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Development of HPLC methods for the determination of water-soluble vitamins in pharmaceuticals and fortified food products

Hung Khiem Trang
Clemson University, tkh3110@hotmail.com

DEVELOPMENT OF HPLC METHODS FOR THE DETERMINATION OF WATER-SOLUBLE VITAMINS IN PHARMACEUTICALS AND FORTIFIED FOOD PRODUCTS

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Master of Science
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by
Hung Khiem Trang
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Accepted by:
Dr. Feng Chen, Committee Chair
Dr. Vivian Haley-Zitlin
Dr. Kurt Young

ABSTRACT

Though many HPLC methods have been developed and reported in literature for vitamin analysis for the past two decades, applying certain methods directly from literature more than often fails to reproduce the results reported due to many variables of liquid chromatography. This issue was targeted in this project through the examination of chromatographic behaviors of water-soluble vitamins in order to help the analysts better modify methods from literature or even develop new methods from scratch to fit their analytical need with the resources available (e.g., columns, detectors, etc.) in their lab.

The first part of the project investigated the chromatographic behaviors of five vitamins: thiamine (B1), riboflavin (B2), pyridoxine (B6), cyanocobalamin (B12) and ascorbic acid (C) using different reversed-phase columns. Type-B-silica columns with novel reverse bonded phase compatible with 100% aqueous phase were found to be best suited for the analysis of water-soluble vitamins. With a simple mobile phase system using 0.1% formic acid (A) and acetonitrile (B), the five analytes mentioned above could be conveniently separated in 2 groups. Group 1 with vitamin B1, B6 and C can be eluted under 100% phase A, while group 2 with vitamin B2 and B12 can be eluted under 85% phase A, 15% phase B. Approaches to enhance the retention of the three fast-eluting vitamins (B1, B6 and C) were investigated. Perfluorinated acids such as TFA or HFBA proved to be efficient in improving the retention of B1 and B6 in reversed-phase columns. An alternative is to use buffered mobile phase with pH from 5.0 to 7.0. Ammonium acetate buffer pH 5.8, which is compatible with LCMS, was found to be able

to improve B1 and B6 retention significantly. HILIC column was another alternative to enhance the retention of not only B1 and B6 but also C.

The second part of the project was expanded to include the other four water-soluble vitamins (niacinamide B3, pantothenic acid B5, biotin B7 and folic acid B9). The goal was to develop HPLC methods for the analysis of all nine water-soluble vitamins using DAD-ELSD and LCMS. ELSD is a universal detector that responds more or less similar to all vitamins. However, its sensitivity is too low to even allow the analysis of samples with high concentration of target analytes such as dietary supplements. DAD is more sensitive but subject to possible background interferences and noisy baseline at low wavelengths (e.g., 210 nm) that were needed to obtain response from non-chromophoric vitamins like pantothenic and biotin. Therefore, the use of DAD for simultaneous multi-vitamin analysis was limited to simple samples like dietary supplements. LCMS has the highest sensitivity and specificity among the three detectors. It was proven to be effective for the simultaneous analysis of all nine analytes in fortified food products with more complicated matrices like fortified cereals and infant formula powder.

DEDICATION

I would like to dedicate this thesis to my parents, Truong Thi Kim and Trang Truong Chau, my siblings, Trang Khiem Giang and Trang Le Hoa and my grandmother To Thi Hia. This long journey would not have been possible without their endless love and support.

ACKNOWLEDGMENTS

I would like to send my deep gratitude and appreciation to my advisor, Dr. Chen for guiding me through my research project and giving me the opportunity to further my knowledge and expertise in food chemistry and food analysis. I would like to thank my committee members, Dr. Haley-Zitlin and Dr. Young for their helpful guidance, assistance and encouragement through my graduate study. Thanks also go to all members in Dr. Chen's lab for their help and support.

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CHAPTER ONE

LITERATURE REVIEW

Vitamins are essential nutrients that must be provided to the body in small amounts on a regular basis to perform various chemical and physiological functions in the human body *(1)*. They are widely distributed in natural food sources and can be easily introduced into the diets to satisfy daily needs. Though vitamins are a group of organic compounds that have different structural and chemical properties, they can be conveniently categorized into two groups based on their solubility: fat-soluble vitamins and water-soluble vitamins *(2)*. While the former includes vitamins A, D, E, and K and other carotenoids with varying degrees of vitamin A activity, the latter is composed of vitamin C and 8 B-vitamins, namely thiamin (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pyridoxine (vitamin B6), pantothenic acid (vitamin B5), biotin (vitamin B7), folate (vitamin B9) and cyanocobalamin (vitamin B12). Vitamin solubility not only decides their distribution in various food groups, but also is an important factor to be considered for their analysis and quantification.

Vitamins have a variety of uses in foods as colorants, antioxidants and especially nutritive additives *(3)*. Although the vitamin requirement of the human body can be easily satisfied by a balanced diet, certain subpopulations in the US are more susceptible to low micronutrient intakes and hence a higher risk of vitamin deficiency. Enrichment and fortification of vitamins in foods such as infant foods, fruit juices, milk, cereal, etc. have helped address this issue in the US. Accurate information on vitamin content in food

sources consumed frequently is critical to assessing dietary adequacy and planning a healthy and balanced diet for optimal nutrient intakes *(1)*. That is where the significance of vitamin analysis comes into play. As many vitamins are unstable and easily degraded, monitoring their loss during processing is important in the development of appropriate processing and storage schemes for optimal nutrient content in the final food products *(4)*. Moreover, addition of vitamins into food needs to be properly controlled to satisfy the guidelines set by the governmental authorities *(3)*. Therefore, rapid and reliable analysis of vitamins in foods is in high demand by food manufacturers.

Measurement of vitamins in foods is complicated by many factors *(5)*, which include: (1) diverse chemical structures and properties of vitamins render it difficult to develop a single universal method for their simultaneous determination. Moreover, each vitamin can occur in different forms called vitamers that possess the same biological activity upon ingestion; (2) Vitamins often occur in food at relatively low levels; (3) Foods are complex matrices, from which the vitamin extraction presents many challenges; (4) Vitamins are susceptible to degradation by exposure to light, air, heat and high pH.

This research project aims to develop fast, reliable and sensitive HPLC methods for the analysis of five water-soluble vitamins in the B-vitamin group: thiamine, riboflavin, pyridoxine cyanocobalamin and ascorbic acid.

1.1 Vitamin B1 (Thiamine)

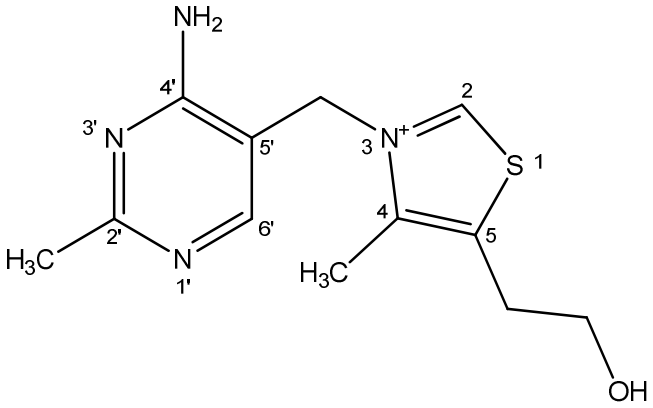
1.1.1 Nomenclature, structure and physiochemical properties

First isolated in 1926 and characterized later on in the 1930s, thiamine was the first water-soluble vitamin structurally characterized and was formally assigned the name vitamin B1 by the British Medical Research Council in 1927 *(6-9)*. The chemical structure of the free base thiamine molecule and other related compounds are shown in Figure 1.1. It is comprised of a pyrimidine ring (4′-amino-2′-methylpyrimidinyl-5′-ylmethyl) connected to the 3-nitrogen atom in a substituted thiazole moiety (5-(2-hydroxyethyl)-4-methylthiazole) by a methylene bridge *(10)*. In nature, thiamine normally occurs in three phosphorylated forms, among which thiamine pyrophosphate (TPP) is the most dominant form contributing up to 90% of the total thiamine in cells *(11)*.

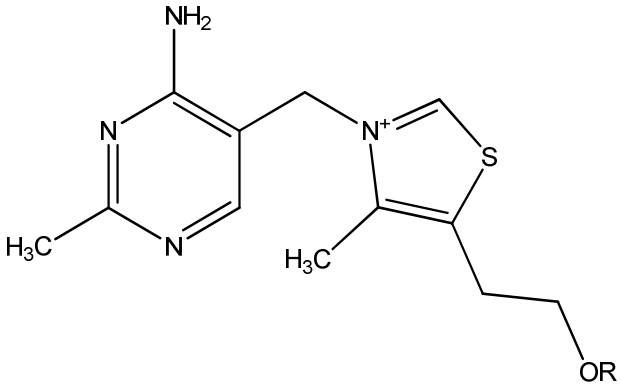
TPP acts as a coenzyme in the conversion of pyruvate to acetyl CoA, an essential step in TCA cycle *(11)*. Besides the metabolically active TPP, thiamine can be found in the organism's tissues as other phosphorylated forms (thiamine monophosphate or thiamine triphosphate) or dephosphorylated forms *(12)*. Inter-conversion of thiamine to other phosphorylated forms in tissues is catalyzed by different phosphokinase and phosphatase enzymes *(13)*.

Two commercially available forms of thiamine are thiamine hydrochloride and thiamine mononitrate *(2)*. Thiamine hydrochloride is a colorless, crystalline powder with a yeasty odor and a salty nut-like taste. It melts at about 207^{o}C, with decomposition. While the hydrochloride form is more soluble in water (1 g/ml) and therefore used in

injectable and parenteral pharmaceuticals and food for food fortification, the mononitrate form is much less soluble (0.027 g/ml) and finds its use in dry blends, multivitamins, and dry products such as enriched flour *(9)*. As thiamine hydrochloride is often used as the standard reagent in vitamin B1 analysis, it is worth noting that this form is sparingly soluble in methanol, ethanol and glycerol and insoluble in fat solvents (ether, acetone, benzene, hexane and chloroform) *(14)*.



Free-base thiamine
3-((4′-Amino-2′-methyl-5′pyrimidinyl)methyl)-5-(2-hydroxyethyl)-4-methylthiazole



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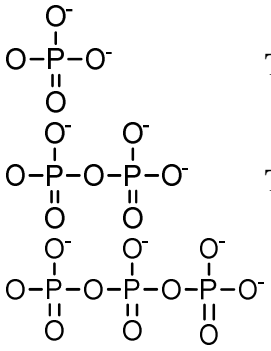
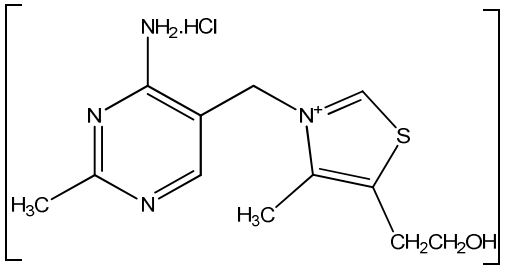


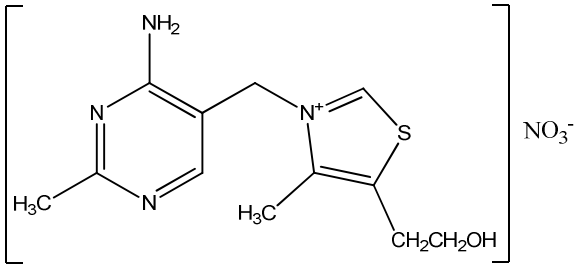
Thiamine monophosphate (TMP)

Thiamine pyrophosphate (TPP)

Thiamine triphosphate (TTP)



Thiamine hydrochloride



Thiamine mononitrate

Figure 1.1 Structures of thiamine and related compounds.

1.1.2 Stability and degradation

Thiamine is generally stable in the dry state and can sustain high temperatures up to 100^{o}C *(15).* Aqueous solutions of thiamine itself are acidic, and at pH below 5.0, they are even stable to autoclaving at 120^{o}C-130^{o}C and not susceptible to oxidation *(2).* Being most stable at pH 2.0 to 4.0, thiamine gradually loses its stability as the matrix pH approaches neutrality and alkaline *(16).* When it reaches pH 8 and above, thiamine solution rapidly turns yellow and various complex degradation products are formed *(17).* Free thiamine bases are very unstable and very easily oxidized. Mild oxidation results in the formation of thiamine disulfide without loss of thiamine activity. Under more vigorous conditions, thiamine is oxidized to other biologically inactive derivatives, among which thiochrome is an important compound utilized in analytical chemistry for thiamine analysis *(9).* In the presence of sulfite ions at pH 6.0 and above, thiamine is rapidly cleaved at the methylene bridge, splitting apart thiazole and pyrimidine moieties. A similar reaction happens in thermal degradation with thiazole being further decomposed to hydrogen sulfide *(3).*

1.1.3 Nutritional and physiological functions

Thiamine, in the form of TPP, acts as a coenzyme in the three closely related dehydrogenase enzyme complexes that catalyze the oxidative decarboxylation essential for carbohydrate metabolism: pyruvate dehydrogenase complex, alpha-ketoglutarate dehydrogenase complex, and branched-chain ketoacid dehydrogenase complex *(8, 10, 13).* Another complex, transketolase, plays a role in the transfer of glycoaldehyde moiety

between sugars and together with ketoaldose, connecting the pentose phosphate pathway with glycolysis *(18)*. Recent research has reported that peroxisomal enzyme also requires TPP in the alpha-oxidation of 3-methyl branched-chain or straight chain fatty acids *(19, 20)*. In addition to its vital roles in energy metabolism, thiamine has also been known to occupy a special site on nerve cell membranes and function in neurotransmission; however, the exact mechanism is not yet elucidated *(21, 22)*.

The RDA of thiamine is 1.2mg/day for men and 1.1mg/day for women *(12)*. This recommended intake is easily met if one consumes a sufficient amount of nutritious food to meet energy needs. However, as thiamine is not stored in the body for long-term use, failing to provide adequate intake leads to thiamine deficiency. A mild form of this deficiency can occur within 10 days after thiamine intake is stopped, accompanied by symptoms such as poor sleep, malaise, weight loss and confusion *(11)*. Severe thiamine deficiency results in two classical conditions known as beriberi and Wernicke-Korsakoff syndrome *(18)*. The former condition is further categorized into dry, wet and infantile beriberi *(23)*. Another acute pernicious form was recorded in the 1980s and named shoshin beriberi *(24, 25)*. Dry beriberi is characterized by damage to the nervous system with symptoms such as poor appetite, fatigue and peripheral neuritis *(23)* while wet beriberi patients may develop cardiac failure and edema *(8)*. Alcohol abusers are susceptible to another severe thiamine deficiency condition called Wernicke-Korsakoff due to the poor diet and the impairment of thiamine absorption by alcohol *(21)*. Mental disorders characteristic of Wernickle-Korsakoff includes confusion, hallucinosis, psychosis, and even coma *(13)* . No upper limit (UL) has been determined due to the lack

of supporting evidence of toxicity for excess thiamine intake *(12)*. This is a common occurrence with water-soluble vitamins that are readily excreted in the urine.

1.1.4 Occurrence and distribution in food

Yeast and yeast extract, whole grain cereals and cereal products, beans, nuts, egg yolk, poultry, fish, meat (especially liver and lean pork) are among the richest dietary sources of thiamine *(26)* while refined and processed foods without fortification are poor sources *(18)*.

Prolonged thermal processing may lead to a significant loss of thiamine due to thermal breakdown *(12)*. Moreover, as a water-soluble compound, thiamine leaches into water during boiling or blanching. Cooking may cause a substantial loss of up to 90% of thiamine content following the boiling of rice and green vegetables *(18, 27)*. Therefore, cooking methods with little or no water such as steaming are recommended to conserve thiamine content in food.

1.1.5 Analytical methods

1.1.5.1 Extraction

The extraction protocols are dependent upon the goals of the analyst to quantify total thiamine in the free forms or thiamine phosphate native forms. Regardless of the form being analyzed, the first step of the extraction involves the liberation of thiamine and thiamine phosphate esters from their association with proteins though acid hydrolysis under high temperature *(2, 8)*. If the free form is of interest to the investigator, the sample

is then subjected to enzyme hydrolysis to convert the phosphorylated thiamine forms into the free forms *(28, 29)*. The optimal extraction procedure outlined in The AOAC International Method 942.23 *(30)* is similar to the method published by The European Union Measurement and Testing Program *(28)* and includes the following steps: (1) autoclaving 0.2-5 g sample in 0.1N HCl for 30min at 121°C, (2) adjusting the solution to pH 4.0 with 4.0 M sodium acetate buffer (pH 6.1), (3) incubating the mixture with takadiastase at 37-45°C for 4hrs. This autoclaving temperature is not high enough to degrade the phosphorylated thiamine, the predominant forms in samples of animal origin *(28)*. However, for cereal products, the temperature is recommended to be lowered to 108°C to protect the non-phosphorylated forms which are more susceptible to degradation *(2)*. Another common alternative condition is to digest the sample at 95°–100°C in a steam bath, or in boiling water, with frequent mixing for 30 minutes *(30)*. If the analysis targets a native phosphorylated thiamine species then these thermal treatment in acidic conditions is sufficient without the enzymatic digestion *(31)*. The main purpose of the enzymatic step is to dephosphorylate thiamine, which can be performed by commercial diastases such as Takadiastase, Claradiastase or Mylase *(28, 32)*. Besides α-amylase and phosphatase activity, these enzymes also possess some protease activity which is useful for digesting proteinaceous samples such as meat. For milk and dairy product samples, the extraction procedures can be simplified to include only the precipitation of protein by acidification followed by filtration or centrifugation *(2)*.

***1.1.5.2 Non-HPLC methods*:**

One of the earliest methods used to determine vitamin B1 in biological samples is microbiological assays *(9)*. The most commonly used microorganism species for this method are *Lactobacillus fermenti* (ATCC 9338) and *Lactobacillus viridescens* (ATCC 12706) *(2)*. The former, though originally used, is subject to inhibitory and stimulatory substances *(33)*. Therefore, *L.viridescens*, which requires intact thiamine for growth, is favored due to its higher specificity for the assay *(34)*. The extraction protocol in AOAC Official Method 942.23 (Thiochrome analysis procedures) can be followed in preparation for microbiological assays. In order to ensure the complete utilization of total vitamin B1 by *L. viridescens*, both acid and enzyme hydrolysis are required to liberate and dephosphorylate all the bound forms in order to prevent differential growth response to TMP, TPP and TTP *(2, 34)*. Another alternative method utilizing the protozoan *Ochromonas danica* was developed by Baker for thiamine assessment in blood-based samples *(35)*.

Official standard procedures by AOAC, AACC (American Association of Cereal Chemists) and European Committee for Standardization all involve the conversion of thiamine into a highly fluorescent product named thiochrome through alkaline oxidation with cyanogen bromide or potassium ferricyanide *(9)*. This reaction (Figure 1.2) was first described by Barger et al. in 1935 and has been widely used in a variety of procedures reported throughout the literature *(36)*. Phosphorylated thiamine can also be converted into thiochrome phosphate esters with the intact phosphate moieties and share the same fluorescent properties with thiochrome *(37)*. They all have similar excitation and

fluorescence maxima at 375nm and 435nm, respectively *(10)*. Without the interference of other fluorescent components, the fluorescence intensity of thiochrome is correlated to total thiamine content in a sample *(9)*. This reaction when combined with HPLC fluorescence either pre or post-column can enhance the selectivity and sensitivity of thiamine analysis significantly *(10)*.

