic acid (70 + 30, v/v) Flow rate: 0.3 mL/min</td>
<td>Mass spectrometry: single quadrupole ESI in negative ion mode</td>
<td>Garrido Frenich et al. (2005)</td>
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<td>Fortified juices and cereals</td>
<td>Folic acid</td>
<td>Juice centrifuged, filtered through nylon membrane filter. Cereals ground in mortar and pestle, solution of DI water, NH₄-formate, methotrexate, ascorbate and NH₃ added. After sonication, centrifugation and membrane filtration</td>
<td>HPLC-UV: Diamond Hydride™ column (75 X 4.6 mm, 4.2 µm particle size). LC-MS: Diamond Hydride™ column (150 X 2.1 mm, 4.0 µm particle size)</td>
<td>A: DI water + 10 mM NH₄-formate B: 90:10 acetonitrile: DI water + 10 mM NH₄-formate Gradient elution: 0-5 min: 90% B 5-9 min: 50% B 9-10 min: 100% B. Flow rate: 1 mL/min</td>
<td>HPLC-UV: UV at 384 nm, LC-MS: MSD-TOF with dual sprayer ESI, operated in negative ion mode</td>
<td>Young et al. (2011)</td>
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<td>Fruit and vegetables</td>
<td>Thiamine</td>
<td>Material cut into small pieces and heat extracted in 0.1 M HCl and filtered through 0.45 µm filter</td>
<td>Supelco Discovery C18 column (25 cm X 0.45 internal diameter).</td>
<td>Isocratic elution with 50 nM phosphate and methanol (70:30) at 1 mL flow rate with 10 µL injection volume</td>
<td>UV at 254 nm</td>
<td>Ismail et al. (2013)</td>
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<td>Fruit juice, infant formula,</td>
<td>Riboflavin</td>
<td>Fruit juice: Dilute with water, add 1 mg DTT. Hold for more than 2 h, filter (0.45 µm). Fruit and processed food: Homogenise with water add 1 mg DTT, filter after 2 h. Add 5%TCA to protein containing samples filter or centrifuge. Filter again after 2 h</td>
<td>polymeric silica-based C18 (250 X 4 mm, 5 µm)</td>
<td>Isocratic: 0.1% DTT in 0.5% potassium phosphate buffer, pH 2.5. 0.5–1.0 mL/min</td>
<td>UV at 254 nm</td>
<td>Brause et al. (2003)</td>
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<td>mango, kiwi fruit</td>
<td>Niacinamide</td>
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<td>Pyridoxine</td>
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<td>Folic acid</td>
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<td>Fruit juices, packed</td>
<td>Ascorbic acid</td>
<td>5 mL juice + 5 mL mobile phase (20 mM K$_3$PO$_4$ / MeOH). Centrifuge at 5000 rpm for 5 min, filter through 0.45 μm PVDF Millipore filters</td>
<td>ODS-3 C$_{18}$ column</td>
<td>Flow rate at 1 mL/min. Mobile phase: 20% MeOH + 80% buffer</td>
<td>UV-Vis detector (Hitachi), wavelength at 240 nm.</td>
<td>Shafqat et al. (2012)</td>
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<td>Green, leafy vegetables</td>
<td>Thiamine, Riboflavin, Nicotinamide, Pantothenic acid, Pyridoxine, Folic acid, Ascorbic acid (and fat-soluble vitamins)</td>
<td>0.25 g freeze-dried and milled sample was extracted with 16 mL 10 mM ammonium acetate/methanol (50:50 v/v) containing 0.1% BHT, shaken and then sonicated for 15 min. The centrifuged at 14 000 g for 15 min and supernatant passed through a 0.45 μm nylon filter. 1 mL supernatant was concentrated in a nitrogen stream and then injected to determine water-soluble vitamins</td>
<td>ACE-100 C$_{18}$ column (100 X 2.1 mm, 3 μm particle size)</td>
<td>HPLC-MS/MS (via ESI interface). Injection volume = 10 μL and flow rate was 0.2 mL/min. Gradient extraction using 3 buffers viz. 10mM ammonium acetate (pH 4.5), methanol containing 0.1% acetic acid and methanol with 0.3% acetic acid</td>
<td>DAD recording spectra from 200 to 680 nm</td>
<td>Santos et al. (2012)</td>