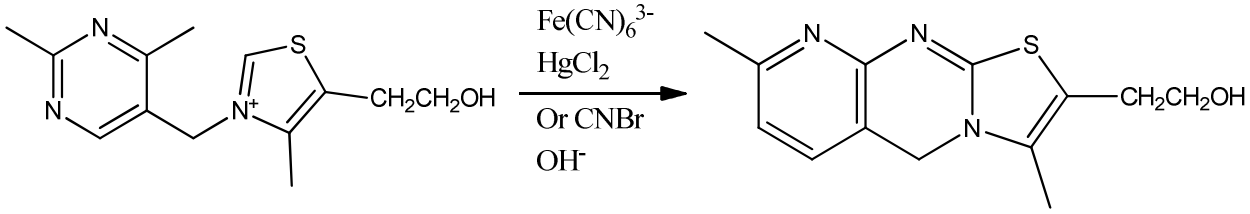


Figure 1.2 Thiochrome reaction

Paper partition chromatography (PPC) was the first chromatographic technique used for the analysis of thiamine phosphates in biological materials. Elution of the targeted compounds by several different solvent systems followed by photometry at 270 nm of the eluted spots was used to quantitate each thiamine compound down to the 10-µg level *(10)*. Separation of thiamine phosphates by PPC was also reported to be followed by a microbiological assay, which allowed a detection limit of about 0.02 µg (60 pmol) of thiamine *(10)*. It is worth mentioning that this limit is at least three orders of magnitude higher than that of HPLC with a fluorescence detector. Another similar technique, thin layer chromatography (TLC), allowed the separation of more complex mixtures of thiamine and its metabolites and precursors when used on two dimensional with two different solvents. These TLC procedures have been used to analyze thiamine in pharmaceutical samples at levels of 20 µg to 10 mg *(38)*.

Ion exchange chromatography has also been a common technique used to separate thiamine and its phosphates in both pharmaceuticals and biological samples. AOAC official method 953.17 uses Bio-Rex 70 cation exchange resin to clean up the sample extract after acid and enzyme hydrolysis *(39)*. A sephadex cation exchange column was also reportedly used in thiamine analysis as well *(40)*.

In comparison to spectroscopic and especially liquid chromatographic methods, other approaches are less popular. Electrochemical *(41, 42)* and capillary electrophoretic *(43, 44)* methods have not been extensively used for routine analysis of thiamine though their feasibility has been proven. Another less popular technique is flow injection analysis (FIA) which has emerged as a promising technique applicable to pharmaceuticals and biologicals. When coupled with fluorimetric or chemiluminescent detection, the method exhibits high sensitivity and selectivity as well as provides high sample throughput. Fluoresence-based methods requires thiamine be converted to either thiochrome or other closely related fluorescent derivatives *(45-47)*. Chemiluminescence-based methods make use of thiamine's ability to decrease chemiluminescense produced by luminol-ferricyanide or luminol-potassium periodate reaction *(48, 49)*.

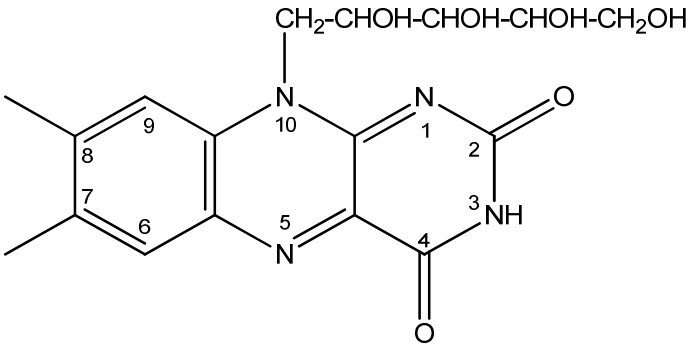
1.2 Vitamin B2 (Riboflavin)

1.2.1 Nomenclature, structure and physiochemical properties

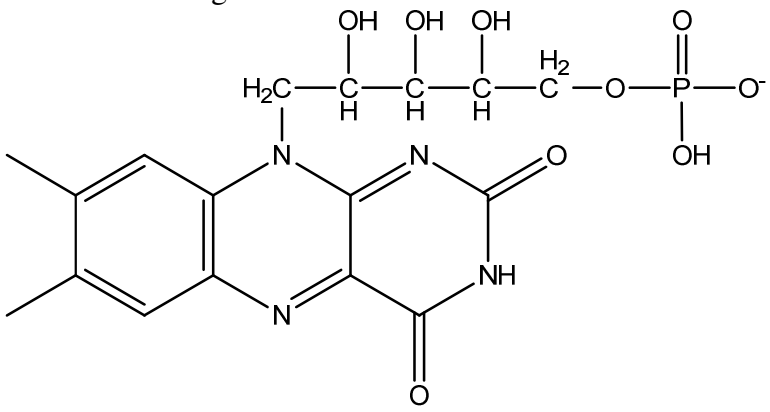
First isolated, though not purified, as yellow fluorescent compounds from whey and different biological matrices more than 100 years ago, riboflavin was originally known as lactochrome or lactoflavin *(50*, *51)*. It was not until the 1930s that the structure

and synthesis of riboflavin were determined by Kuhl *(52)* and Karrer *(53)*. In the same decade, the coenzyme forms of riboflavin, namely flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were isolated and structurally identified *(54)*. In fact, these terms are misnomers because FMN and FAD are basically not mono and dinucleotide respectively *(50)*. However, the names are still accepted and widely used. In the scientific context, the term “riboflavin” is used to refer to the parent riboflavin molecule and in many cases can be used synonymously with vitamin B2 *(2)*.

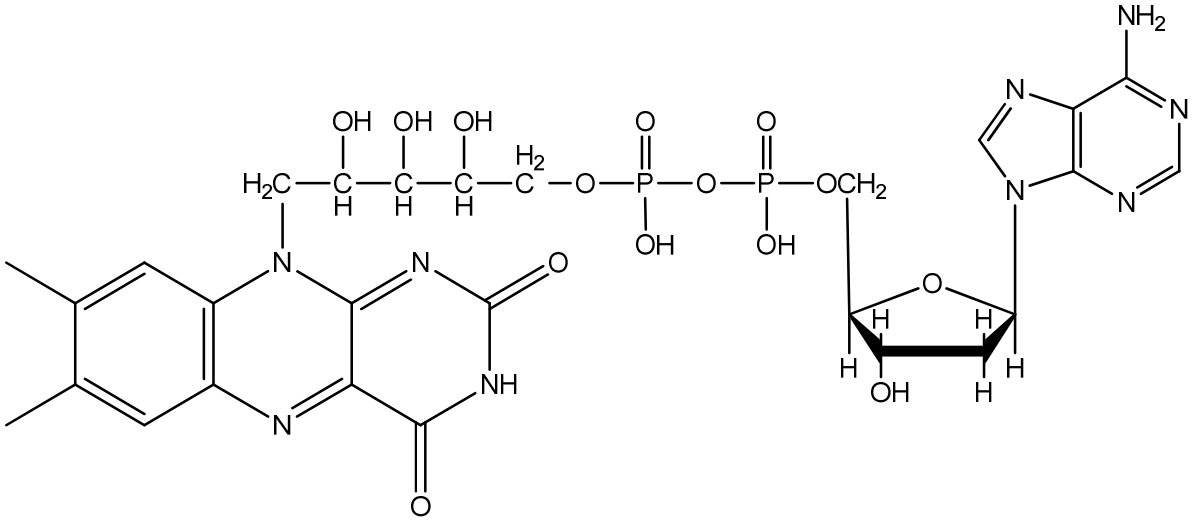
The official IUPAC name of riboflavin is 7, 8-dimethyl-10-(1′-d-ribityl) isoalloxazine *(3)*. The name "riboflavin" originates from the sugar moiety (ribitol) which is the reduced form of the pentose sugar ribose and the isoalloxazine ring moiety (commonly referred to as flavin ring) which imparts the yellow color to the oxidized molecule *(9)*. The reduced form, which occurs in metabolism along with the oxidized form, is colorless. The addition of a phosphate group or adenosine-5′-diphosphate at the 5′ position of the ribityl side yields FMN and FAD respectively *(9)*. These groups can be removed through acid hydrolysis, which is utilized in analytical procedures to liberate the free form for the quantitation of the total riboflavin. The structure of riboflavin and other related compounds are shown in Figure 1.3.



Riboflavin
7,8-dimethyl-10(19-D-ribityl)isoalloxazine
Figure 3 Structure of riboflavin



Riboflavin 5'-phosphate (FMN)



Flavin Adenine Dinucleotide (FAD)

Figure 1.3 Structures of flavin coenzymes

Riboflavin is an odorless orange crystalline powder with an unpleasant bitter taste and a melting point of about 280°C *(14)*. In neutral aqueous solution, riboflavin exhibits a strong yellow-green fluorescence *(15)*. Though classified as a water-soluble vitamin, riboflavin has a low solubility in water (10–13 mg/100 ml at 25–27.5°C; 19 mg/100 ml at 40°C; 230 mg/100 ml at 100°C) *(14, 54)*. It is sparingly soluble in absolute ethanol (4.5 mg/100 ml at 27°C) and not at all in acetone, diethyl ether, or chloroform *(2)*. Riboflavin solubility can be enhanced in dilute acid or alkali though it is not very stable in alkali *(2)*. The presence of aromatic compounds is known to make riboflavin more soluble in aqueous solution, which is utilized in pharmaceutical preparations *(2)*. In contrast, FMN and FAD are much more soluble than riboflavin *(2, 14)*.

The well-studied characteristic spectral properties of flavins in different oxidized and reduced states have laid a foundation for their chemical analysis *(55)*. In the oxidized state, flavins are yellow pigments that have two characteristic absorption bands at 370 and 450 nm *(51)*. Affected by solvent properties, the maxima around 370nm tend to shift to lower wavelengths as the solvent polarity decreases *(56)*. The reduced forms of flavins, on the other hand, show untypical and variable absorption spectra above 300nm *(57)*.

Analytical procedures employing these UV-Vis spectral properties have been adapted for vitamin B2 analysis. However, their popularity pales in comparison to fluorimetric methods considering riboflavin possess a strong native fluorescence without the need of derivatization (Ex λ = 440–500, Em λ = 520–530) *(55)*. Among the three forms, riboflavin and FMN have similar fluorescence capacity, which is much stronger than FAD's *(58)*. It is due to the fluorescence quenching of the adenine and the

isoalloxanzine ring. It is worth mentioning that the reduced forms of flavins do not fluoresce *(57)*.

1.2.2 Stability and degradation

In crystal form, riboflavin is stable when stored in dry conditions *(2)*. Stability becomes a concern when riboflavin occurs in solution as it is easily degraded by exposure to both UV and visible light *(9)*. The rate of this photodegradation process is sped up with elevated temperature and pH with the wavelength range of 350–520 nm exerting the greatest destructive effects *(59)*. As shown in Figure 1.4, the nature of this degradation is the reduction of the isoalloxanzine ring by the ribityl side chain, resulting in the formation of lumichrome and lumiflavin under alkaline and acidic conditions, respectively *(58, 60)*. Both of these degraded products do not exhibit vitamin B2 activity.

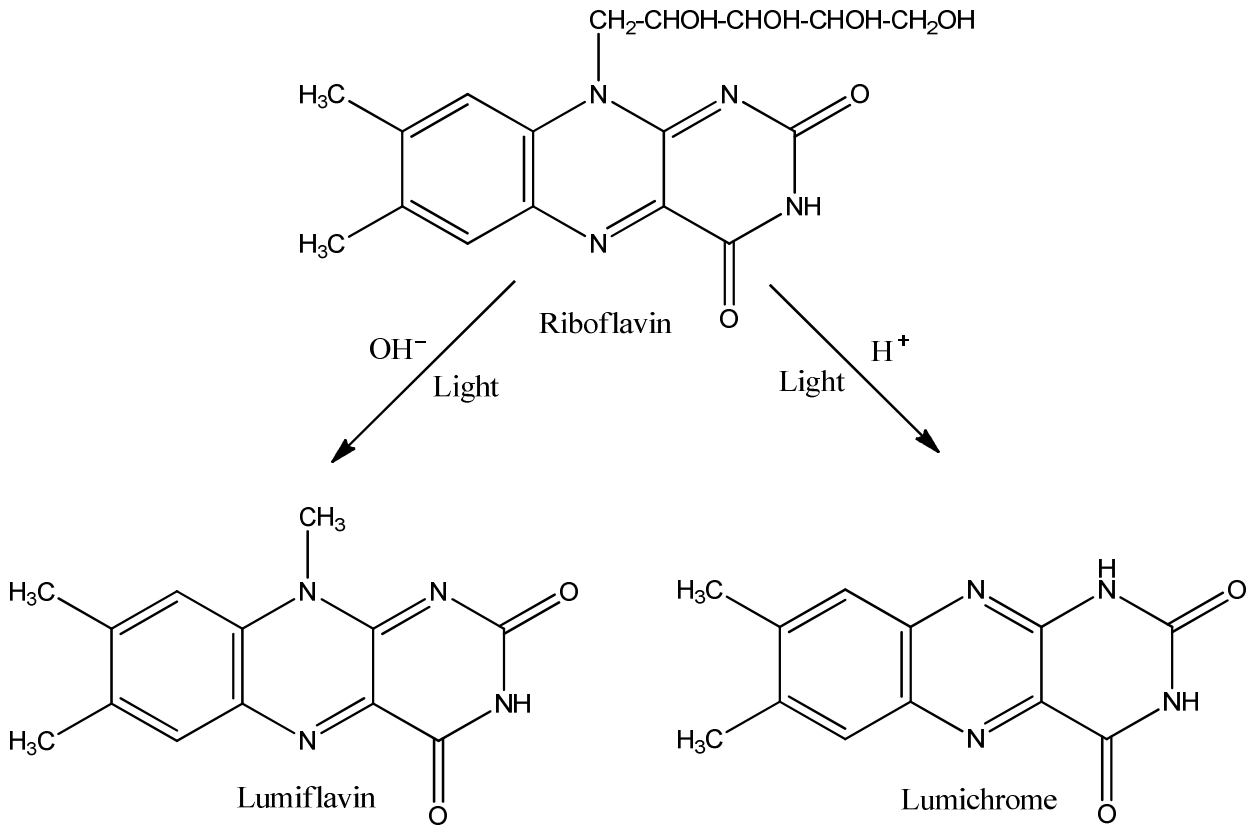


Figure 1.4 Photodegradation of riboflavin under basic and acidic conditions

Except for being light sensitive, riboflavin is generally stable to heat and oxidation *(2)*. If light is excluded, most food processing operations or normal cooking have little effect on riboflavin content. Its stability increases as acidity increases with optimal stability to heat degradation being between pH 2.0 and 5.0 *(2)*.

Low stability of flavins upon exposure to light is taken into consideration in food packaging *(9)*. Containers made of glass or other translucent materials are subjected to sunlight, which induces significant loss of riboflavin in the food products such as milk and juices *(12)*. The same phenomenon even occurs in dry products such as enriched pasta over a prolonged exposure to light during storage *(9, 26)*. The use of blow molded

polyethylene containers can provide a sufficient barrier against photodegradation of riboflavins in food products *(61)*.

1.2.3 Nutritional and physiological functions

The three major biologically active forms of vitamin B2 found in nature including riboflavin, riboflavin-50-phosphate (FMN) and riboflavin-5'-adenosyldiphosphate (FAD) have equal vitamin activity in human *(21, 26)*. With the ability to participate in either one- or two-electron redox reactions, FMN and FAD can either act as cofactors for several flavoprotein enzymes that catalyze redox reactions in cells or serve as electron carriers in the mitochondrial electron transport system *(23, 51)*. Some typical reactions in intermediary metabolism that require riboflavin include dehydrogenation, hydroxylations, oxidative decarboxylations, dioxygenations, and reductions of oxygen to hydrogen peroxide *(62, 63)*. Acting as coenzymes of dehydrogenases, FMN and FAD are essential to both glucose and fatty acid metabolism *(21)*. Besides their role in energy metabolism, they contribute to drug and steroid metabolism in conjunction with cytochrome P450 enzymes *(64)*. Other important functions of riboflavin also include the activation of pyridoxine (vitamin B6) and the conversion of tryptophan to niacin *(26)*.

Riboflavin does not have much, if any, antioxidant activity by itself. However, its reduced form as a precursor to FMN and FAD exhibits protective effects against oxidative damage to cells *(54)*. Together with NADPH, riboflavin coenzymes help recycle glutathione peroxidase, an enzyme that breaks down reactive lipid peroxides *(64, 51)*. Riboflavin has been found to be related to a number of disease states *(51)*. Some

studies suggest that there is an association between cataract and riboflavin deficiency and nutritional supplements (including riboflavin) may help to improve cataracts *(65)*. Riboflavin deficiency may also increase plasma homocysteine concentration, which is thought to be linked to an increased risk of cardiovascular disease *(66)*. Moreover, impaired iron absorption and even night blindness have been found to be associated with riboflavin deficiency *(51)*.

Like other water soluble vitamins, urinary riboflavin excretion occurs on a daily basis, therefore deficiency can happen when the dietary intake is low *(64)*. Symptoms of riboflavin deficiency (ariboflavinosis) may include glossitis, angular stomatitis, angular cheilitis and dermatitis *(26)*. However, these classical symptoms are not characteristic of riboflavin deficiency but closely associated with other vitamin deficiencies as well. Actually, when ariboflavinosis does occur, it does not do so in isolation but rather accompanies other nutrient deficits *(54)*. RDA of vitamin B2 for men and women are 1.3mg/day and 1.1mg/day respectively *(12)*. No toxicity symptoms have been reported therefore no UL has been established *(12)*.

1.2.4 Occurrence and distribution in food

As FMN and FAD are essential for the enzymatic activity in living cells, flavins are prevalent in all natural unprocessed foods *(15)*. Yeast extract, liver and kidney are exceptional sources of vitamin B2 *(2)*. However, the primary contribution to the dietary intake is from milk and dairy products, which contains mainly free riboflavin with a small amount of protein-bound flavins (about 14%) *(67, 68)*. Due to a significant loss of

up to 60% during milling, cereal products are enriched with the vitamin *(2)*. Together with whole grain, enriched cereal products are important sources in regions where they are a part of the staple diet *(9)*. In terms of nutrient density, dark green leafy vegetables such as spinach, broccoli, asparagus, etc. top the list *(12)*. They are an excellent source for vegans who avoid consumption of milk products.

Riboflavin is generally heat stable, therefore cooking does not destroy it. However, it is susceptible to degradation upon exposure to UV light and irradiation *(9)*. Milk is therefore packaged in cardboard or opaque polyethylene containers, instead of translucent glass bottles to provide protective barriers for vitamin B2 *(26)*.

1.2.5 Analytical methods

1.2.5.1 Extraction

Riboflavin extraction from foods is similar to that of thiamine in that it requires both an acid and enzymatic treatment. The acid hydrolysis step is normally performed by autoclaving samples with 0.1 M HCl at 121^{o}C for 30 minutes to liberate the flavins from the protein complex. In this process, FAD is converted into FMN which is partially hydrolyzed to riboflavin or goes through isomerization to form 2'-, 3'-, and 4'-phosphates *(69)*. For food samples with high-starch content, this step also helps to convert the polysaccharide into soluble sugars *(28)*. Subsequent dephosphorylation to produce free riboflavins can only be accomplished by enzymatic hydrolysis using the same diastatic enzymes commonly used for thiamine extraction procedures (e.g., Takadiastase, Takadiastase and Mylase) *(28)*. Due to variable phosphatase activity,

parameters of incubation condition such as time, temperature and pH should be empirically adjusted for the optimal conversion *(70)*. If the goal of the study is to analyze the native forms of riboflavin or if the predominant forms in the samples are free or loosely bound riboflavins, then mild extraction protocol with simple precipitation of protein suffices *(2)*. That is normally the case for the analysis of milk, eggs, and dairy products. Detailed extraction procedures can be found in AOAC Official Method 970.65 and 981.15 *(71, 72)*.

1.2.5.2 Non HPLC methods

AOAC Official method 970.65 is based on the native fluorescence ability of riboflavin *(71)*. It has been widely used to determine the riboflavin content of food products for routine nutritional label compliance analysis. The basic procedure of the methods involves the acid hydrolysis of FMN and FAD to release free riboflavin and oxidation to remove the interfering fluorescent components with potassium permanganate. The oxidation step does not affect riboflavin and is essential to increase the analysis specificity. Fluorescence measurements are made at Em λ=565nm and Ex λ=440nm. Sodium hydrosulfite is used to reduce riboflavin to nonfluorescent leuco form and the sample is measured again as a blank to correct for the non-riboflavin fluorescence left over after the sample preparation. This manual method was modified for semiautomated flow-injection analysis procedure outlined in AOAC Method 981.15 which was originally published by Egberg and Potter *(73, 74)*.

Another fluorometry method is approached indirectly by converting riboflavin into lumiflavin through photolysis under alkaline condition *(75)*. The sensitivity and specificity of the method is greatly enhanced by strong fluorescence capacity of lumiflavin and an extra step of lumiflavin extraction with chloroform. This method is not as popular as the indirect fluorometry analysis by AOAC; however, this sample preparation protocol can be applied to LC methodology *(76)*.

Lactobacillus rhamnosus (ATCC 7469) has been extensively used for the determination of vitamin B2 *(2)*. Since this organism cannot use FAD and responds to FMN and riboflavin differently, microbiological methods utilizing *L. rhamnosus* is recommended for total riboflavin analysis *(77)*. The procedures therefore require the hydrolysis step to release free riboflavin from FMN and FAD. *L. rhamnosus* growth is affected by common components in food including starch and protein degradation products *(2)*. Fatty acids, on the other hands, show contradictory effects with either stimulatory or inhibitory activity. Therefore, it is recommended that fat extraction be done before the acid hydrolysis step. Some other microorganisms that have been proposed to use over the time include *Leuconostoc mesenteroides*, *Tetrahymena pyriformis* and *Enterococcus faecalis (9)*. Among them, *E. faecalis* and was found to be less affected by matrix effects and offer much higher sensitivity than *L. rhamnosus (9)*. However, it is not used as extensively due to the lack of commercial media needed for its growth.

Identification of flavins can be done by TLC or its advanced form HPTLC *(78-81)*. Quantitative analysis of the separated flavins is then followed by the use of

densitometry. Commercial availability of high-quality precoated plates with various stationary phases and particle sizes allows the separation of different flavin compounds from each other. This technique was even applied to the simultaneous determination of riboflavin with other water soluble vitamins as well. One advantage worth mentioning is that TLC can be utilized for multidimensional separation.

Conventional column chromatography with Silica gel C18 or Florisil used to be utilized for sample clean-up in LC methods in previous decades before SPE gained its popularity *(82, 83)*. Ion exchange chromatographic methods with DEAE-Sephacel (acetate form) and DEAE-cellulose (chloride form) were reported to separate FMN efficiently from riboflavin and FAD, which is useful for the preparation of substantial amounts of purified FMN and FAD *(69)*.

Flow injection analysis (FIA) when coupled with fluorescence provides a highly sensitive and selective analytical approach *(84, 85)*. Due to the strong fluorescence of riboflavin and its metabolites, the derivatization step can be skipped, making the method simple and feasible enough for routine analysis. In addition, FIA can also be combined with chemiluminesence *(86, 87)*. Capillary electrophoresis is another technique that takes advantage of riboflavin spectral properties. The procedures are applicable to pharmaceutical samples with high vitamin levels and adaptable to multianalyte analysis *(88, 89)*.

1.3 Vitamin B6

1.3.1 Nomenclature, structure and physiochemical properties

Vitamin B6 was first identified as a curative factor distinct from riboflavin and niacin for a characteristic dermatitis in rats by Gyorgy in 1934 *(90, 91)*. He referred to the compound as pyridoxine, which was isolated and structurally characterized by several researchers in the late 1930s *(92)*. Later research found other derivatives of pyridoxine including pyridoxal and pyridoxamine that also demonstrated vitamin B6 activity in 1944 *(93)*. The commonly used term 'vitamin B6' is a generic descriptor for all derivatives of 3-hydroxy-2methylpyridine that are referred to as vitamin B6 vitamers and show the same biological activity of pyridoxine (PN) in rats *(94)*. Generally used synonymously with vitamin B6, PN is one of the three forms occurring naturally besides pyridoxal (PL) and pyridoxamine (PM). They can exist in free or phosphorylated forms and be bound to proteins. Enzymatically phosphorylated and converted to the metabolically active pyridoxal-5′-phosphate (PLP), these three forms are interconvertible and considered to be biologically active equivalents *(12)*. The end product of vitamin B6 metabolism, 4-pyridoxic acid (4-PA) is also a common form occurring naturally in biological samples *(9)*. Another important form is the glucosidically bound form of PN (PN glucoside), which has a low bioavailability and only occur in plant sources *(95)*. All the above structures are demonstrated in Figure1.5

Free vitamers of B6 are commercially available as crystalline hydrochlorides including pyridoxine hydrochloride (PN·HCL), pyridoxal hydrochloride (PL·HCL) and pyridoxamine dihydrochloride (PM·2HCL) *(96)*. Among them, PN·HCl is the UPS

reference standard and the only form used in food fortification and pharmaceutical preparations due to its higher stability than PL and PM. PN·HCl is a white, odorless, crystalline powder with a slightly salty taste and a melting point of 204–206^{o}C (with decomposition) *(9)*. It is readily soluble in water (22g/100ml), sparingly soluble in ethanol (1g/100ml) and practically insoluble in diethyl ether and chloroform. PN·HCl has pK values of 5.0 and 9.0 (25^{o}C) and its 5% solution has the pH of 2.3–3.5 *(2)*.

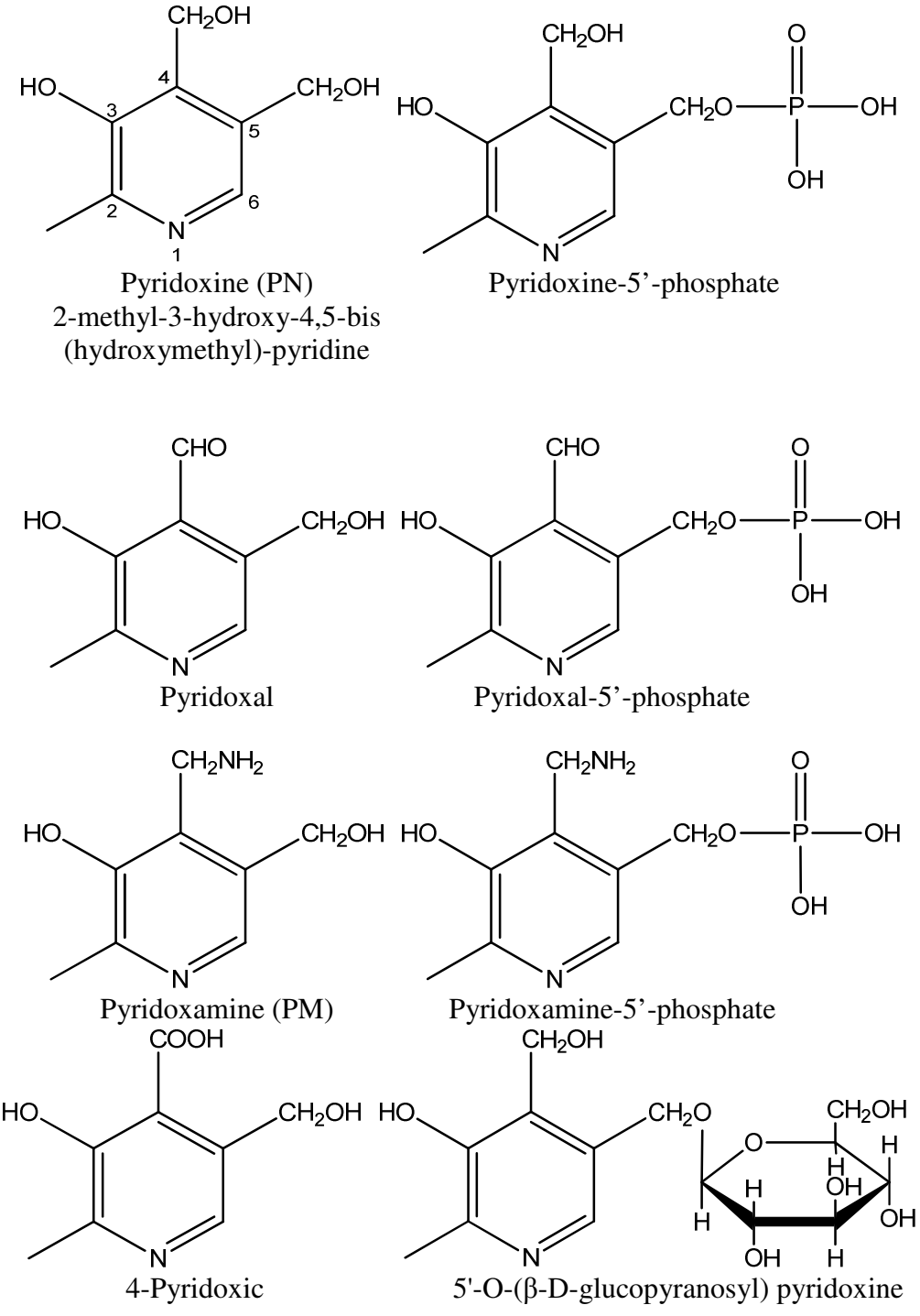


Figure 1.5 Structures of pyridoxine and related compounds

1.3.2 Stability and degradation

When protected from light, aqueous solution of PN·HCl shows no significant loss even at elevated temperature (40 and 60°C) for up to 140 days at pH 4 to 7 *(97)*. PN·HCl and PM·2HCl were reported to be the most and least stable forms of the three free B6 vitamers *(98)*. In general, all forms of vitamin B6 are quite stable at acidic pH when light is excluded, but exposure to light and especially UV or near UV irradiation results in significant degradation, the degree of which increases as the pH approaches alkalinity *(97, 99)*. In order to avoid photodegradation during sample preparation for an accurate analytical determination, low-actinic amber glassware and gold fluorescent light should be used in the analytical laboratory *(9)*. Heat also causes vitamin B6 degradation, the rate of which increases with elevated pH levels *(26, 100)*. Phosphorylated vitamers even when stored in the dark are still susceptible to hydrolysis to free counterparts *(101)*. Their stability can be greatly enhanced in acidic solution and low temperature (-20°C or lower) storage before the analysis.

1.3.3 Nutritional and physiological functions

PLP has been reported to have functions in more than 100 enzymatic reactions in the body, many of which are majorly involved in protein and urea metabolisms *(2, 102)*. The class of vitamin B6 dependent enzymes includes those for trans-aminations, α decarboxylations, α,β eliminations, β,γ eliminations, aldolizations, and racemizations *(100)*. Decarboxylases play a role in synthesizing important neurotransmitters (converting the amino acid tryptophan to niacin or to the neurotransmitter serotonin); therefore

vitamin B6 intake is necessary for healthy brain function *(103)*. Aminotransferases play an important role in anabolism and catabolism of amino acids. PLP is also critical to the activity of glycogen phosphorylase *(104)* and lipid metabolism *(105)*. The conversions of tryptophan to niacin or to the neurotransmitter serotonin are also dependent on PLP *(26)*. Moreover, PLP functions in the synthesis of heme, nuleic acids and phospholipid in the body as well *(12)*. Several studies conducted in the last decade have suggested that vitamin B6 may play important roles in cognitive performance, immune function, and steroid hormone activity *(21)*. Recent reports have shown yet another role of vitamin B6 as an effective antioxidant *(106)*.

Due to the role of PLP in amino acid metabolism, vitamin B6 requirement increases with increased protein intake. RDA of vitamin B6 for adults (19-50yrs) is 1.3mg *(12)*. As vitamin B6 naturally occurs in foods high in protein, adequate protein intake can easily achieve the recommended vitamin B6. Inadequate vitamin B6 intake leads to interrupted synthesis of key neurotransmitters in the nervous systems *(92, 107)*. Tryptophan metabolism is upset with undesirable compounds being formed and accumulated in the brain. Early symptoms of depression and confusion may progressively advance to more severe conditions including abnormal brain wave patterns and convulsions if deficiency prolongs. Alcoholics have a higher risk of vitamin B6 deficiency as alcohol can antagonize the vitamin *(12, 21)*. Acetaldehyde produced during alcohol metabolism can remove PLP coenzymes from the enzyme complex, which are then catabolized and excreted.

Unlike other water soluble vitamins discussed here, overconsumption of vitamin B6 can lead to toxicity *(12)*. It was reported that neurological damage was experienced by those studied subjects having the daily intake of 2 grams of vitamin B6 (20 times higher than the Upper Limit (UL) of 100mg) for 2 months or longer *(12)*. Symptoms of vitamin B6 toxicity may include fatigue, headaches, irritability, skin lesions, convulsions, muscle weakness and even irreversible nerve degeneration *(107)*.

1.3.4 Occurrence and distribution in foods

Vitamin B6 occurs in a wide variety of natural unprocessed foods with yeast extract, liver and wheat bran being excellent sources *(15)*. Other important sources include whole-grain cereals, meats, fish, poultry, potatoes and other starchy vegetables, legumes, noncitrus fruits, fortified cereals and soy products *(2, 12)*. Over 90% of vitamin B6 content is found in the germ and bran of cereal grains *(108)*. However, a significant loss (up to 90%) can occur during the milling process to produce flour, which results in a much lower vitamin B content in white bread compared to whole wheat bread *(109)*. The major form of vitamin B6 in plant-derived foods is β-glucoside of PN, which has a low bioavailability *(95)*. PN glucoside was found to be poorly metabolized and converted into the active PN form in rats, leading to its incomplete utilization and low bioavailability (only 20-30% efficiency of pyridoxine).

Besides loss through leaching, vitamin B6 is also susceptible to heat degradation in cooking and processing *(9)*. This is more of a concern in animal-derived foods as the

main forms of vitamin B6 in these products are PL and PM which are less stable than PN, the prevalent form in plants.

1.3.5 Analytical methods

1.3.5.1 Extraction

Extraction approaches for vitamin B6 for liquid chromatographic (LC) analysis can involve (1) hydrolysis of phosphorylated forms to liberate the free vitamers PL, PM, and PN; (2) release of all the vitamers native forms including the phosphorylated and glycosylated forms (PN-glucoside), and metabolites such as 4-PA or (3) conversion of all forms into PN. Therefore, depending on the ultimate goal of the study, extraction conditions may vary *(9)*. Common extraction process involves acid hydrolysis under heating condition (either in a boiling water bath or in an autoclave) followed by enzyme hydrolysis *(28)*. The purpose of the acid hydrolysis step is to partially release free vitamers from phosphorylated and glycosylated forms and convert polysaccharide in high starch-content food into soluble sugars *(92)*. Mineral acids are normally used in this vigorous treatment step with hydrochloric acid being the most common. Specifically, in AOAC official method 961.15, animal-derived foods are autoclaved with 0.055N HCl for 5h at 121^{o}C to dephosphorylate vitamin B6 as well as release PL from its bound forms while plant-derived samples are treated with a stronger acid concentration (0.44 N HCl) to liberate PN from its glycosylated form *(110)*. Coupled with enzymatic hydrolysis of phosphate ester bonds, the release of free vitamers satisfies the requirement of the approach (1) mentioned above. The conversion of free vitamers into PN for approach (3)

can be performed right after the hydrolysis steps. PM, via the reaction with glyoxylic acid, is first converted to PL, which in turn is reduced to PN by sodium borohydride in alkaline medium *(111)*. The extraction of vitamin B6 in the native forms for approach (2) requires a milder treatment. Organic acids without heat and enzymatic treatment are often employed to denature proteins and release protein-bound vitamers in their phosphorylated and glucosylated forms *(9, 28)*.

1.3.5.2 Non-HPLC methods

Common subjects for vitamin B6 analysis are foods (natural or supplemented), pharmaceutical and biologicals (blood, plasma and urine). Vitamin B6 determination is challenging because vitamin B6 (1) occurs naturally in six different forms at relatively low levels in most biological samples; (2) has low solubility in organic solvents, making their use irrelevant in extraction and purification/enrichment procedures; (3) is highly photosensitive, which requires the analysis to be performed in a subdued light condition and (4) is tightly bound to the protein matrix in the samples *(9, 100)*. Numerous methods developed over the years satisfy different requirements of vitamin B6 analysis, but have their own disadvantages. Enzymatic and immunological assays are ideal for biological samples, they also have certain limits in application. Enzymatic assays can only detect PLP *(112)*. As to immunoassays, different forms of vitamin B6 require different antibodies and even then the chance of cross reactivity is likely to happen, thereby compromising the selectivity of the methods *(113)*. Microbiological assays are sensitive and specific but they are time consuming and require strictly followed protocols to obtain

accurate results. Moreover, they are not suitable for biological fluids such as blood and plasma as complicated substances in those samples can either promote or inhibit the growth of micro-organisms, thereby invalidating the results *(114)*.The ideal organism for the microbiological assay of vitamin B6 should produce equal growth response to PN, PL and PM *(2)*. However, there are no single strains that satisfy this requirement as they all show differential response to the three vitamers. Commonly used microorganisms include yeast (*Saccharomyces uvarum*, *Saccharomyces cerevisiae*), bacteria (*Streptococcus faecalis*, *Lactobacillus helviticus*, *Lactobacillus casei*) and protozoan (*Tetrahymenia pyriformis*). The most commonly used microorganism is *S. uvarum* (ATCC 9080) introduced in 1943 *(115)*. *S. uvarum* responds similarly to PN and PL but less to PM. Therefore, a chromatographic procedure is employed to separate PN, Pl and PM before the microbiological assay, which forms the basis for AOAC Method 961.15 *(110)*. Another strain, *Kloeckera apiculata* (ATCC 9774), was suggested by Barton-Wright and later by Guilarte *(116, 117)*. Both studies showed equal response to PN PL and PM by K. apiculata. However, this is not conclusive among other research groups.

Spectrophotometric and fluorometric methods are mainly developed for the analysis of pharmaceuticals. Spectrophotometric methods are based on the UV absorbance of the analytes. Absorption spectra of all B6 vitamers in 0.1M HCl are similar and they show maximum absorption at around 290nm *(92*, *118)*. At different pH values, the spectra vary and may have maximum absorption at more than one wavelength. This fact can be used to increase selectivity not only in spectrophotometric methods but also in HPLC-UV. Derivatization is also helpful in enhancing the sensitivity and selectivity of

the spectrophotometric methods *(9)*. Fluorimetric methods utilizing the fluorescence capacity of vitamin B6 compounds are more sensitive and selective. PL, PN, PM and their phosphorylated counterparts PNP and PMP exhibit significant fluorescence while only PLP is weakly fluorescent *(100)*. Fluorimetric methods are ideal for pharmaceuticals, but can be limited when used for others due to the complex sample matrix. Application of the fluorimetric methods to quantitate PLP and PL has been reported but these methods are considered unreliable due to interference from other fluorescent compounds in the samples *(119, 120)*. Rigid sample cleanup procedures are therefore required *(100)*. Spectrophotometric and spectrofluorometric procedures have been coupled with flow injection or sequential injection analysis to automate the determination of vitamin B6 in pharmaceuticals *(121, 122)*.

Early electrochemical assays for the analysis of vitamin B6 were based on the oxidation of PN at a carbon paste electrode *(9)*. Modifications made to these assays such as combining with flow injection and sequential injection techniques or using potentiometric membrane sensors and modified glassy carbon electrodes have made these assays more selective and efficient *(123, 124)*.