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<td>Green vegetables</td>
<td>Folates</td>
<td>To 1-2 g ground sample, 10 mL MES buffer added. I.S. mix contained four deuterated vitamers. Samples boiled for 10 min at 100°C, cooled, Chicken pancreas + Rat serum conjugase preparations added and incubated overnight at 37°C. Deconjugated samples heated for 10 min at 100°C, cooled and centrifuged. Purification performed on SAX SPE cartridges</td>
<td>Phenomenex HyperClone BDS C18 column (150 X 3.2 mm, 3 µm particle size)</td>
<td>A: 10 mL/L acetic acid; B: Acetonitrile, acidified with 10 mL/L acetic acid. Gradient elution: 0-2 min: 2% B; 98% A 2-7 min: 10% B; 90% A 7-10 min: 10% B; 90% A 10-18 min: 15% B; 85% A 18-20 min: 100% B 20-21 min: 100% B 22-23 min: 2% B; 98% A Flow rate: 0.2 mL/min</td>
<td>HPLC coupled with triple quadrupole MS, operated in positive ESI mode using MRM</td>
<td>Delchier et al. (2013)</td>
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<td>Green tea extracts with folate</td>
<td>Folate as 5-CH₃THF</td>
<td>Extracts + folate added to 0.04 M Britton-Robinson buffer at pH 5.5 in water</td>
<td>Agilent Zorbax SB-C18 (150 X 4.8 mm, 1.8 µm particle size)</td>
<td>Linear gradient elution (details not published), A: 0.2% formic acid in water; B: 0.1% formic acid in acetonitrile Flow rate 1 mL/min</td>
<td>HPLC-DAD at 230 nm, LC-MS Single quadrupole MS with API-ES source (in positive ion mode)</td>
<td>Rozoy et al. (2013)</td>
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<td>Honey</td>
<td>Ascorbic acid</td>
<td>Various – see original article</td>
<td>µBondapack C18 10 µm(10 X 3.9 mm), one of which was saturated in G TAB (hexadecyl trimethyl-ammonium bromide) Second version with surfactant</td>
<td>Many permutations of gradient elution</td>
<td>Programmable UV/Vis detector and fluorescence detector</td>
<td>León-Ruiz. Et al. (2013)</td>
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<td>Honey</td>
<td>Riboflavin</td>
<td>10 mL honey homogenized in 10 mL water to which added 1 mL 2 M NaOH and 12.5 mL phosphate buffer (pH 5.5). Filtered through 0.45 µm PVDF membrane and stored.</td>
<td>Reversed phase column</td>
<td>Multi-step gradient elution using TFA (0.025%) and acetonitrile</td>
<td>UV detection: 254 nm for nicotinic acid and 210 nm for the rest</td>
<td>Ciulu et al. (2011)</td>
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<td>Nicotinic acid</td>
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<td>Pantothenic acid</td>
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<td>Ascorbic acid</td>
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<td>Malt</td>
<td>Thiamine</td>
<td>Milled samples (0.5 g) were vortexed in 5 mL 0.5 M trichloroacetic acid, agitated for 15 min, centrifuged at 2 000g and the supernatant removed to a clean tube. 1 mL of 10 mM phosphate was added and the pH adjusted to 6.5 – 6.6 with 2 M KOH, diluted to 10 mL with buffer and filtered through 0.45 µm regenerated cellulose filter. For Riboflavin, samples were injected at this stage. For thiamine, 300 µL alkaline potassium ferricyanide was added to 1 mL degassed sample, vortexed for 15 seconds and neutralized with 600 µL 1.33 M phosphoric acid and filtered through 0.45 µm regenerated cellulose filter</td>
<td>Varian Pursuit C18 (250 mm X 4.6 mm) with C18 guard cartridge (4 X 30 mm)</td>
<td>Gradient elution with A: 10 mM phosphate, pH 6.5; B: methanol. Gradient programme: 0–0.5 min with A:B = 95:5, 0.5–10 min at 63:35, 10–15 min at 63:35, 15–16 at 95:5</td>
<td>Fluorescence detection with excitation emission being 360/425 nm for thiamine and 270/516 nm for riboflavin</td>