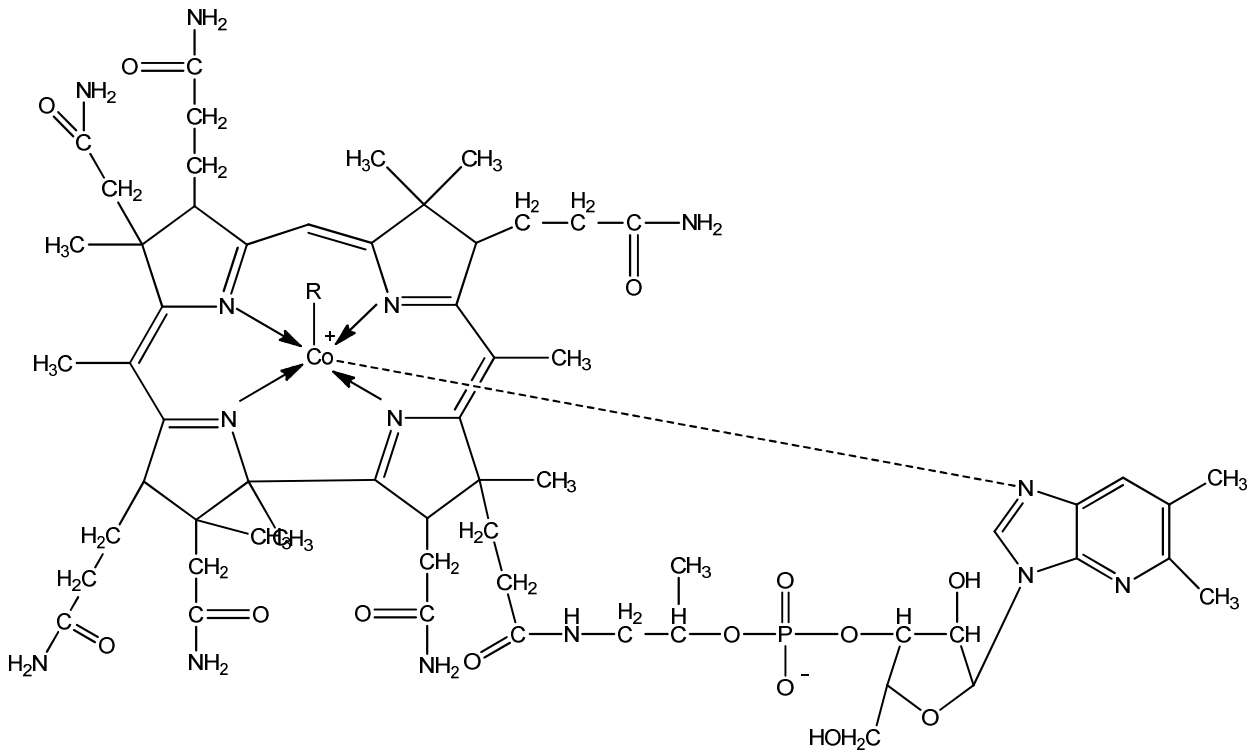
1.4 Vitamin B12

1.4.1 Nomenclature, structure and physiochemical properties

Vitamin B12 deficiency (pernicious anemia) was originally described in 1855 *(125)*. Not until 1925 was the treatment reported when Whipple discovered the curative effects of raw liver for anemic dogs *(126)*. One year later, Minot and Murphy claimed

that this treatment was successfully applied to human patients *(127)*. The identification and isolation of the active compound was simultaneously reported by Folkers *(128)* in the US and Smith in Britain *(129)*. In 1956, Dorothy Hodgkin determined the structure of vitamin B12 by X-ray crystallography *(130)*.

Belonging to the corinoid family, vitamin B12 is a collective term for cobalt-containing compounds that have anti-pernicious anemia activity *(9, 26)*. The structures of cyanocobalamin and other related compounds are shown in Figure 1.6. Cobalamin is composed of a corrin backbone with a six-coordination cobalt as the center *(131)*. The corrin structure has four reduced pyrrole rings with three being joined by methylene bridges and two linking directly. It is similar to heme structure, with the central cobalt coordinating with the nitrogen atoms in the pyrrole rings. Two biologically active forms of vitamin B2 acting as coenzymes in human are methylcobalamin and 5'-deoxyadenosylcobalamin (or adenosylcobalamin) in which the methyl and adenosyl ligands bind to cobalt at the X position, respectively. Other forms shown in Figure 1.6 include aquocobalamin, hydroxycobalamin and cyanocobalamin. The latter two are available for use in medical fields and food fortification respectively *(9)*. Among them, cyanocobalamin is more stable and used more in pharmaceutical preparations and fortified foods.



-R	Name	Abbreviation
-CN	Cyanocobalamin	CN-Cbl
-OH	Hydroxycobalamin	OH-Cbl
-H2O	Aquocobalamin	HOH-Cbl
-5'-Deoxyadenosyl	5'-Deoxyadenosylcobalamin (Coenzyme B12)	AdoCbl
-CH3	Methylcobalamin	MeCBl
-SO3	Sulfitocobalamin	SO3Cbl

Figure 1.6 Structure of vitamin B12

CNCbl is an odorless, dark red crystalline hygroscopic powder which can take up more than 12% by weight of moisture *(3, 9)*. It is quite soluble in water (1.25g/100 mL at 25°C), lower alcohols, phenols and other hydroxylated polar solvents while insoluble in acetone, ether, chloroform and benzene *(2, 14)*. Crystals of cyanocobalamin decompose above 200°C without melting *(9)*.

1.4.2 Stability and degradation

Cyanocobalamin is stable in crystalline forms and aqueous solution in air at room temperature when protected from light *(2)*. On exposure to light, the cyano group is cleaved to produce OHCbl, which occurs as aquocobalamin in neutral or acidic solution *(9, 131)*. Because these two forms are equally biologically active, this photolytic reaction does not result in a loss of vitamin B12 activity. Other cobalamins such as sulfito-, chloro-, cyanato-, nitrito-, bromo-, thiocyanato-, and azido- can result from the binding of corresponding ions or groups to the central cobalt.

CNCbl exhibits optimal stability at pH 4.5-5.0 and can sustain autoclaving at 120^{o}C for 20 min in solution of pH 4.0-7.0 *(2)*. Hydrolysis under heating in acidic and alkaline conditions results in biologically inactive degraded products. Vitamin B12 is also susceptible to deactivation in the presence of strong oxidizing agents or reducing agents, such as ascorbic acid, sulfite, and iron (II) salts *(132)*.

1.4.3 Nutritional and physiological functions

Vitamin B12 and folate are interdependent as they activate each other through the transfer of the methyl group from the latter to the former *(12, 133)*. Acting in conjunction, they both play important roles in the regeneration of methionine and the synthesis of DNA and RNA *(134)*. Moreover, vitamin B12 also helps maintain the sheath surrounding nerve fibers and participate in bone cell activity and metabolism *(12)*. The two biologically active forms of vitamin B12, adenosylcobalamin and methylcobalamin,

function as coenzymes for methylmalonyl-CoA mutase and methionine synthase which play key roles in the metabolism of propionate and amino acids *(21)*.

The RDA for vitamin B12 is the lowest among all the vitamins listed with only 2.4 micrograms per day for adults *(132)*. Unlike other water soluble vitamins, vitamin B12 can be reserved in the body up to 2-5mg, about 50% of which is stored in the liver *(2, 133)*. Approximately 0.1%-0.2% of this amount is excreted via renal and biliary routes per day. As most of the vitamin B12 secreted in the bile is recycled via enterohepatic circulation, the liver can efficiently store a sufficient amount during long periods of deprivation. Therefore, vitamin B12 deficiency is rarely seen.

Vitamin B12 deficiency mostly results from inadequate absorption which is due to the lack of either hydrochloric acid or intrinsic factor *(132, 133)*. These conditions are normally observed in the elderly who are prone to atrophic gastritis causing damage to the stomach cells or those with a defective gene for the intrinsic factor. Subsequent vitamin B12 deficiency is called pernicious anemia and characterized by large, immature red blood cells due to the inhibition of DNA synthesis *(12)*. The factor responsible for slowing down DNA synthesis is actually folate deficiency itself. Because vitamin B12 and folate activation is closely related, folate can mask vitamin B12 deficiency anemia when it is given instead of the needed vitamin B12. However, in this case, other symptoms of vitamin B12 are not treated. As vitamin B12 is needed to protect and promote the normal growth of nerve fibers, its deficiency can cause severe damage to the nervous system. Preliminary symptoms such as fatigue, depression, impaired cognition and poor memory may ensue a marginal deficiency *(26)*. However, these symptoms are

not specific enough to diagnose the deficiency, especially if sufficient folate is provided in the diet. Excess vitamin B12 consumption appears to cause no harm so no UL has been established *(134)*.

1.4.4 Occurrence and distribution in foods

Vitamin B12 is synthesized solely by bacteria and other microorganisms found in soil, water, sewage or the intestinal tract of animals *(132)*. Only animal-derived food contains vitamin B12 though a small amount may be detected in plants due to microbial contamination from soil or manure *(2)*. Meat, fish, egg, cheese and milk are good sources of vitamin B12 *(12)*. Contrary to common belief, yeast is not a good source of vitamin B12 *(135)*. When grown in a vitamin B12-enriched media, yeast may provide some amount but yeast itself does not contain active vitamin B12. Fermented soy products and sea algae such as spirulina do not contain active vitamin B12 either *(135)*. As vitamin B12 is found exclusively in food of animal origin, vegans are likely to have suboptimal vitamin B12 intake. In this case, vitamin B12–fortified products or supplements are reliable sources. Except for possible loss through leaching, vitamin B12 is stable to most food processing and cooking techniques *(12)*. However, unlike other water-soluble vitamins, it is susceptible to microwave heating. Degradation of up to 40% of vitamin B12 content was reported in beef, pork, and milk by microwave processing *(136)*.

1.4.5 Analytical methods

1.4.5.1 Extraction

AOAC Official method 952.20 is a microbiological assay recommended for the determination of vitamin B12 in vitamin preparation *(137)*. However, the extraction protocol in that method has been proven to be applicable to food samples as well *(138)*. The procedure involves homogenizing the sample in an extraction solution containing disodium phosphate, citric acid and sodium metabisulfite, which is then autoclaved at 121^{o}C for 10 minutes. The purpose of the heat treatment is to release protein-bound cobalamins and catalyze the conversion of those cobalamins into more stable sulfitocobalamin form *(2)*. Modifications of the AOAC procedure using various extraction buffers and protein denaturant acids have been reported. Alternative conversion of liberated cobalamins into cyanocobalamin is also commonly used.

Procedures may also vary depending on the nature of the samples. Simple matrix such as pharmaceuticals can be easily prepared by solubilization into water without further treatment while more complicated food matrices may require treatment with various buffers *(139, 140)* or trichloroacetic acid *(141)* and even enzymatic hydrolysis with α-amylase and pepsin to better release the protein-bound cobalamins *(142, 143)*. Because vitamin B12 normally occurs only in trace amounts, its extraction often requires a cleanup and/or concentration step to enhance the analysis selectivity and sensitivity *(139, 140)*. Commonly used tools include solid phase extraction, ion-exchange and immunoaffinity chromatography.

1.4.5.2 Non-HPLC methods

Paper chromatography and thin-layer chromatography are among the earliest techniques used to separate cobalamins and corrinoids *(144, 145)*. Cellulose-based adsorbent materials were frequently used with complicated solvent mixtures *(145)*. Two-dimensional TLC was reported for cobalamin separation in blood plasma by Linnel *et al (146)*. The bioautography technique first developed by Linstrand and Stalberg allowed the visibility of cobalamins in the chromatograms utilizing the growth response of cobalamin dependent E. coli *(147)*. Areas on the agar dish inoculated with this strain of bacteria exhibit growth on exposure to cobalamins separated in the TLC or paper chromatograms.

Microbiological assays published by AOAC use *Lactobacillus delbrueckii* (ATCC 7830) for the determination of vitamin B12 in pharmaceutical preparations and food matrices *(137)*. It is sensitive enough to quantitate CNCbl at the level of 1.0pg/ml *(9)*. However, *L. delbrueckii* has similar growth response to hydroxocobalamin, sulfitocobalamin, dicyanocobalamin, and nitritocobalamin while lower and greater response was observed with adenosylcobalamin and methylcobalamin respectively *(148)*. Therefore, it is recommended that these two cobalamins be converted to hydroxycobalamin by exposure to fluorescent light before the microbiological assays *(148)*. Specificity of the assays is compromised due to the interference of other vitamin B12 biologically inactive analogs to the growth response of *L. delbrueckii (2)*. However, such compounds are mainly found only in fermented materials and their occurrence in foods is low enough not to cause significant interferences in the analysis. Another

microorganism used in a procedure developed by Ford is *Poterioochromonas malhamensis (149)*. Having similar growth response to that of *L. delbrueckii*, this protozoan is more specific and responds only slightly to nonbiologically active cobalamins. However, a comparative study of the two microorganisms on the analysis of various food products did not show much difference in the obtained results *(150)*. Considering the longer incubation time of the *P. malhamensis* method, *L. debrueckii* is preferable in most laboratories.

Radio-ligand binding assays are more often routinely used for blood and tissue analysis than for food analysis. The methods utilize intrinsic factor (IF) as the binding protein and [Co]CNCbl as the radiolabeled ligand *(151, 152)*. Besides IF, other binding proteins used include transcobalamin I (TC I), transcoblamin II (TC II) and haptocorrin or R-binder *(9)*. However, one disadvantage of the radio-ligand binding assay is that the binding proteins can bind other biologically inactive cobalamins, compromising the assay specificity *(9)*. More specific procedures available for CNCbl and AdoCbl are developed using monospecific antisera can overcome the non-specific binding with other cobalamins *(152)*. In food analysis, though radio-ligand binding and microbiological assays show some degree of agreement for most foods, they are not interchangeable. In many cases, radio-ligand procedures gave lower values than microbiological assays *(148)*.

Aqueous solution of cobalamins has maximal absorbance in the region of 350-370nm *(2, 132)*, which is utilized in reported spectrophotometric procedures. However,

because cobalamin absorption is not intense, these methods are only applicable to high concentration pharmaceutical products *(153, 154)*.

Cobalamins are relatively weak fluorescent compounds; therefore, few reported methods utilize their native fluorescence *(155)*. Watanabe et al. proposed the conversion of vitamin B12 into a highly fluorescent derivatives using 6, 7-dimethoxy-1-methyl-2(1H)-quinoxaline-3-propionylcarboxylic acid hydrazide (DMEQ) followed by the binding of the derivative with hog intrinsic factor *(156)*.

Several chemiluminscence methods have been published. The method proposed by Liu *et al*. makes use of the energy transfer fluorescence quenching of Acridino orange-Rhodamine B and the ability of vitamin B12 to diminish the fluorescence intensity of the Rhodamine B *(157)*. Another chemiluminesence-based method by Song and Hou relied on the enhancement of cobalt (II) on the chemiluminescence reaction between luminol and dissolved oxygen . When combined with flow injection analysis (FIA), these methods not only offer high sensitivity but also provide a high sample throughput.

1.5 Vitamin C (Ascorbic acid)

1.5.1 Nomenclature, structure and physiochemical properties

Vitamin C is the generic descriptor referring to a class of compounds that exhibit the same biological activity as ascorbic acid and exert the preventive effects against scurvy, a vitamin C deficiency disease *(158)*. Although the curative effects of citrus fruits were first documented by James Lind, a British naval physician in 1747, it had not been

until the 1930s that the active antiscorbutic component was isolated from natural sources by Szent-Györgyi group and Haworth and King *(159, 160)*.

Ascorbic acid occurs naturally in two stereoisomer forms, L-ascorbic and D-isoascorbic (also known as erythorbic acid), among which, only the former shows vitamin C biological activity. The terms L-ascorbic acid and ascorbic acid can be used interchangeably as the trivial names accepted by IUPAC for vitamin C *(158)*. Its systematic chemical designator is 2, 3-didehydro-l-threo-hexano-1,4-lactone (C6H8O6, MW =176.1) *(3)*. Other active compounds include esters of ascorbic acid like ascorbyl palmitate, the synthetic form 6-deoxy-l-ascorbic acid or the oxidized form L-dehydroascorbic acid. The structure of ascorbic acid and its related compounds are all provided in Figure 1.7.

Ascorbic acid is an odorless, white to pale yellow crystalline powder with a pleasant sharp taste. It has a high water solubility (33g/100ml at ambient temperature) and an mp of about 190^{o}C (with decomposition) *(2, 14)*. Ascorbic acid has two ionization sites at the hydroxyl groups on C3 and C2 with pKa of 4.17 and 11.79 respectively *(3)*. The most prominent chemical property of ascorbic acid is its strong reducing ability of the carbonyl enediol group, which leads to it being used as the antioxidant agent in food technology *(9)*. Other important functional roles of ascorbic acid include its application as a nutritional food additive, browning inhibitor and stabilizer especially in the processing of beverages, wines, and meat products *(9)*.

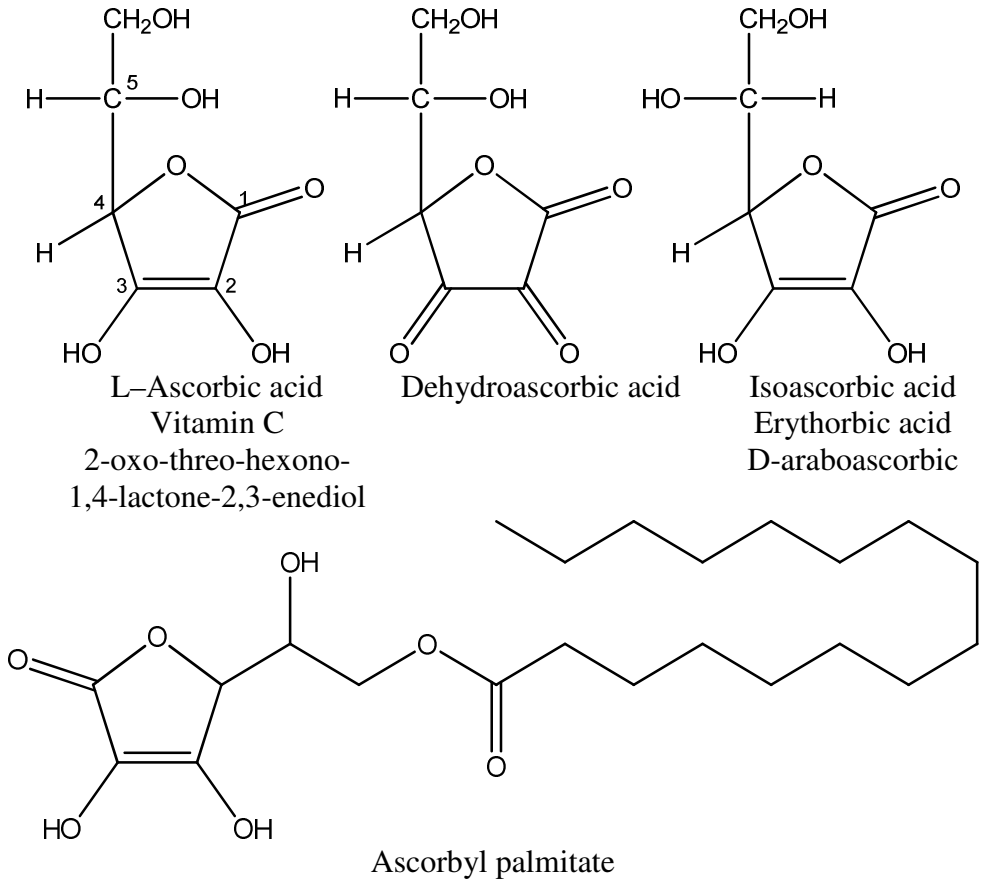


Figure 1.7 Structure of L-ascorbic acid and related compounds

The synthetic lipid-soluble form of ascorbic acid, ascorbyl palmitate exhibits 100% relative antiascorbutic activity of ascorbic acid and can be used synergistically with other fat soluble antioxidants such as tocopherols *(2)*. Another synthetic form of ascorbic acid is erythorbic acid that possesses similar reductive properties to ascorbic acid. However, erythorbic acid only exhibits about 5% of antiascorbic activity of ascorbic acid *(161)*. Being commercially cheaper to manufacture, erythorbic can be used as a substitute for ascorbic in some countries when the antioxidant and not the nutritional properties is required *(2)*.

Ascorbic acid is readily oxidized to dehydroascorbic acid in a reversible reaction. In the human body, this oxidized form is easily reduced back to ascorbic acid; therefore full vitamin C activity is maintained *(2)*. Dehydroascorbic acid is a misnomer as it is not an acid per se due to the lack of dissociable protons at C2 and C3.

1.5.2. Stability and degradation:

Pure crystalline ascorbic acid and sodium ascorbate are highly stable even in the presence of oxygen and on the exposure to daylight at normal room temperature for long periods of time as long as it is kept in dry conditions *(9)*. One study found that commercial form of ascorbic in vitamin C tablets can have their potency intact even after a storage period of 8 years at 25^{o}C *(162)*.

Ascorbic acid is much less stable in solution due to its strong reducing ability which results in rapid oxidation to dehydroascorbic acid. The process is slower in the pH range of 3.0-4.5 than 5.0-7.0 *(163)*. At neutral and alkaline pH, ascorbic acid is highly unstable due to not only the much faster conversion to dehydroascorbic acid, but also further degradation of dehydroascorbic acid in a non-reversible reaction to the biologically inactive straight-chained product named 2,3-diketo-1-gulonic acid *(2)*.

The stability of ascorbic acid in food is quite dependent on the pH level. At low pH, ascorbic exists in the fully protonated form which is less susceptible to degradation. Optimal pH range for ascorbic stability is between pH 4.0 and 6.0 *(158)*. However, the whole oxidative degradation of ascorbic in food is a complicated process influenced by many factors including oxygen availability, thermal processing conditions, oxidizing

lipid effects, the presence of transition metal catalysts, antioxidants and ascorbic acid oxidase *(9)*.

1.5.3 Nutritional and physiological functions

With its strong reducing ability, vitamin C works as an antioxidant to protect the body from free radicals which are highly unstable and reactive molecules with one or more unpaired

electrons *(164)*. Figure 1.8 shows how vitamin C can readily donate its electrons to neutralize free radicals, preventing the chain reactions from damaging other substances; and then the reactive vitamin C radical itself reacts with another radical to become reactivated. Vitamin C is stored mostly in the adrenal glands and is released together with hormones into the blood stream when the body is exposed to stresses such as infections, burns, ingestion of toxic heavy metals, extremely high/low temperatures and cigarette smoking *(12)*. It is believed that the vitamin C antioxidant property plays an important role especially when stress triggers the immune system into action *(158)*. As the immune system relies on free radicals to attack the invasive microorganisms and remove the damaged cells, vitamin C comes into play as an antioxidant to keep this oxidative activity in control. The reducing property of vitamin C also plays an important role in enhancing iron absorption in the body by protecting iron from oxidation *(26)*.

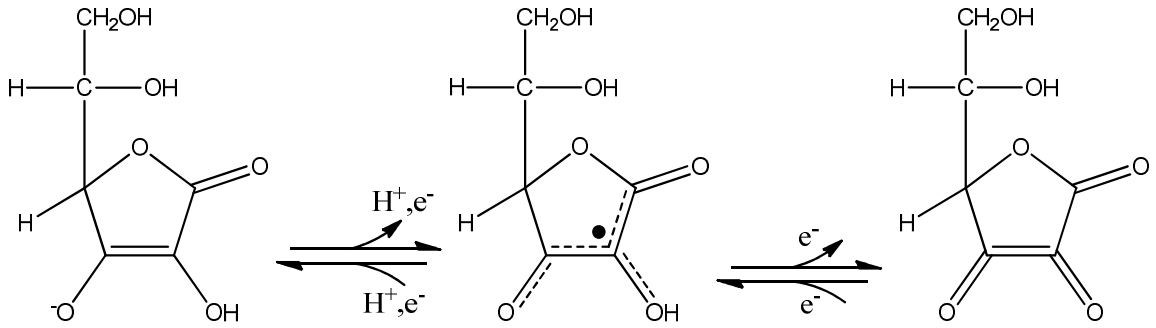


Figure 1.8 Oxidation of ascorbate
L-ascorbate anion AH^- loses an electron to a free radical like $^{\bullet}OH$, resulting in the formation of ascorbyl radical $A^{\bullet-}$ which then reacts with another radical to yield dehydroascorbic acid. Vitamin C is therefore recycled and the reservoir of antioxidants is maintained in the body.

Vitamin C plays an important role as a cofactor in the synthesis of collagen which is a fibrous structural protein of connective tissues *(21)*. Collagen formation requires the conversion of proline and lysine to hydroxyproline or hydroxylysine, allowing the collagen molecule to take up its shape as a triple helix with a strong, ropelike structure. This hydroxylation process is facilitated by the activity of hydroxylase enzymes for which vitamin C acts as a co-factor *(165)*.

Vitamin C is needed for the synthesis of other compound as well. It aids in the hydroxylation of carnitine which is important for the transport of long-chain fatty acids across mitochondrial membranes in cells *(12)*, and the conversion of tryptophan and tyrosine to neurotransmitters serotonine and norepinephrine as well as the production of the metabolic rate regulating hormone thyroxin require vitamin C *(12)*.

In human adults, classic symptoms of scurvy occur after 45 to 80 days of vitamin C deprivation *(165)*. The early notable signs of deficiency include gum bleeding around the teeth and skin lesion *(12)*. As the deficiency worsens, scurvy symptoms start to

escalate. Malfunctioning collagen synthesis results in further hemorrhaging, muscle degeneration, impaired wound healing, tooth loss, edema and bone weakness *(26, 158)*. Anemia, infections and psychological changes, including hysteria and depression are also commonly observed *(134)*. As little as 10 milligrams daily can prevent scurvy but that amount is not enough to maintain the healthy reserve of vitamin C in the body *(21)*. The RDA for adult women and men are 75 and 90mg/day respectively *(9)*.

1.5.4 Occurrence and distribution in foods

Humans are among the few species that cannot synthesize vitamin C; therefore, it is an essential nutrient that needs to be provided in human diets. Beside citrus fruits which have long been known to be an excellent source of vitamin C, other fruits and vegetables such as strawberry, blackcurrant, bananas, broccoli, spinach, bell pepper, etc. can also potentially provide a generous amount of the vitamin to the human diets *(2, 12)*. Cereal grains and legumes are examples of poor sources of vitamin C *(12)*. While human milk is an adequate source to prevent scurvy in infants, cow's milk is significantly lower in the vitamin due to the oxidative loss during processing *(2)*. Organ meats (liver, kidneys, heart) have some vitamin C but muscle meats contain literally none *(165)*. In cured meats such as luncheon meats, the manufacturer may add erythorbic acid, which is another isomer of ascorbic acid to prevent oxidation and spoilage *(12)*. It is worth mentioning that this compound has only little vitamin C activity in the human body.

Losses in cooking are not limited to leaching into the cooking medium and the degree of heating. Further degradation upon the exposure to water and oxygen in

combination with other factors such as pH and transition metal catalysts can result in significant loss of the vitamin. Baking also potentially reduces vitamin C content because it can participate in the Maillard browning reaction *(9)*. Therefore, in general, cooked foods usually have lower vitamin C content than their raw counterparts.

1.5.5 Analysis

1.5.5.1 Extraction

Due to its labile nature, the key success to extraction procedures of vitamin C is to stabilize the compound in the sample *(9)*. Ideally the extraction solution should provide an acidic medium (preferably below 4.0) to ensure the stability of both ascorbic and dehydroascorbic acid *(166)*. Moreover, it is also expected that the extractant chelate metals, denatures and precipitates proteins (thereby inactivating all enzymes including ascorbic acid oxidase) and limit soluble oxygen *(166)*. Over the years, there have been many procedures developed to better suit different sample matrices and determinative analytical methods. Metaphosphoric acid at 3% concentration dissolved in 8% glacial acetic acid suggested in AOAC Official Method 967.21 has been the most commonly used extractant *(167)*. Modification of the procedure with the addition of EDTA to enhance metal chelation is made in AOAC Official Method 985.33 *(168)*. In those samples where starch could interfere with colorimetric titrations or fluorometric assays, ethanol or acetone can be added to the metaphosphoric extract to remove solubilized starch *(169)*.

1.5.5.2 Non-HPLC methods

One of the most common and simplest method of vitamin C determination is the AOAC titration method with 2, 6-Dichlorophenolindophenol (DCPIP) *(167)*. The principle of the method is based on the reduction of DCPIP by ascorbic acid *(170)*. In its oxidized form, DCPIP has a deep blue color at neutral or alkaline pH and pink in acid solution. Upon reacting with ascorbic acid, this dye is reduced into a colorless form. The endpoint is therefore signaled when excess DCPIP display the pink color in the acid extract of the sample. If the endpoint is difficult to be detected in colored samples, absorbance at 518 nm can be measured to alternatively determine the endpoint. The method cannot measure dehydroascorbic acid and distinguish between l-ascorbic acid and isoacorbic acid *(9)*. Moreover, its specificity is open to question due to the fact that all reducing agents contained in the sample can react with DCPIP, leading to possible overestimation of ascorbic acid content *(2)*. Interfering compounds include cuprous, ferrous ions, sulfite, thiosulfate, tannins, betanin, cysteine and glutathione. Many modifications such as adding chelating agents to suppress the interference from the metal ions *(168)* or using SPE *(171)* to remove significant interferences from the sample matrix were suggested to minimize the interfering effects on the titration.

Another method that utilizes the redox reaction is the metal ion reduction method, the principle of which is to form a stable colored complex between the reduced ion and a chelator *(172)*. The complex is then spectrophotometrically measured. The most commonly used metal ion redox reaction is the reduction of Fe(III) to Fe(II) for which

2,29-dipyridine, 2,4,6-tripyridyl-5-triazine, and ferrozine are usually used as the chelating agents. Nobrega employed Fe (III) and hexacyanoferrate (III) as chromogenic complexing reagents and combined the method with flow injection technique to quantitate ascorbic acid *(173)*.

Derivatization methods require the conversion of L-ascorbic acid to L-dehydroascorbic acid using activated charcoal *(174)* or DCPIP *(175)* as the first step. AOAC International Method 967.22 developed by Deutsch and Weeks is based on the condensation reaction between o-phenylenediamine (OPD) and L-dehydroascorbic acid to form a highly fluorescent quinoxaline product which is then determined fluorimetrically at Ex λ = 350, Em λ = 430 *(174)*. Another less extensively used derivatization method reacts 2,4-Dinitrophenylhydrazine (DNPH) with L-dehydroascorbic acid under acidic conditions to form a red osazone derivative, the absorbance of which is then determined at about 520nm *(176)*. Developed in 1943, this DNPH method is suitable for the analysis of total vitamin C in samples with low sugar content.

Enzymatic treatment with ascorbate oxidase or ascorbate peroxidase can be used instead of chemical treatment to convert L-ascorbic acid to L-dehydroascorbic acid before the OPD derivatization step *(177, 178)*. Tsumura *et al.* reported the efficient use of guaiacol peroxidase, a commercially available enzyme extracted from horseradish for the oxidation step. The enzymatic treatment was then combined with the direct spectrophotometric assay to determine ascorbic acid in various foods *(179)*.

The most noticeable modification in L-ascorbic acid analysis in recent literature is the coupling of flow injection analysis and sequential injection analysis with proven approaches such as spectrophotometric *(180)*, fluorescence *(181)*, chemiluminescence *(182)*, and electrochemical *(183)* determinations to provide rapid and efficient analytical methods.

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CHAPTER TWO

CHROMATOGRAPHIC BEHAVIORS OF THIAMINE, RIBOFLAVIN, PYRIDOXINE, CYANOCOBALAMIN AND ASCORBIC ACID

2.1 Introduction

Playing an essential role in normal growth and maintenance of the body, vitamins are needed only in a small amount daily that can be easily provided with proper diets *(1)*. However, the human diet sometimes fails to meet the daily vitamin requirements so some people are more prone to vitamin deficiency than others *(2)*. In those cases, fortification of food products such as infant formula, cereal, low-calorie foods, juices, etc. becomes important in ensuring an adequate intake of vitamins *(3)*. Therefore, a rapid and simple vitamin analysis in supplemented food products would benefit the proper regulation of fortification. The current regulatory and standard methods for water-soluble vitamin analysis are mostly based on microbiological techniques developed more than 50 years ago *(4, 5)*. Although they can offer high sensitivity, these methods are time-consuming and sometimes not sufficiently specific *(6)*. Other proposed methods involve spectrophotometric or fluorimetric techniques which are sometimes time-consuming and not adaptable for simultaneous determination of water-soluble vitamins with different chemical and physical properties *(4-6)*.

Another popular current method of choice is HPLC which was first utilized for the analysis of vitamins in the 1970s. It is favored due to the convenience, specificity, sensitivity and accuracy, especially with the current improvement in the chromatography

technology. United States Pharmacopeia (USP) and Association of Official Analytical Chemists (AOAC) both have included standard chromatographic methods for vitamin analysis in their handbook, which are summarized in Table 2.1 and 2.2. However, most of these methods only focus on the analysis of a single vitamin at a time. Moreover, in order to improve retention and peak shape of the vitamin analytes, they require the use of many complicated mobile phase components, most of which are not directly transferable to other detectors, especially MS without significant modifications.

For the past two decades, there have been many HPLC methods developed and reported in the literature for vitamin analysis. The trend is to develop multi-vitamin analysis methods which are simple and easy to transfer. Thanks to the chromatography literatures, it is unnecessary to reinvent the wheel when it comes to developing HPLC methods for routine vitamin analysis. However, applying certain methods directly from literature more than often fails to reproduce the results reported due to many variables in liquid chromatography. In the present study, chromatographic behaviors of the five water-soluble vitamins including thiamine, riboflavin, pyridoxine, cyanocobalamin and ascorbic acid were studied. The ultimate goal of the project is to help the analysts better modify methods from literature or even develop a novel method to fit the need of their analysis with the resources available in their lab (columns, detectors, etc.)

Table 2.1 Chromatographic methods for vitamin analysis suggested by AOAC

Vitamin (Form)	Method and application	Approach
Vitamin B6 (Pyridoxine)	50.1.26 AOAC Official Method 2004.07 Vitamin B6 in baby foods and reconstituted infant formula	Column: Phenomenex Luna 5 µm phenyl-hexyl column, 250x4.6 mm id or other equivalent reversed-phase C8 and C18 columns Mobile phase: Methanol-0.01M phosphoric acid (26:74) with 0.05% 1-heptanesulfonic acid (w/v), pH 2.50-2.60. Condition: flow rate 1.0 mL/min; fluorescence detection λ_{ex}=290 nm and λ_{em} =395 nm
Vitamin B12 (Cyanocobalamin)	50.1.31AOAC Official Method 2011.08 Vitamin B12 in baby foods, infant formula and adult nutritionals	Column: Macherey-Nagel Nucleosil 100-3 C18 HD, 125x3.0mm, or C18 ACE 3AQ, 150x3.0 mm Mobile phase: (A) 0.025% TFA in water, (B) 0.025% TFA in acetonitrile. Conditions: flow rate 0.25ml/min; injection volume 100 µL; detection UV 361 nm; and gradient elution
	AOAC Official Method 2011.09 Vitamin B12 in baby foods, infant formula and adult nutritionals	Column: ACE 3AQ, 150x3.0 mm or equivalent Mobile phase: (A) 0.025% TFA and (B) acetonitrile Conditions: flow rate 0.25ml/min; injection volume 100 µL; UV detector 361 nm; gradient
	AOAC Official Method 2011.10 Vitamin B12 in baby foods, infant formula and adult nutritionals	HPLC system: Gradient system with switching valve and isocratic pump on side and a UV-Vis detector. Autosampler capable of injecting 2 mL sample. Column: 1/ Analytical size-exclusion column Zorbax GF-250 4µm, 250x9.4mm or Shodex Protein KW 5µm, 300x8mm or equivalent; 2/ Thermo Scientific Aquasil C18 3µm, 100x4.6mm or equivalent Mobile phase: 1/Isocratic pump: 2.5% acetonitrile in water. Flow rate: 1.1-1.2ml/min. 2/ Gradient pump: (A) 0.4% TEA in water, pH 5-7; (B) 0.4% TEA and 25% acetonitrile in water pH 5–7; (C) 0.4% TEA and 75% acetonitrile in water pH 5–7 Conditions: UV 550nm

Source: The Official Methods of Analysis of AOAC International, 19th Edition, 2012

Table 2.2 Chromatographic methods for vitamin analysis suggested by USP

Vitamin forms	Approach
Ascorbic acid Method 1 (pg 1670)	Titration with standard dichlorophenol-indophenol solution
Ascorbic Method 2 (pg 1738)	Titration with 0.1N sodium thiosulfate
Biotin Method 1(pg 1671)	Column: 4.6x150mm, 3 µm packing L7 Mobile phase: Mixture of 85 ml acetonitrile, 1 gram sodium perclorate and 1mL phosphoric acid diluted to 1 L with water Conditions: flow rate 1.2 mL/min, UV detection at 200nm
Biotin Method 3 (pg 1701)	Column: 4.6x250mm, packing L1 Mobile phase: solution A with 100 ml triethylamine, 80ml phosphoric acid 85% diluted to 1L. Mix 80 ml acetonitrile and 10 mL solution A and dilute to 1 L. Sample clean-up with SPE Conditions: flow rate 2.0 mL/min, UV detection at 200 nm
Cyanocobalamin Method 1(pg 1672)	Column: 4.6x150mm, 5µm packing L1 Mobile phase: Methanol:Water (7:13) Conditions: flow rate 0.5 mL/min, UV detection at 550 nm
Folic acid Method 1(pg 1675)	Column: 3.9x300mm, packing L1 Mobile phase: 2 gram of monobasic potassium phosphate in 650 mL water + 12 mL of tetrabutylammonium hydroxide 25% in methanol +7.0 mL phosphoric acid + 240ml methanol. Adjust with phosphoric acid or ammonia to pH 7.0 then dilute with water to 1 L. Conditions: flow rate 1.0 mL/min, UV detection at 280 nm
Folic acid Method 2 (pg 1675)	Column: 4.6x250mm, packing L7 Column temperature: 50^{o}C Mobile phase: Mixture of 0.4 mL triethylamine, 15 mL acetic acid, 350ml methanol diluted with 8 mM sodium 1-hexanesulfonate to 2 L. Conditions: flow rate 2.0 ml/min, UV detection at 270 nm
Calcium pantothenate Method 1(pg 1677)	Column: 3.9x150 mm, packing L1 Mobile phase: phosphoric acid in water (1:1000) Conditions: flow rate 1.5 mL/min, UV detection at 210 nm
Calcium pantothenate Method 3 (pg 1679)	Column: 3.9x300mm, 5 µm packing L1 Column temperature: 50^{o}C Mobile phase: methanol and phosphate buffer (1:9) (phosphate buffer contains 10g monobasic potassium phosphate in 1L water adjusted with phosphoric acid to pH 3.5