<td>Hucker et al. (2012)</td>
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<td></td>
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<td>Meat (cooked sausages)</td>
<td>Ascorbic acid</td>
<td>Homogenise with 20 mM monosodium L-glutamate, pH 2.1; filter through 0.45 μm</td>
<td>Spherisorb NH2 (250 X 4.0 mm, 5 μm (Teknokroma) at 35°C</td>
<td>Isocratic: Acetonitrile + 20 mM phosphate buffer, pH 3.6 (60+40, v/v) at 1 mL/min</td>
<td>UV absorbance, 248 nm</td>
<td>Valls et al., (2002)</td>
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<td>Meat products (salami, sausages, bacon, ham)</td>
<td>Vitamin B₁₂</td>
<td>To 30 g sample 50 mL mM Na-acetate buffer (pH 4.0), 2 g pepsin, 0.5 g α-amylase and 2 mL KCN (1%) added. Homogenisation with Polytron, incubated for 30 min at 37°C, then heated for further 30 min at 100°C. After cooling, centrifugation and filtration, sample loaded onto immunoaffinity column. Vitamin B₁₂ eluted with methanol. Eluate is concentrated to dryness at 60°C, then reconstituted to 300 μL with mobile phase</td>
<td>Nucleosil RP C18 HD 100 column (250 X 4.6 mm, 5 μm particle size)</td>
<td>Water/acetonitrile (87:13) with 0.025% TFA (isocratic elution) Flow rate: 1.2 mL/min</td>
<td>UV at 361 nm</td>
<td>Guggisberg et al. (2012)</td>
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<tr>
<td>Milk, human</td>
<td>Total vitamin C as AA, DHAA by difference</td>
<td>Total vitamin C: Reduce DHAA to AA with 100 mM DTT, keep in the dark for 15 min, add 0.56% MPA, centrifuge at 10°C. Ascorbic acid: Mix 0.56% MPA, centrifuge at 10°C. Injection volume = 50 μL</td>
<td>Precolumn: Tracer C18, 5 μm (Tracer Analytica). Analytical: Tracer Spherisorb ODS C18 (250 x 4.6 mm, 5 μm, Tracer Analytica), 25°C</td>
<td>Isocratic: Methanol + 0.1% acetic acid (5 + 95, v/v) 0.7 mL/min</td>
<td>UV at 254 nm</td>
<td>Romeu-Nadal et al. (2006)</td>
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<tr>
<td>Milk</td>
<td>Thiamine, Riboflavin, Niacin, Pyridoxine, Folic acid</td>
<td>Vitamin extraction by adding 0.1 M HCl and digesting while mixing in boiling batch, cooling, adjusting pH to 4.0 – 4.5 with 2.5 M sodium acetate. Digestion with 10% taka-diastase for 3 hrs at 45 – 50°C, mixing every 30 min. Cooled, filtered, diluted and then passed through 0.2 µm filter.</td>
<td>Zorbax SB-C8 (4.6 X 150 mm, 5 nm) column) at ambient temperature</td>
<td>Gradient elution at 1.2 mL/min using (A) 50 mM phosphate buffer in water and (B) 50 mM phosphate buffer in methanol (10:90). Started run on A and increased B from zero to 70% within 18 min</td>
<td>UV at 245 nm</td>
<td>Khair-un-Nisa et al. (2010)</td>
</tr>
<tr>
<td>Milk powder SRM 1846, milk powder, infant formula, breakfast cereal, orange juice, fortified soup</td>
<td>Total vitamin C as ascorbic acid</td>
<td>Sample with no starch: Mix with TCEP solution. Dilute with 1%TCA, filter and dilute with mobile phase. Samples with starch: Mix with 10mg taka-diastase, incubate (42°C) for 30 min, add 1%TCA and filter. Dilute with mobile phase</td>
<td>Analytical column: LiChrospher RP-18 (250 X 4.6 mm, 5 µm)</td>
<td>Isocratic: Acetonitrile + 25 mM acetate-phosphate buffer, pH 5.4, containing 10.17 mM decylamine and 0.17 mM TCEP at 1 mL/min</td>
<td>UV at 265 nm</td>
<td>Fontannaz et al. (2006)</td>
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**Ref.**
Khair-un-Nisa et al. (2010)
Fontannaz et al. (2006)
<table>
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<tr>
<td>Milk-based infant formula (powdered), infant cereals with milk and fruit, infant cereals without milk, milk-based infant formula, hypoallergenic infant formula, milk powder SRM 1846, meat SRM 1546, milk powder CRM 421</td>