	Conditions: flow rate 2.0 ml/min, UV detection at 205 nm
Niacinamide Method 1 (pg 1679)	Column: 3.9x300 mm, packing L1 Mobile phase: 1L mixture of methanol, acetic acid and water (27:1:73) containing 1.4 mg sodium 1-hexanesulfonate Conditions: flow rate 1.0ml/min, UV detection at 280 nm
Niacinamide Method 2 (pg 1680)	Column: 4.6x250mm, packing L1 Mobile phase: 0.1M sodium acetate solution adjusted to pH 5.4 with acetic acid. A small amount of methanol (up to 1%) may be used for improved resolution. Conditions: flow rate 1.0 mL/min, UV detection at 254 nm
Pyridoxine hydrochloride Method 2 (pg 1680)	Same as above (Niacinamide Method 2)
Riboflavin Method 2 (pg 1680)	Column: 4.6x250 mm, packing L1 Mobile phase: Mixture of sodium acetate (6.8 g/L) and methanol (13:7). Add 2 mL triethylamine per L of mixture, adjust pH with acetic acid to 5.2. Conditions: flow rate 2.0 mL/min, UV detection at 254 nm
Thiamine Method 2 (pg 1681)	Column: 4.6x250mm, packing L1 Mobile phase: Solution A (1.88 mg/mL of sodium 1-hexanesulfonate in 0.1% phosphoric acid) : Acetonitrile (46:9) Conditions: flow rate 1.0 mL/min, UV detection at 254 nm
Niacinamide, pyridoxine HCl, riboflavin and thiamine Method 3 (pg 1682)	Column: 4.6x250 mm, packing L7 Column temperature: 50°C Mobile phase: Mixture of 0.4 mL triethylamine, 15 mL acetic acid, 350ml methanol diluted with 8 mM sodium 1-hexanesulfonate to 2 L Conditions: flow rate 2.0 mL/min, UV detection at 270 nm
Niacinamide, pyridoxine HCl, riboflavin and thiamine Method 1 (pg 1706)	Column: 3.9x300mm, packing L1 Column temperature: 50°C Mobile phase: 1L mixture of methanol, acetic acid and water (27:1:73) containing 1.4 mg sodium 1-hexanesulfonate Conditions: flow rate 1.0 mL/min, UV detection at 280 nm
Calcium pantothenate Method 1 (pg 1742)	Column: 4.0x100mm, packing L1 Mobile phase: methanol and 0.2 M monobasic sodium phosphate (3:97). Adjust with 1.7 M phosphoric acid to a pH of 3.2 +/- 0.1 Conditions: flow rate 1.0 mL/min, UV detection at 210 nm
Niacinamide (pg 1745)	Column: 4.6x250mm, packing L7 Mobile phase: Mixture of 0.4ml triethylamine, 15ml acetic acid, 350ml methanol diluted with 8mM sodium 1-hexanesulfonate to 2 L Conditions: flow rate 2.0 mL/min, UV detection at 270 nm

Pyridoxine HCl (pg 1746)	Column: 4.6x250mm, packing L7 Mobile phase: Mixture of 0.4 mL triethylamine, 15 mL acetic acid, 350ml methanol diluted with 8mM sodium 1-hexanesulfonate to 2 L Conditions: flow rate 2.0 mL/min, UV detection at 270 nm

Source: U.S. Pharmacopeia, National Formulary 2013, USP36/NF31 Dietary Supplements Oficial Monographs

2.2 Materials and Methods

2.2.1. Standards and reagents

Vitamin standards were purchased from different suppliers/manufacturers: thiamine hydrochloride, pyridoxine hydrochloride and cyanocobalamin from Enzo Life Sciences (Farmingdale, NY); riboflavin from Eastman Kodak Co. (Rochester, NY) and ascorbic acid from Fisher Scientific (New Jersey, USA). All reagents were of analytical grade.

HPLC grade acetonitrile, certified ACS o-phosphoric acid 85% and trace metal grade glacial acetic acid were purchased from Fisher Scientific (New Jersey, USA). Trifluoroacetic acid 99% (TFA), heptafluorobutyric acid 99% (HFBA) and formic acid 99% were obtained from Acros Organics (New Jersey, USA). Water was purified using a Millipore Synergy UV system (Millipore Billerica, MA, USA). Mobile phase pH was measured using UB-10 pH meter from Denver Instrument (New York, USA).

2.2.2. Standard preparation:

Individual stock solutions of thiamine, pyridoxine, cyanocobalamin and ascorbic acid were prepared monthly at 1000 ppm (1mg/mL) in Millipore-purified water. Riboflavin was prepared at 50 ppm by dissolving 25 mg of the component into 500 mL of

0.05 M formic acid. The solution was then sonicated in the dark for one hour for complete dissolution. These stock solutions were kept in 1.5 mL Eppendorf tubes and stored at -80^{o}C to avoid degradation. Working solutions of vitamin standards were prepared daily by mixing and diluting the individual stock solutions in deionized water to desired concentrations. Preparation steps were performed in the subdued light condition using glasswares covered with foil to keep vitamins from degradation, especially vitamin B2, B6 and B12.

2.2.3. Instrumentation:

The LC system consisted of Shimadzu SIL-20A HT auto-sampler, Shimadzu LC-20AT liquid chromatograph, Shimadzu DGU-20A5 degasser and Shimadzu SPD-20A UV-Vis detector. All samples were filtered through Fisher Brand Nylon 25mm Syringe filters 0.22 µm and 0.45 µm Fisher Scientific before being loaded onto the HPLC system for analysis.

2.2.4 Column testing:

The five vitamins of interest were divided into two groups based on their retention: group 1 includes thiamine, pyridoxine and ascorbic acid while group 2 included riboflavin and cyanocobalamin. Working standard solutions of each group was prepared at 100 ppm level each (except for vitamin B2 at 5 ppm level). Both groups were eluted with isocratic runs programmed by adjusting the percentage of phase B coming to the mixing chamber. Chromatographic conditions are listed in Table 2.3. Mobile phase

with 0.1% formic acid (phase A) and acetonitrile (phase B) were used. Chromatographic separation of the analytes was performed on different columns, the characteristics of which are shown in the Table 2.4.

Table 2.3 Summary on HPLC conditions for two groups of vitamin analytes

Group 1 (Vitamin B1, B6 and C)	Group 2 (Vitamin B2 and B12)
Mobile phase: Isocratic with 100%A:0%B Flow rate: 0.8 ml/min Injection volume: 10µL Column temperature: ambient	Mobile phase: Isocratic with 85%A:15%B Flow rate: 0.8 ml/min Injection volume: 10µL Column temperature: ambient

2.2.5 Acid modifier testing for vitamin B1, B6 and C

The effects of different acid modifiers on chromatographic selectivity of thiamine, pyridoxine and ascorbic acid were studied. Aqueous mobile phase containing either formic acid, acetic acid, phosphoric acid or TFA with concentrations of 0.01%, 0.025%, 0.05%, 0.1% and 0.25% were prepared. Analyses were performed on an Agilent Zorbax SB-Aq column (5µm, 250 x 4.6 mm) with isocratic condition of 100% aqueous phase at the flow rate of 1.0 mL/min. Detection was set at two wavelengths of 254 nm and 280 nm. Working standard solutions of each group were prepared at 100 ppm level each for analysis.

2.2.6 Enhancing retention of B1, B6 and C

2.2.6.1 Heptafluorobutyric acid (HFBA)

Isocratic condition with 0.1% HFBA (~7.7mM) in water-acetonitrile (85:15) at flow rate of 0.5 mL/min was tested for the separation of a mixture of thiamine (B1),

riboflavin (B2), pyridoxine (B6) and ascorbic acid (C) on Agilent Zorbax Eclipse Plus C18 column (3.5µm, 150 x 3.0 mm).

2.2.6.2 Buffer with higher pH

Ammonium acetate buffer at pH 5.76 and acetonitrile were used as the mobile phase to improve the retention of thiamine and pyridoxine. Both isocratic and gradient conditions were tested on Agilent Zorbax SB-Aq column (5µm, 250 x 4.6 mm) at the flow rate 0f 1.0 mL/min for the separation of the mixture of thiamine, riboflavin, pyridoxine, cyanocobalamin and ascorbic acid.

2.2.6.3 HILIC column

The method development using HILIC (Hydrophilic Interaction Liquid Chromatography) column was performed on Agilent Technologies 1200 Series LC system consisted of G1379B Degasser, G1312A Binary pump, G1329A Autosampler, G1316A Thermostatted column compartment and G1314B Variable wavelength detector. The mobile phase included (A) 100 mM ammonium acetate buffer, pH 4.8 and (B) acetonitrile. Both isocratic and gradient conditions were tested on Phenomenex Luna HILIC column (3µm, 100 x 3.0 mm) for the separation of different mixtures of vitamin analytes.

2.2.7 Column performance calculations

Column performance was evaluated with two main factors: retention times (tR) of the five vitamin analytes and tailing factor (T_f). Tailing factor describes the asymmetry of peak shape and is calculated as follows:

$$T_f = \frac{W_{0.05}}{2f_{0.05}}$$

with $W_{0.5}$ as the width of the peak and $f_{0.5}$ as the distance from the peak center line to the front slope, both measured at 5% of the maximum peak height. For further discussion on tailing factors as well as other column performance factors, please refer to the Appendix B.

2.2.8 Testing the applicability of hydrophobic subtraction model to the prediction of chromatographic behaviors of the vitamins

Column characterization parameters obtained from "PQRI Database" on USP for selecting columns of equivalent selectivity are provided in Table 2.1. Detailed information of the hydrophobic subtraction (H-S) model behind this database is provided in Section 2.4. The retention times of the five vitamins obtained by protocols in section 2.2.4 are correlated with the five column selectivity parameters including hydrophobicity (H), steric interaction (S*), hydrogen-bond acidity (A), hydrogen-bond basicity (B) and relative silanol ionization or cation-exchange capacity at pH 2.8 (C2.8). Six columns included in this study are Ypro, YAq, ZoAq, SyPo, SyHy and UlAq.

Table 2.4 List of all columns used in the study

Abbreviation	Column name	Column size (mm)	Pore size (Å)	Particle size (µm)	Surface area (m^2/g)	Total carbon content (%)	pH range	Comments	USP classification
Type-A-silica columns									
	NovaPak (Waters)	3.9x150	60	4	120	7.3	2.0-8.0		L01
	Ultrasphere (Beckman)	4.6x250	80	5		12	2.0-7.0		L01
Type-B-silica columns with novel reverse bonded phase compatible with 100% aqueous phase									
YPro	YMC Pro C18	4.6x250	120	5		16	2.0-8.0		L01
YAq	YMC ODS-AQ	4.6x250	120	5		10,14	2.0-7.5		L01
ZoAq	Zorbax SB-Aq	4.6x250	80	5	180		1.0-8.0	No endcapped	
SyPo	Synergi Polar-RP	4.6x250	80	4		11	1.5-7.0	Ether-linked phenyl with polar endcapping	L11
SyHy	Synergi Hydro-RP	4.6x250	80	4			1.5-7.5	C18 with polar endcapping	
UlAq	Ultra Aqueous (Restek)	4.6x250	100	5		15	2.5-8.0	No endcapped	
Type-B-silica columns with conventional C18 bonded phase									
	Zorbax StableBond 80A C18	3.0x150	80	3.5	180	10	0.8-8.0		
	YMC Basic	4.6x150	Wide pore	3		7	2.0-7.5		NA
	Zorbax Eclipse Plus C18	3.0x150	95	3.5	160	9	2.0-9.0		
	Gemini C18 110A	4.6x250	110	5	375	14	1.0-12	C18 with TMS endcapping	

2.3 Overview of chemical behaviors of the five vitamins in solution

2.3.1 Thiamine

With a pKa of 9.2, the quarternary N on the thiazole ring (N-3) of the thiamine molecule remains cationic over a wide pH range. Another pKa (~4.8) is due to the protonated pyrimidine N-1', which yield uncharged pyrimidyl moiety of thiamine free base as shown in Figure 2.1 *(7, 8)*.

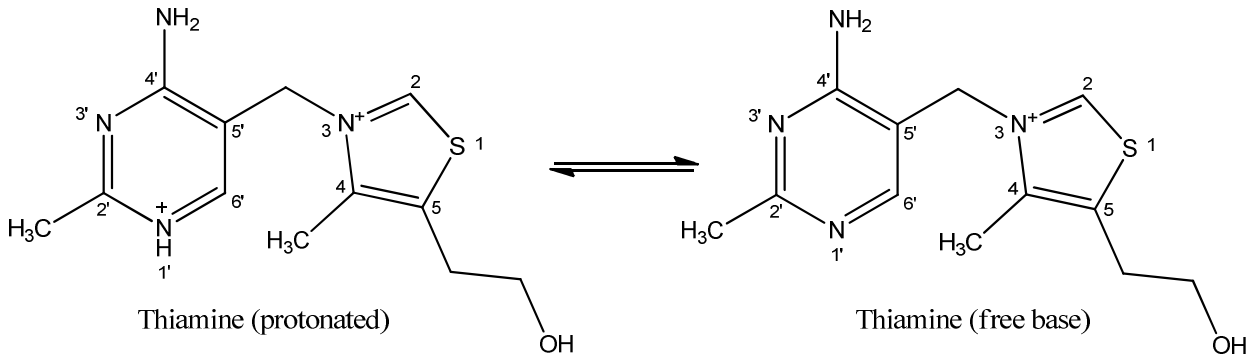


Figure 2.1 Protonation of thiamine

2.3.2 Pyridoxine

Vitamin B6 compounds occur in different ionic forms in aqueous solution depending on the pH *(9, 10)*. They exist either as cations in acidic solutions and or as anions in alkaline solutions *(1)*. Due to the opposite nature of the basic pyridinium N (pKa~8) and acidic hydroxyl groups (pKa~3.5–5.0), vitamin B6 mostly occurs in Zwitterionic form at neutral pH *(9)*. The net charge on B6 vitamers varies as a function of pH *(10)*. Shown in the figure below are the four predominant forms of pyridoxine in aqueous solution *(1, 10)*.

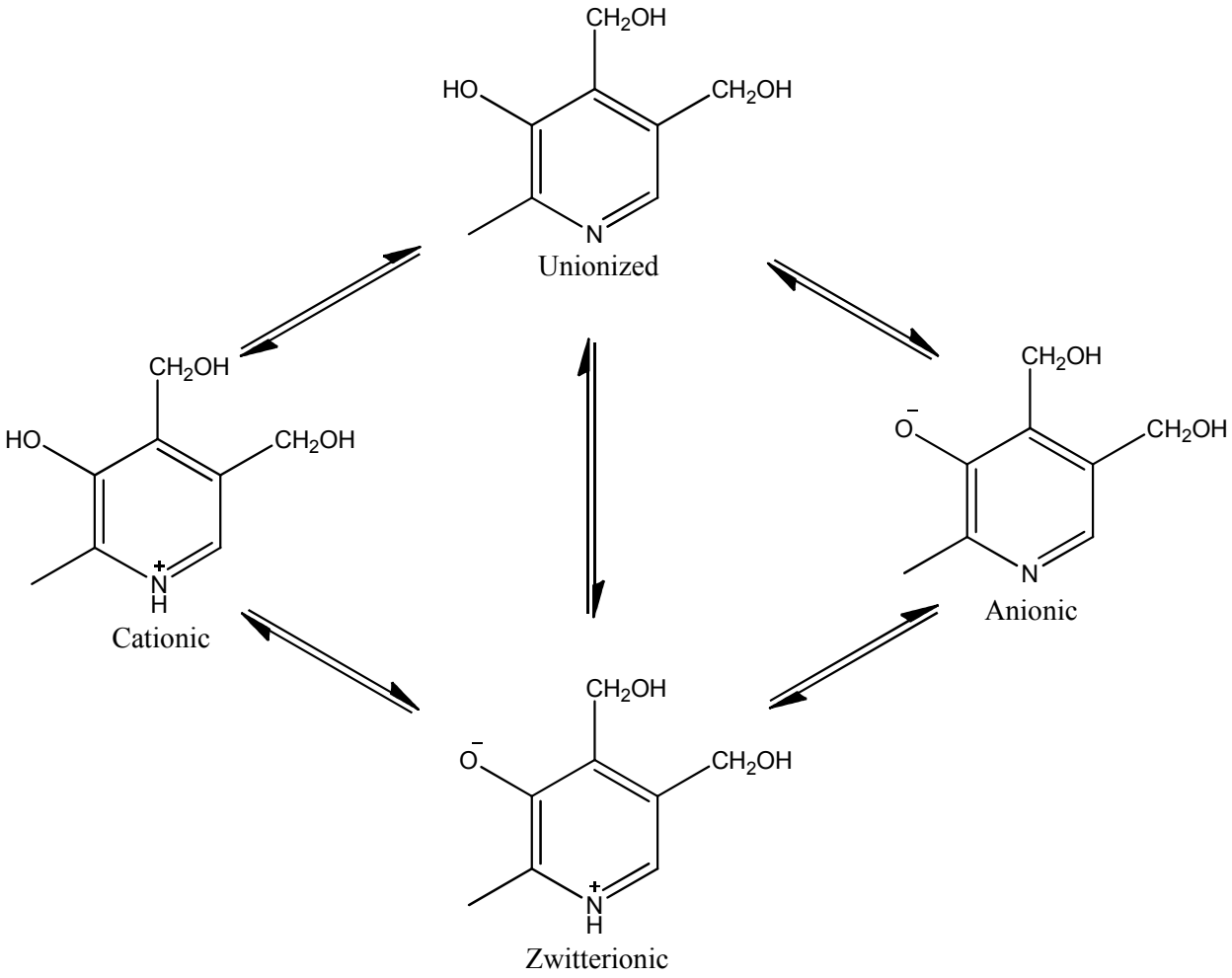


Figure 2.2 Different forms of pyridoxine in solution

2.3.3 Riboflavin

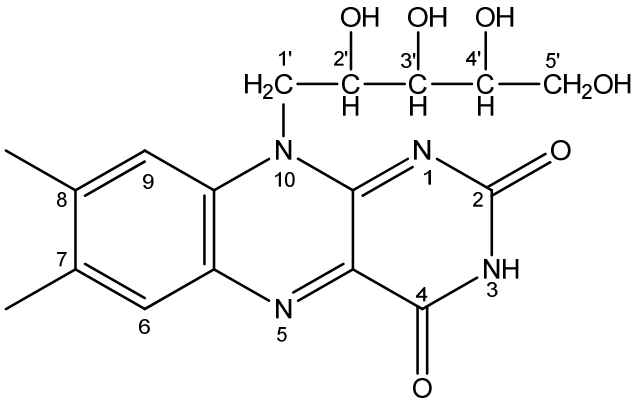


Figure 2.3 Riboflavin structure

Riboflavin itself contains various potential ionic sites that are theoretically suggested to produce different ionic forms, including the anions formed from the deprotonation of hydroxyl groups attached to C-2',3',4' and 5' of the ribityl side chain or cations formed from the protonation at N-1,3,5 and 10 (Figure 2.3) *(11)*. Empirical studies reported pKa of 10 for the protonation of riboflavin at N3 position *(12)*. Therefore, riboflavin is predicted to be non-ionized and its chromatographic behavior does not depend on the pH of the buffer within the pH range of 2.0-7.0, the normal working pH range of conventional reversed-phase silica based columns.

2.3.4 Cyanocobalamin

Though there have been many studies on the chemistry of vitamin B12 and related compounds in the literature for the past century *(13-15)*, only a few sources cited the pKa values of cobalamins and these values lack consistency across the references found *(16-18)*. According to Ladd et al., vitamin B12 is a weak base that stays

approximately neutral at pH from about 5 to 10 while it is positively charged in acidic environment and negatively charged above pH 11 *(19)*. That seems to agree with the pKa of 3.3 and 9.3 reported in two sources found *(17, 18)*.

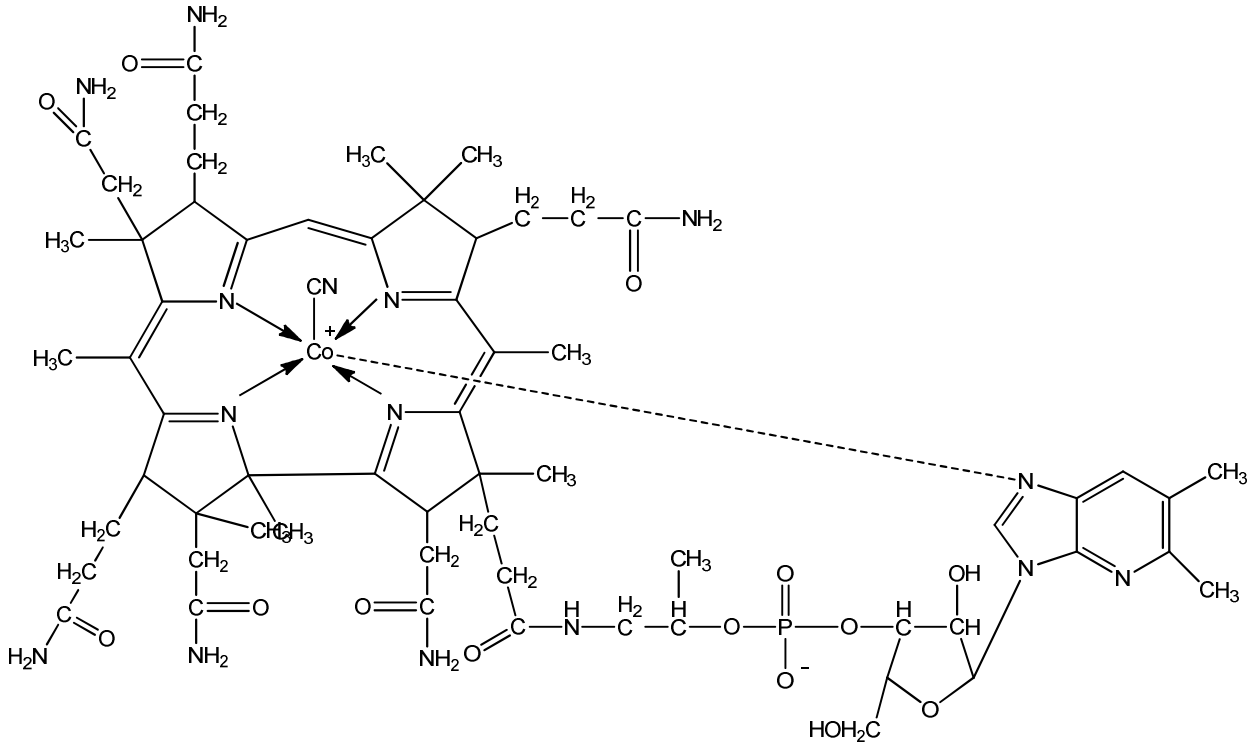


Figure 2.4 Cyanocobalamin Structure

2.3.4 Ascorbic acid

The acidic and reducing nature of L-ascorbic acid (AA) is contributed by the 2, 3-enediol moiety. Ionization of the C-3 hydroxyl group (pKa_1= 4.04) is more favorable than that of the C-2 hydroxyl (pKa_2 =11.4) *(7)*. Figure 2.5 shows the two possible ionic forms for ascorbic acid that can occur in the solution at different pH values. It is worth mentioning that even though the oxidized form L-dehydroascorbic acid retains its vitamin

C biological activity, it behaves differently in terms of chromatography *(1)*. This compound is not shown under UV-Vis detection.

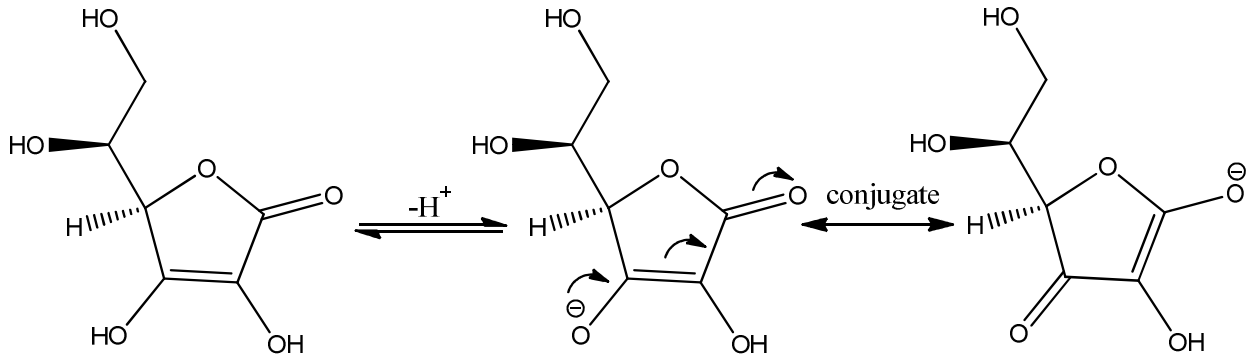


Figure 2.5 Ionic forms of ascorbic

2.4 Hydrophobic-Subtraction (H-S) model of RP column selectivity

2.4.1 Brief introduction

The theory behind the H-S model originates from the recognition that retention in RP chromatography is primarily attributed to the hydrophobic interaction among sample molecules, the mobile phase and the stationary phase, as described by solvophobic theory *(20-22)*. However, as the column could contribute to retention in additional ways, other than by hydrophobic interaction between solute and column, it became apparent that the solvo-phobic model is incomplete to describe the RPC retention and selectivity *(23)*. The hydrophobic-subtraction model proposed by Snyder and Dolan started with the assumption the major contribution of hydrophobicity to RP-LC retention is subtracted to better understand the remaining contributions to retention from other solute-column interactions *(23-30)*. Retention is quantitatively described as a function of solute and column in the following equation *(26, 30)*:

$$log\alpha = \log\left(\frac{k}{kEB}\right) = \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C \text{ (Eq.1)}$$

In this equation, k and kEB are the retention factors of a given solute and a non-polar reference solute (ethylbenzene) obtained on the same column under the same condition (50%, v/v, acetonitrile/buffer; buffer is pH 2.80, 60 mM potassium phosphate) respectively *(23)*. The separation factor α is related to the complementary properties of the solute and the column. The five terms η'H, σ'S*, β'A, α'B and κ'C in this equation refer to the five solute-column interactions shown in Figure 2.6 (a–e), respectively *(25, 31)*. The Greek letters η', σ', β', α', κ' denote complementary solute properties, where η' is solute hydrophobicity, σ' is molecular bulkiness or resistance to insertion of the solute into the stationary phase, β' is solute hydrogen-bond basicity, α' is the solute hydrogen-bond acidity, and κ' is the effective charge on the solute molecule. The other five capital letters H, S*, A, B and C refer to column properties, which are of primary practical interest because they determine the selectivity and applicability of most RPC columns.

Column hydrophobicity H increases with an increase in ligand density and ligand length attached to the particle. Small-pore packings which result in the compression of the ends of the alkyl chains, also increase ligand density, hence the value of H. End-capping of free silanols does not lead to a significant increase in total carbon, therefore it only affects H slightly.

Column steric interaction S* describes the resistance to the penetration of bulky solutes into the stationary phase. Behaving the same way as H, this parameter exhibits an increase as the bonded phase becomes more crowded (ie. longer chain length or denser

concentration of the bonded phase) and a decrease with an increase in particle pore diameter. End-capping also shows a minor effect on values of S*. However, in contrast to H, an increase in S* corresponds to bulky solute molecules being more difficult to penetrate the crowded bonded phase attached to the RP particles. This leads to less interaction between the solutes and the stationary phase, resulting in smaller k values.

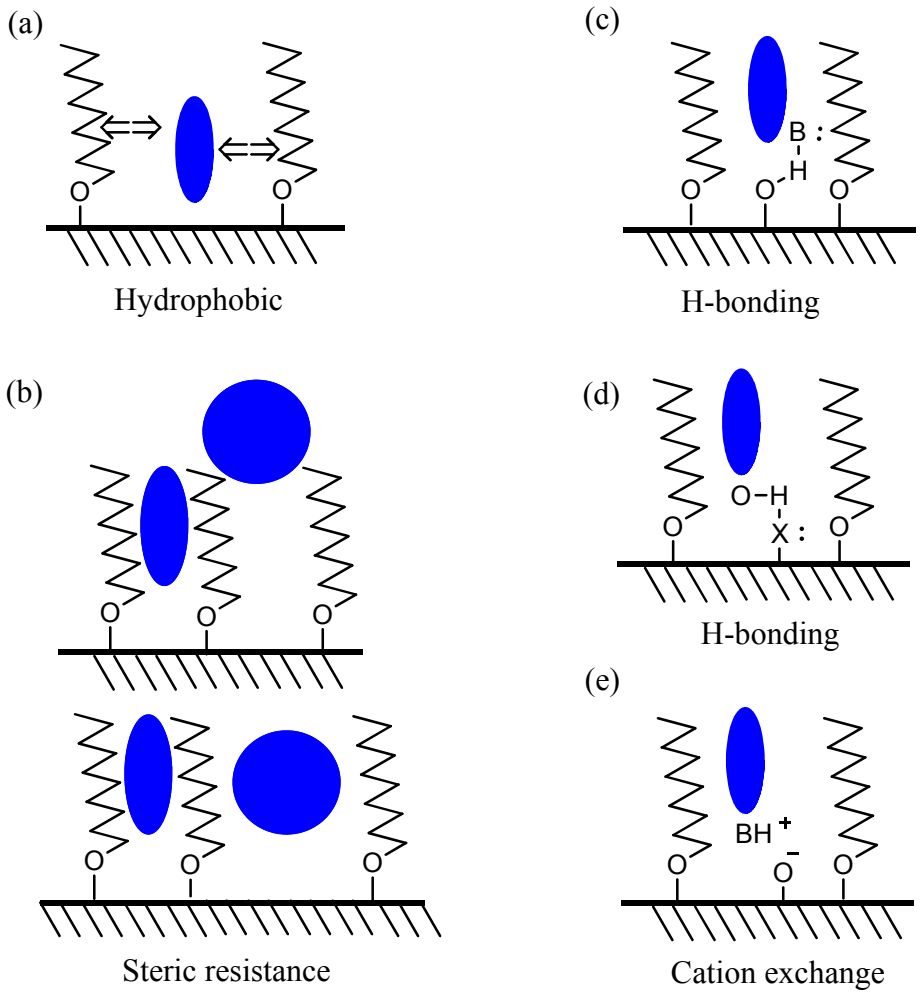


Figure 2.6 Cartoon representation of five solute–column interactions of H-S model (Adapted and reconstructed in modified forms from references 22 and 30)
Note: Figures in blue are analyte molecules. B, hydrogen-bond acceptor group of the analyte (e.g., NH_2); BH^+, protonated group of the analyte (e.g., NH_3^+); X, hydrogen-bond acceptor group of the stationary phase.

Column hydrogen-bond acidity is attributed to non-ionized residual silanols on the stationary surface. In this case, these underivatized silanols in the non-ionized form acted as a proton donor responsible for the retention of hydrogen-bond acceptor molecules as illustrated in Figure 2.6c. This parameter is therefore expected to exert significant selective effects on nonionized basic molecules such as amines and amides, especially aliphatic derivatives. Column hydrogen-bond basicity is believed to originate from various functional groups within the bonded phase. Forming a permanent part of the column surface, silanols and siloxane groups seem to be potential acceptor sites contributing to the column basicity. If that is the case, then end-capping, which reduces the number of free silanols on the surface and restricts the silanol accessibility of the solutes, is expected to cause a pronounced decrease in B. However, empirical data indicates instead a slightly positive effect of end-capping on the values of B, which negates the speculation that silanol and siloxane groups are responsible for column hydrogen-bond basicity. Supporting evidence has suggested that water from the mobile phase apparently sorbs onto the bonded phase, interacting with and binding to non-ionized carboxylic acids. This sorbed water is believed to play an important role in column hydrogen-bond basicity. Columns with greater B values preferentially retain acidic compounds. Polar-embedded columns fall in this category. With a polar functional group (urea, amide, carbamate) inserted within the alkyl ligand attached to the silica surface, these columns preferentially bind both phenols and carboxylic acids. Some type-A columns with high metal impurities also exhibit a larger value of B.

Column cation-exchange capacity C arise from the dissociation of underivatized silanols -SiOH → $-SiO^-$ + H^+. As the pH of the mobile phase increases, the silanol ionization increases, imposing more negative charges on the column, which tend to attract ionized (positively charged) bases and repel ionized (negatively charged) acids. Type B columns are less acidic than Type A columns; therefore, the C value of the former is expected to be lower than that of the latter. End-capping restricts the access to ionized silanols, resulting in a significant decrease in C. Silanol ionization results in a negative charge on the column, and this charge attracts ionized (positively charged) bases and repels ionized (negatively charged) acids. For samples that contain ionized acids or (especially) bases, the column parameter C is a very important contributor to column selectivity. For samples that do not contain acids or bases, C is unimportant. Column ionization and values of C increase as mobile-phase pH is increased. End-capping results in decreased access to ionized silanols and a large decrease in C.

2.4.2 Application of the H-S model to equivalent column selection

HPLC columns need to be replaced from time to time for routine analysis due to deterioration. Also, when a method is transferred to another laboratory, a particular column is needed for the procedure. However, in either case, problems may be encountered. Although manufacturers now manage to maintain column performance reproducibility from batch to batch, a new column of the same designation may not result in the same (or acceptable) separation, especially when the chromatographers are dealing with samples that are difficult to be separated *(23)*. Moreover, the same column may not

be supplied by the original manufacturer anymore or not be readily available at the new site where the method is transferred *(25)*. These cases require the chromatographer to choose alternative columns that are equivalent in selectivity to the original one. That is when the H-S model can come into play as it allows the quantitative comparison of two columns and selects those of equal selectivity to the column one would like to replace. The function for column comparison has been derived for this purpose as follows:

$$F_S = \{[12.5(H_2–H_1)]^2 +[100(S^*_2–S^*_1)]^2 +[30(A_2–A_1)]^2+[143\ (B_2–B_1)]^2 +[83(C_2–C_1\ )]^2\}^{1/2}$$

(Eq.2)

where H_1 and H_2 refer to values of H for columns 1 and 2 respectively, and similarly for the remaining column parameters S*, A, B and C. The equation also considers the differences of the relative contributions of each parameter by adding weighting factors (12.5, 100, etc.) which were determined empirically *(24)*. Depending on the nature of the solute, C-term and B-term can be omitted for samples that do not contain ionized compounds (acids or bases) and carboxylic acid respectively. F_S can be interpreted as the distance between two columns in a plot of the five parameters in a five-dimensional space. The smaller the value of F_S, the closer in selectivity the two columns of interest is. In general, if $F_S \leq 3$ then the two columns are considered excellent matches and expected to have similar selectivity and band spacing for any sample or set of conditions *(24, 31)*. On the other hand, Fs values above 5 indicate poor matches.

The Impurities Working Group of the Product Quality Research Institute (PQRI) Drug Substance Technical Committee applies the H-S subtraction model to the evaluation of several hundred RP-LC columns including from C1–C30 alkyl-silica (both

type-A and-B), embedded-polar group, polar-end-capped, cyano, and most other commonly used column types *(23)*. Results obtained from this project have been collected and continually updated in a searchable database referred to as PQRI database by USP. The list of equivalent columns to those used in this study is put together using this PQRI database and included in the Appendix C.

2.5 Results and discussion

2.5.1 Mobile phase choice for column testing procedure

Some analytes, especially thiamine, pyridoxine and ascorbic, are ionic compounds that are not well retained in reversed-phase chromatography. Therefore official methods by USP and other reported methods in literature for these vitamins usually involve the use of ion pairing reagent for reversed-phase chromatography to enhance their retention on the column. The addition of amphiphilic ions in the mobile phase such as alkyl sulfonates or sulphates for basic solutes and quaternary amines for the acidic ones can greatly enhance the retention and separation of ionizable vitamin analytes through a dual mechanism: (a) the adsorption of the amphiphilic counterion on the stationary phase surface introduces the ionic interaction to the analytes; (b) the formation of the ion pair between the amphiphilic counterion and the analyte, resulting in an increased retention of the complex on the hydrophobic bonded phase *(32, 33)*. However, the biggest drawback of this method is that the ion-pair reagents are hard to be fully washed from the column, which requires the dedication of a particular column to ion-pair applications *(33)*. Moreover, trace levels of those reagents can change the column selectivity when it is

used for non-ion-pair applications, making column-to-column reproducibility a problem *(20, 24)*. Therefore, the use of amphiphilic ion-pairing reagents is usually recommended as a last resort in chromatography practice.

Because these vitamins can occur in aqueous solutions in various ionized states, pH of the mobile phase is an important factor that can affect the reproducibility of the method. It is highly recommended that the pH of the mobile phase should be about 2 pH units away from the pKa of the analytes *(32, 34)*. The reasoning behind this is conveniently explained based on the Henderson-Hasselbalch equation which states:

$$pH = pKa + \log\frac{[A^-]}{[HA]} \quad \text{(Eq.3)}$$

where A^- and HA should be extensively understood as deprotonated and protonated species of the same chemical, and does not necessarily only refer to acidic compounds. At pH of 2 units away from the pKa, it is guaranteed that one species exists in the solution predominantly with the percentage of 99%. In fact, throughout literature, many reported methods used phosphate or acetate buffers to control the pH of the mobile phase *(35)*. However, there is a tradeoff for good chromatographic separation of water-soluble vitamins resulted from this practice. In order to maximize the retention of some highly polar, ionizable vitamin analytes (especially vitamin C, vitamin B1 and vitamin B6), the analysis must be run at a very high aqueous percentage of mobile phase. This high salt content condition is detrimental to the integrity of the HPLC system, causing serious silting of the column and tubings *(34, 36)*. Moreover, methods using these buffer salts are not transferable to mass spectrometry detectors as these salts are non-volatile *(37)*. In some cases when the acidic environment is needed for separation, merely acid modifiers,

instead of a true buffering system with a weak acid and its conjugate base, suffice. To put it simply, their neutralizing capacity allows them to act as mild buffers against possible pH fluctuation on the introduction of the samples. Even when there is a mismatch between the pH of samples and mobile phase, as long as the injection volume is kept reasonably within the neutralizing capacity of the acid additives at certain concentration, a stabilized pH is maintained for chromatographic separation of the analytes. For this preliminary stage of method scouting, a simple aqueous mobile phase with 0.1% formic acid was found to work effectively. Its final pH 2.75 is not only well above the lower limit for most silica-based columns but also more than 2 units away from the pKa's of ionizable thiamine and pyridoxine. Moreover, at this pH, the ionization of residual silanols, which can lead to serious peak tailing for protonated compounds, is suppressed. As UV-Vis detection was used, acetonitrile (ACN) with its low UV cutoff in comparison to methanol (190nm v.s 205nm) was more preferable as the organic phase *(20, 36)*. This fact is advantageous to expanding the method detection scope to other vitamins that are only responsive to low UV wavelength such as pantothenic acid and biotin *(35)*.

2.5.2 Column characteristics

The list of columns used for the preliminary phase of method scouting is provided in Table 2.4. For the purpose of better evaluation, all the columns tested are classified into 3 groups based on their silica types and aqueous phase compatibility: type A columns, conventional type B columns and 100% aqueous compatible type B columns.

2.5.2.1 Type A columns

In 1986, Kirkland and others coined the terms 'Type A' and 'Type B' to refer to two different generations of silica supports used in HPLC column packing *(36)*. Approximately more than 20 years ago, silica-based columns mostly used type A silica which is characterized by a high level of metal impurities causing a heterogeneous acidic surface *(38)*. Together with residual silanols, metal contaminants interact strongly with sample components, leading to poor peak shape, especially asymmetrical and serious tailing for basic compounds *(30)*. Among the columns tested, Waters Nova Pak and Beckman Ultrasphere belong to this category. Aqueous mobile phase containing 0.1% formic acid with the pH of 2.75 cannot overcome the synergistic tailing effects of both residual silanols and metal impurities in these columns. Serious tailing was observed in both columns, as shown in Figure 2.7. Peak overlapping happened to pyridoxine (B6) and niacinamide (B3) in the first chromatogram and to niaciniamide and thiamine (B1) in the second. The tailing factor T_f is 5.936 for B6 in Nova Pak and 6.032 for B1 in Beckman Ultrasphere. Peak shape of the vitamin C, vitamin B2 and B12, however, was not affected and stayed symmetrical because they are not affected by the silanol interaction. Residual silanols are acidic in nature and normally stay uncharged around pH3 *(38)*. However, in the presence of metal impurities, the acidity of silanol surface is greatly increased. Therefore, a lower pH mobile phase was tried. When pH of the mobile phase pH is lowered to 2.3 with 1% formic acid, the tailing is improved a little but overall the peak shape of vitamin B1 and B6 is still unacceptable.