<td>Total and free vitamin B&lt;sub&gt;12&lt;/sub&gt; as CNCbl</td>
<td>Free vitamin B&lt;sub&gt;12&lt;/sub&gt;: 50 mM Na-acetate buffer, pH 4.0 + 0.016% NaCN + α-amylase; digest for 30 min at 37°C; cool, adjust pH to 4.8; reflux under N&lt;sub&gt;2&lt;/sub&gt; for 35 min at 100°C to convert all vitamers to CNCbl; Cool, dilute and filter.</td>
<td>Ace 3 AQ C&lt;sub&gt;18&lt;/sub&gt; (150 mm X 3.9 mm), 3 µm</td>
<td>A: Acetonitrile B: 0.025% aqueous TFA 0-3.5 min: 0% A + 100% B (isocratic) 3.5-11 min: 25% A + 75% B (linear gradient) 11-19 min: 35% A + 65% B (linear gradient) 19-20 min: 10% A + 90% B (linear gradient) 20-26 min: 0% A + 100% B (linear gradient) 26-30 min: 0% A + 100% B (isocratic) Flow rate 0.25 mL/min</td>
<td>UV absorbance: 361 nm (DAD)</td>
<td>Heudi &lt;i&gt;et al.&lt;/i&gt; (2006)</td>
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<tr>
<td>Milk- and soy-based infant formula, cereals, cocoa beverages</td>
<td>Total and free vitamin B₁₂ as CNCbl</td>
<td>All extracts (Total and free vitamin B₁₂): 1% NaCN + α-amylase; incubate for 30 min at 40 ± 5°C; add 20 mM Na-acetate, pH 4; hold for 35 min at 100°C to convert all vitamins to CNCbl; Cool, dilute and filter; clean up filtrate on commercial B₁₂ immunoaffinity column, elute CNCbl with methanol; evaporate eluate under N₂; reconstitute residue in 0.025% TFA in acetonitrile + 0.025% aqueous TFA (10 + 90 v/v) Work under subdued light in low actinic glass ware</td>
<td>Nucleosil 100-3 C₁₈ HD (150 mm X 3.9 mm), 3 μm, Machery-Nagel or Ace 3 AQ C₁₈ (150 mm X 3.9 mm), 3 μm, Ace</td>
<td>A: 0.025% TFA in acetonitrile</td>
<td>UV absorbance: 361 nm (DAD)</td>
<td>Campos-Giménez et al. (2008)</td>
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<tr>
<td>Noodles</td>
<td>Riboflavin</td>
<td>6 g noodles homogenized in 30 mL 1.1 M H₂SO₄ for 2 min and autoclaved for 15 min at 121°C and then cooled and pH adjusted to 4.5 using sodium acetate. Riboflavin was extracted using Clara-diastase for 90 min at 45°C acidified using H₂SO₄ made to volume, filtered through paper and then through 1.2 μm membrane</td>
<td>Spherisorb ODS 2 C₁₈ Column (250 X 4.6 mm, 0.5 μm) with matching guard cartridge</td>
<td>B: 0.025% aqueous TFA 0 min: 10% A + 90% B (isocratic) 0-0.5 min: 10% A + 90% B (isocratic) 0.5-4 min: 25% A + 75% B (linear gradient) 4-5 min: 90% A + 10% B (linear gradient) 5-9 min: 90% A + 10% B (isocratic) 9-11 min: 10% A + 90% B (linear gradient) 11-16 min: 10% A + 90% B (isocratic)</td>
<td>Methanol-water (15:85) with water containing 1-hexsulphonic acid (0.005 M), glacial acetic acid (2.4%) and triethylamine (0.5%). Temperature = 30°C. Flow rate = 1 mL/min. Injection volume = 25 μL</td>
<td>UV detector at 268 nm and 35 min run time</td>
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<td>Rice</td>
<td>Folic acid (FA)</td>
<td>50 mM phosphate buffer + 1% ascorbic acid + 0.5% dithiothreitol, pH + IS. Added to 1 g rice, homogenised, boiled, cooled. 1.5 mL aliquot + 10 µL α-amylase; 10 min at RT. 150 µL protease. Incubation at 37°C for 1 h; 10 min boiling to inactivate; 100 µL rat serum, incubated at 37°C for 2 h. Boiling to inactivate enzyme, centrifugation and ultrafiltration (12 000x g for 30 min on 5 kDa Millipore filter). Subdued light throughout</td>
<td>Polaris 3 µm C18-A column (150 X 4.6 mm)</td>
<td>A: (0.1% formic acid in water); B: (0.1% formic acid in Acetonitrile (acetonitrile). 0-2.5 min: 95% A (isocratic); 2.5-3.5 min 85% A, 15% B; 3.5-5.5 min 75% A, 25% B; 5.5-10 min 100% B</td>
<td>Flow rate 0.8 mL/min</td>
<td>Applied Biosystems API 4000 tandem quadrupole MS operated in positive ion ESI mode. Detection carried out in MRM mode</td>
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<tr>
<td>Rice bran powder</td>
<td>Thiamine</td>
<td>Extraction heated ultrasonic system in water and then passed through 0.45 µm filter</td>
<td>Reversed phase C18 column (YMC, 250 mm X 10 mm)</td>
<td>Flow rate = 1 mL/min. Injection volume = 20 µL. Gradient elution using A: 99.8% methanol and B: 0.2% acetic acid in water. Ratio change from 0:100 to 25:75 in 20 min with flow rate change from 1 to 0.7 mL/min at 8 min</td>
<td>UV at 260nm.</td>
<td>Chen et al. (2011)</td>
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<tr>
<td>Tea, nutritional supplement, dried Lycium barbarum fruit</td>
<td>Total vitamin C as AA in the presence of related compounds</td>
<td>Dried fruit: Homogenize fruit with 200 mg/L DTT in acetonitrile + water (30 + 70, v/v) to reduce DHAA to AA; centrifuge; dilute supernatant with 200 mg/L DTT in acetonitrile + 66.7 mM ammonium acetate (85 + 15, v/v); Other samples: Dilute with 200 mg/L DTT in acetonitrile + 66.7 mM ammonium acetate (85 + 15, v/v) to reduce DHAA to AA; Keep samples at ≤4°C during extraction. Autosampler at 4°C Injection volume = 10 μL</td>
<td>Analytical: Inertsil Diol (250 X 4.6 mm, 5 μm; GL Sciences); 40°C</td>
<td>Isocratic: Acetonitrile + 66.7 mM ammonium acetate (85 + 15, v/v); Flow rate = 0.7 mL/min</td>
<td>UV absorbance: 260 nm External standardization Linearity: 1–50 μg/mL AA (r = 0.9996) Precision: Intra-day CV = 1.0–2.8% for AA in standards and control samples (n = 6); inter-day CV = 1.0–2.0% for AA in standards and control samples (n = 3) Accuracy: 92% AA recovery from standard solutions and control samples LoD = 300 ng/mL AA</td>
<td>Tai &amp; Gohda (2007)</td>
</tr>
<tr>
<td>Various Matrices (e.g. fruits and vegetables, dry foods etc.)</td>
<td>Ascorbic acid</td>
<td>2 g sample + 8 mL extraction buffer (5% MPA/1 mM EDTA/5 mM TCEP, pH 1.8) mix and centrifuge. Supernatant filtered through 0.45 μm PVDF membrane</td>
<td>Phenomenex Synergi 4 μm Hydro-RP (250 X 4.6 mm)</td>
<td>Mobile Phase: 0.05% (w/v) aqueous formic acid or 0.02% aqueous ortho-phosphoric acid (w/v); Flow rate at 0.4 mL/min, 0.7 mL/min or 1 mL/min</td>
<td>DAD (Perkin Elmer), wavelength at 254 nm</td>
<td>Tarrago-Trani et al. (2012)</td>
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<tr>
<td>Wide range of food matrices where polyphenols may or may not occur</td>
<td>Thiamine</td>
<td>Various – see original article</td>
<td>L-column ODS (250 X 4.6 mm i.d., 5 μm particle size). Temperature = 40°C</td>
<td>Injection volume = 20 μL. Flow rate = 1 mL/min. Isocratic elution for post-column derivatization. Mobile phase = [0.01 M sodium phosphate, 0.15 M NaClO₄ buffer pH 2.2]-methanol = 95:5 v/v. Reaction reagent = 0.91 M K₃Fe(CN)₆-3.75 M NaOH</td>
<td>Fluorescence detection excitation wavelength = 375 nm and emission wavelength = 440nm</td>
<td>Yashida et al. (2012)</td>
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<td>Non-food matrices</td>
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<td>Ampoules</td>
<td>Thiamine</td>
<td>Ampoule contents and diluted to 100 mL with 0.1 mL 0.1 M HCl, shaken for 10 min and diluted to range</td>
<td>UPLC: Acuity UPLC® BEH C18 column (50 X 2.1 mm i.d., 1.7 μm particle size)</td>
<td>Gradient elution with methanol and HCl at 0.6 mL/min</td>
<td>UV detection at 220 nm and data treatment</td>
<td>Dinç (2012)</td>
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<td></td>
<td>Pyridoxine</td>
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<td>Lidocaine</td>
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<td>Human serum</td>
<td>Homocysteine</td>
<td>Not included</td>
<td>LiChrocart C18 cartridge (150 mm X 4.6 mm internal diameter) with Purospher endcapped (both 5 μm)</td>
<td>Methanol and 1-heptane sulphonic acid sodium salt (33:67) + 0.5% triethylamine (pH 2.3). Flow rate 0.5 mL/min. Column temperature 28°C</td>
<td>DAD at 210 nm</td>
<td>Shaik &amp; Gan, (2013)</td>
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<td>Vitamin B₁₂</td>
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<td>Vitamin B₉</td>
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<td>Vitamin mixture</td>
<td>Thiamine</td>
<td>Dissolved in methanol and passed through 0.2 μm PTFE filter</td>
<td>RP-WAX Column (125 X 4 mm, 5 μm particle size)</td>
<td>Injection volume = 10 μL. Flow rate = 1 mL/min</td>
<td>IUV at 270 nm</td>
<td>Dabre et al. (2011)</td>
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<td></td>
<td>Riboflavin</td>
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<td>Folic acid</td>
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<td>Cobalamin</td>
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<td>Biotin</td>
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<td>Vitamin/Mineral tablets</td>
<td>Ascorbic acid</td>
<td>Powdered tablets suspended in diluting solutions as per article, sonicate for 25 min at 25°C and filter through 0.2 μm PTFE membrane</td>
<td>Altima C18 column (250 mm X 4.6 mm i.d., 5 μm, ambient temperature)</td>
<td>Injection volume 20 μL. Mobile phase 50 mM ammonium dihydrogen phosphate and acetonitrile delivered at 0.5 mL/min. Set at 95:5 for or 85:15 depending on vitamins being investigated</td>
<td>DAD with 275 nm or 280 nm depending on vitamins</td>
<td>Jin et al. (2012)</td>
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<td>Thiamine (B₁)</td>
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<td>Niacinamide (B₃)</td>
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<td>Pantothenic acid (B₅)</td>
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<td>Riboflavin (B₂)</td>
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<td>Folic acid (B₉)</td>
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<tr>
<td>Vitamin capsules (special study of design space)</td>
<td>Thiamine, Riboflavin, Pantothenic acid, Pyridoxine, Cobalamin</td>
<td>Capsule content dissolved in 25 mL 25mM phosphate buffer (pH 2.6) and filtered through 0.45 µm filter</td>
<td>Hypersil Gold C18 column (250 X 4.6 mm, 3 µm)</td>
<td>Methanol (100%)</td>
<td>UV at 254 nm</td>
<td>Wagdy <em>et al.</em> (2013)</td>
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</table>

AA = L-Ascorbic Acid
DAD = Diode array detector
DTT = 1, 4-dithiothreitol
EDTA = ethylenediaminetetraacetate
ESI = Electrospray Ionization
I.S. = Internal standard
MPA = metaphosphoric acid

MS = Mass Spectrometer
SPE = Solid phase extraction
TOF = Time of Flight
TFA = Trifluoroacetic acid
TCA = trichloroacetic acid
TCEP = Tris[2-carboxyethyl]phosphine
U(H)PLC = Ultra Performance Liquid Chromatography
REFERENCES


Chandra-Hioe, M. V. Bucknall, M. P. & Arcot, J. Folate analysis in foods by UPLC-MS/MS: development and validation of a novel, high throughput quantitative assay; folate levels


fluorescence detection after precolumn conversion to 5-methyltetrahydrofolates.

*Journal of Chromatography A, 928*, 77–90.


Van Wyk, J. (2002). An HPLC evaluation of vitamin B\textsubscript{12} and folate synthesis by the *Propionibacterium freudenreichii* group. Doctoral Dissertation, University of Stellenbosch, Stellenbosch, South Africa.