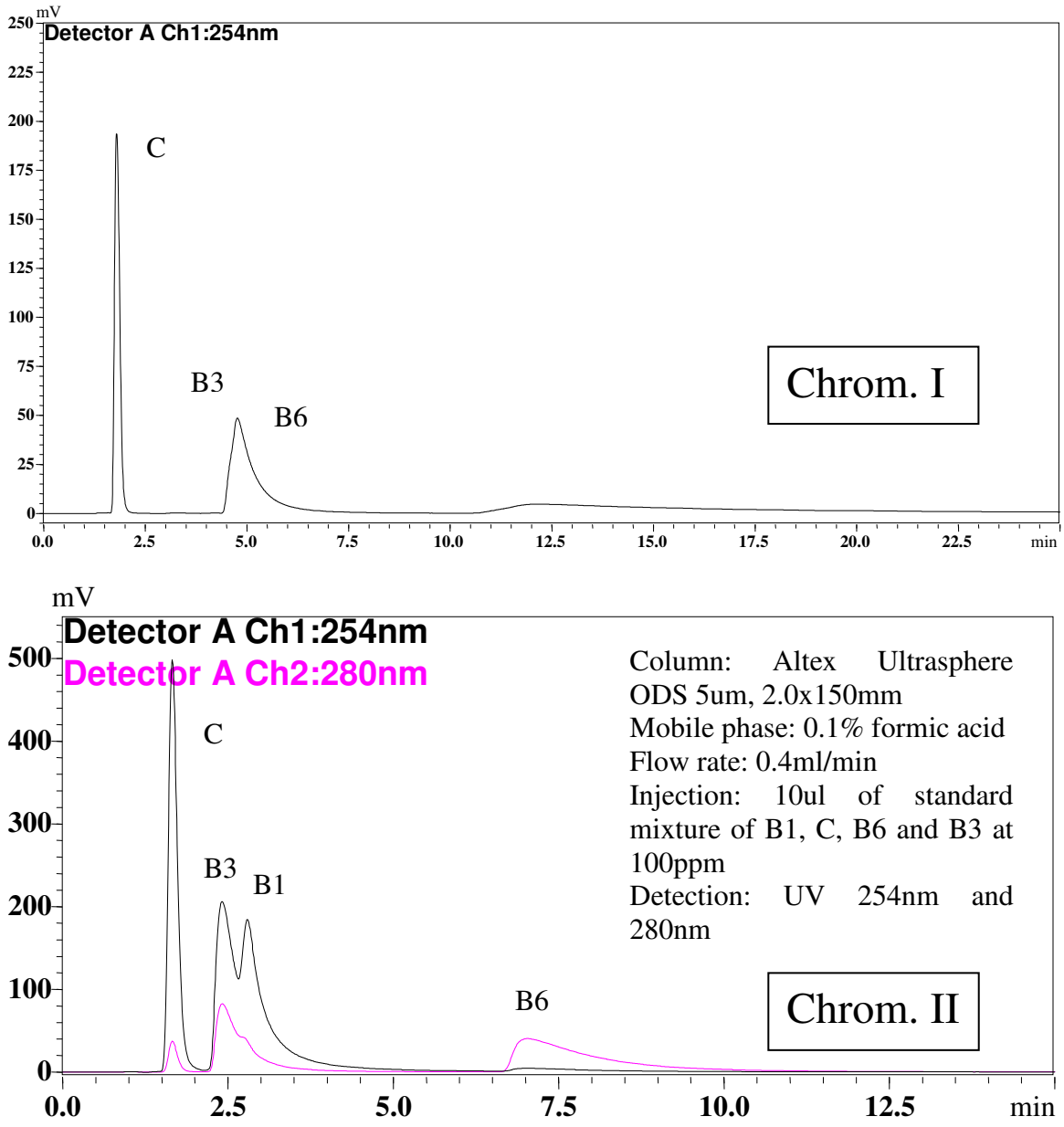


Figure 2.7 Demonstration for chromatographic performance of Type A columns
Notes: Noticeable tailing was observed in both chromatograms above: B6 (T_f= 2.863) and B1(T_f= 6.032) in chromatogram I and B6 (T_f=5 .936) in chromatogram II

Moreover, due to the non-uniform bonded phase coverage and active sites, columns with type A silica support has a high column-to-column variability *(38)*. Putting aside the fact that RP columns have insufficient retention for some water-soluble

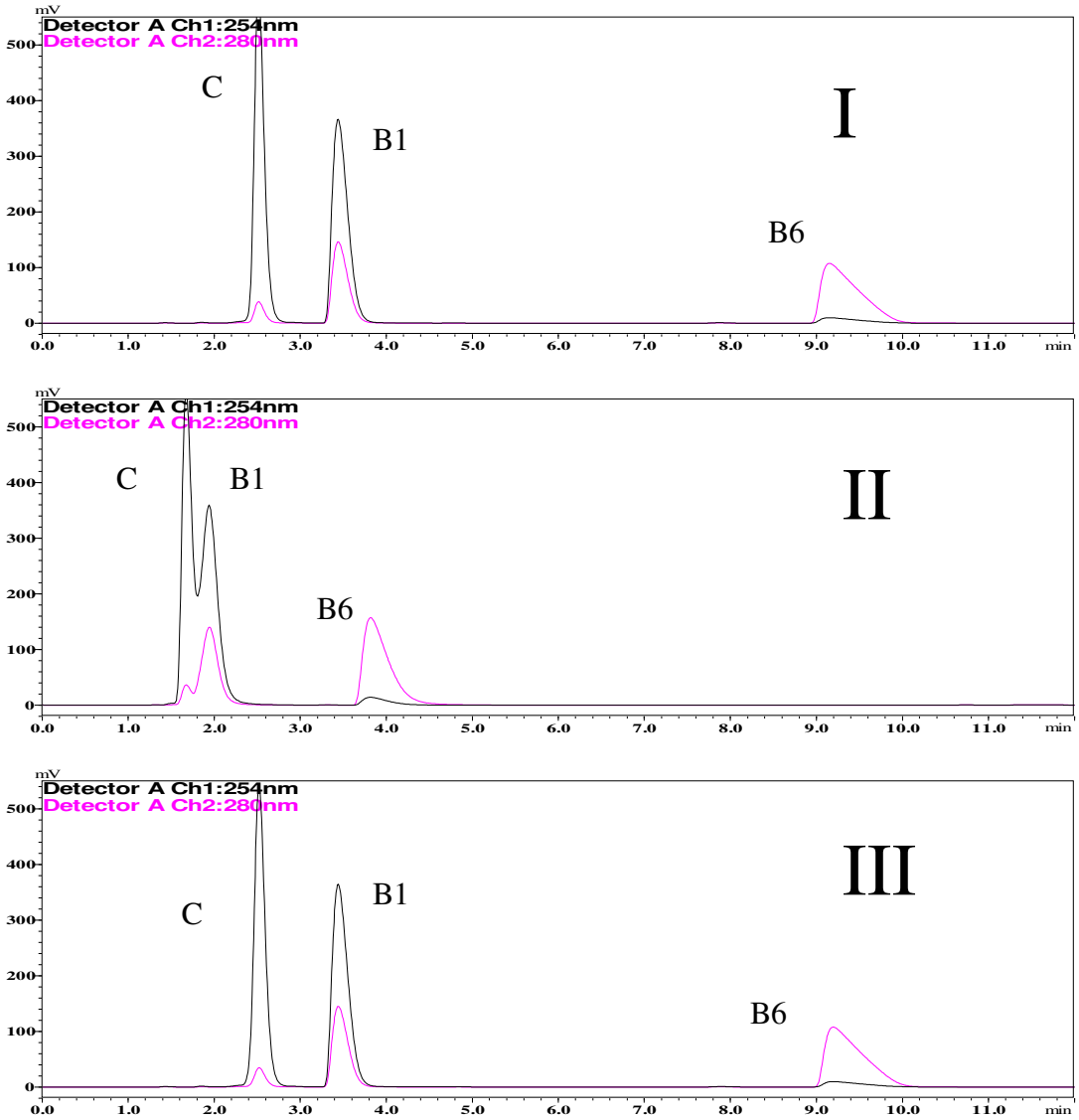
vitamins especially thiamine and ascorbic acid, it is their poor reproducibility and bad peak shape that made chromatographers resort to ion-pair reagents and other complicated additives back in the old days .

2.5.2.2 Conventional type B columns

Type B refers to a newer generation of highly purified and less acidic silicas which offer higher reproducibility and much more improved chromatographic performance of ionizable compounds, especially basic analytes *(29, 39)*. Type B silica is used almost exclusively these days for any new lines of columns. However, as many official methods were established using type A columns, they are still around and continue to be used without the need to revalidate. These days, unless one would like to use the established methods by official organizations, it is recommended that one should use type B columns for method development process.

Due to their polar and ionic nature, vitamin analytes require high aqueous mobile phase to achieve desired retention and separation. Conventional ODS (octadecylsilane) columns with octadecylsilane chemically bonded to porous silica are not compatible with highly aqueous phase and display stability problems without the use of ion-pair reagents *(40, 41)*. It has been reported that retention times decrease gradually when some RP columns are used with highly aqueous mobile phases while in other studies, the loss of retention was only observed when the flow was stopped and then restarted *(42-44)*. This phenomenon was not recorded when the mobile phase containing more than 10% (v/v) of

organic solvents was used *(44)*. Figure 2.8 shows an example of this issue and how it was resolved.



Column: Zorbax Eclipse Plus C18 3.5um, 3.0x150mm
Mobile phase: isocratic with 0.025% TFA
Flow rate: 0.5 ml/min
Injection: 10ul of standard mixture of C, B1 and B6 at 100ppm
Detection: UV 254nm and 280nm

Figure 2.8 Dewetting issue solution
Notes: Restoring retention of the analytes by column can be achieved by reequilibrating the column with high percentage of organic phase (>50%) before running the aqueous mobile phase through

Reid and Henry attributed retention losses to “phase collapse” or “hydrophobic collapse” *(45)*. It is speculated that the long alkyl chains are fully stretched in high percentage of organic solvents while they fold onto each other and onto the silica surface in highly aqueous phase. As a result, the stationary surface becomes less accessible for the partitioning of polar analytes between the mobile and stationary phases, leading to reduced retention. Phase collapse is the most popular explanation for the retention loss effect of RP columns under highly aqueous phase. However, this explanation is propagated more as a speculation rather than an empirically validated theory. There have been many reports on the behaviors of bonded alkyl chains in different solvents, but convincing evidence on such a phase collapse is still elusive *(44)*. One report even raised the contradictory conclusion that all bonded alkyl chains stay “collapsed” in all mobile phases *(46)*.

An alternative explanation for the retention loss of RP columns after flow stoppage and restart was first proposed by Walter, et al. in 1997 *(44)*. Due to their hydrophobic nature, most C18 bonded phases cannot be wetted by water *(47, 48)*. Therefore, in order to push water into the pores of the stationary phase, pressure must be applied *(42, 43)*. The force that keeps water in the pores is the water/solid interfacial tension and the external pressure. This metastable condition is maintained as long as the column is under sufficient pressure. However, once the pressure falls below the needed value or the flow is stopped, water is expelled out of the pores due to the liquid/gas surface tension and partial pressures of water vapor and gases formerly dissolved in water. The pores then are inaccessible to the mobile phase, which results in the loss of

retention *(49, 50)*. However, this issue is reversible. Retention can be regained by rewetting the pores with a mobile phase high in organic solvent (more than 50%) before re-equilibrating the column with 50% ACN before the aqueous mobile phase. However, it is inconvenient because it requires longer time for re-equilibration.

Excessive cases of tailing are the most evident in the cases of Type A silica columns as discussed in the column choice section above. On the other hand, Type B columns produced with newer technology have significantly fewer iron contaminants and free underivatized silanol residues, hence reducing the tailing effects *(39)*. Except for Waters Nova Pak and Beckman Ultrasphere columns, all others used in this section fall into this category.

2.5.2.3 Aqueous compatible type B columns

The past decade has seen a dramatic increase in popularity of polar-embedded and polar-endcapped columns that are specifically developed for the analyses of polar compounds *(28, 51-54)*. These phases involve modifications of the chemistry of classical alkyl phases through either an insertion of a polar functional group (amide, urea, carbamate and ether groups) within the alkyl chain attached to the silica surface for the former or the deactivation of residual silanols with polar functional groups (amino or hydroxyl terminated short alkyl chain) for the latter *(51, 52)*.

Among the six columns designated for this part of the study, Restek Ultra Aqueous C18 is polar-embedded while Synergi Hydro and YMC ODS AQ are polar-endcapped. Synergi Polar RP is a special column in the group as it is both polar-

endcapped and polar-embedded. The bonded phase of the column is stated by the manufacturer as phenyl linked to the silica particle through an ether link. Another special case is that of YMC Pack Pro C18. Marketed as a typical type B C18 column with proprietary endcapping, YMC Pro was unexpectedly found to be compatible with 100% aqueous mobile phase. Though the exact nature of endcapping chemistry is not disclosed, it is thought that the column is partially polar endcapped. Last but not least is the unclassified Zorbax SB-Aq. According to the manufacturer, the column is non-endcapped, which means the free residual silanols can hydrogen bond with water, preventing dewetting issues; therefore, the column is compatible with 100% aqueous phase. This compatibility may also come from the nature of the bonded phase, which can either be embedded with a polar group or be a polar group itself and is undisclosed by the manufacturer. Classification of the six tested columns in this group is illustrated by the Venn diagram in figure 2.9.

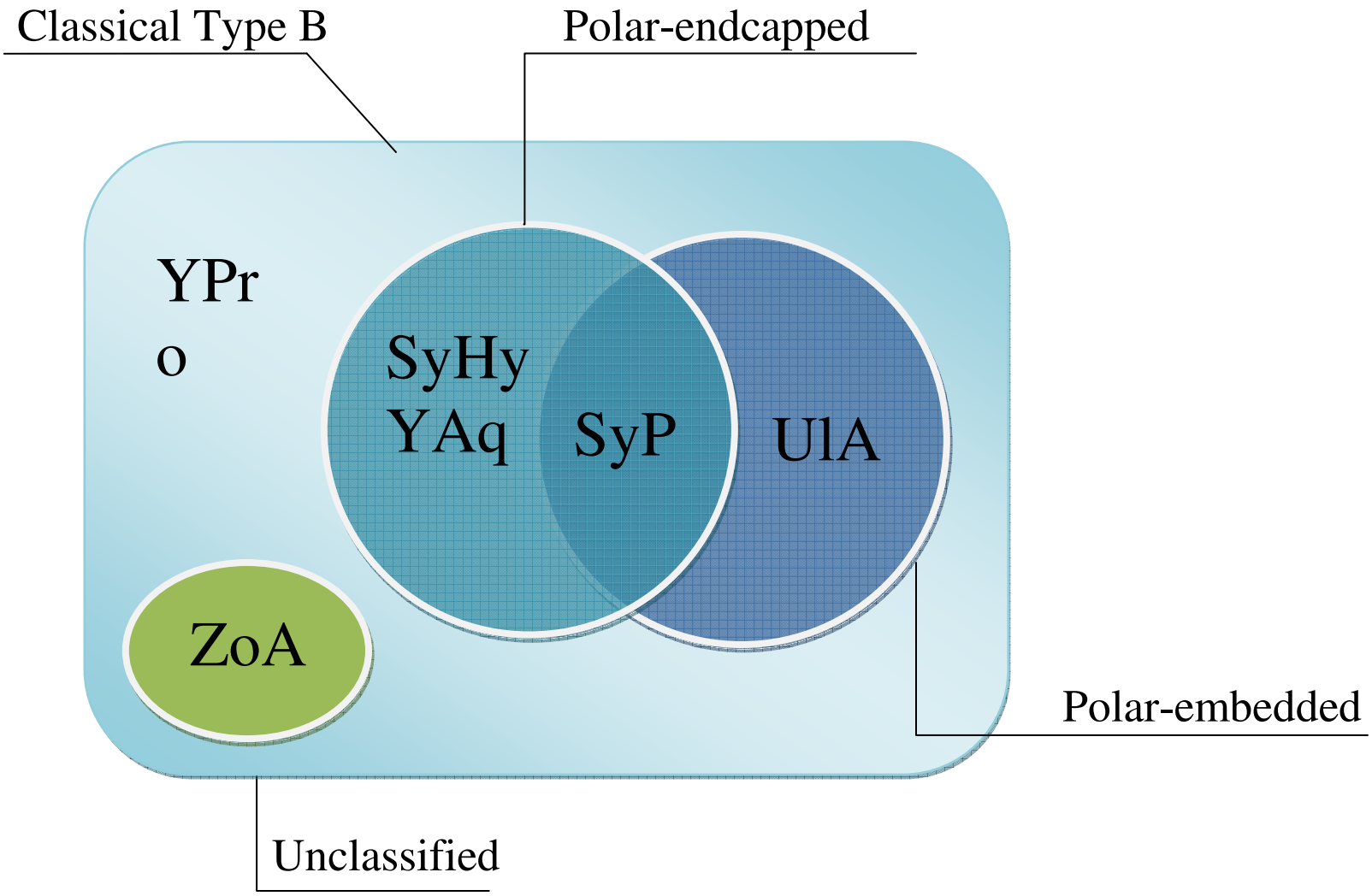


Figure 2.9 Classification of the 6 aqueous compatible columns (Refer to Table 2.4 for abbreviated names)

2.5.3 Chromatographic behaviors of the analytes and performance of aqueous compatible columns

The 5 analytes can be categorized into 2 groups, not necessarily based on polarity, but rather on their relative retention under the mobile phase used. This trend is consistent with all the columns tested. While vitamin B1, B6 and C are eluted at 0% organic solvent (100% aqueous phase of 0.1% formic acid in water), vitamin B2 and B12 require 15% of acetonitrile for their elution. The retention time and tailing factor of all five vitamins in the six aqueous-phase-compatible Type B columns are given in Table 2.5. Graphical illustrations of these data are shown in Figure 2.10.

Table 2.5 Retention time and tailing factor of B1, B6, C, B2 and B12
Notes: Mobile phase: (A) 0.1% formic acid and (B) Acetonitrile. Group 1 (B1, B6 and C) were separated under 100% A while group 2 (B2 and B12) were separated under 85%A:15%B. Flow rate at 0.8 ml/min.

Columns		B1	B6	C	B2	B12
YMC Pack pro	t_R (min)	2.79	5.97	6.75	13.69	10.31
	T_f	1.99	1.36	1.19	1.13	1.18
Synergi Hydro	t_R (min)	3.54	11.14	6.15	14.08	10.04
	T_f	1.39	3.65	1.09	1.08	1.09
Zorbax SB-Aq	t_R (min)	4.30	8.98	5.19	10.52	14.70
	T_f	2.64	2.47	1.19	1.09	1.34
Synergi Polar	t_R (min)	4.49	8.14	5.10	13.32	22.32
	T_f	1.50	1.58	1.27	1.07	1.34
YMC ODS AQ	t_R (min)	7.31	7.38	5.11	23.56	39.35
	T_f	4.23	2.06	1.27	0.91	1.54
Restek Ultra Aqueous	t_R (min)	8.10	14.17	6.35	19.43	19.45
	T_f	4.09	2.22	1.02	1.07	1.14

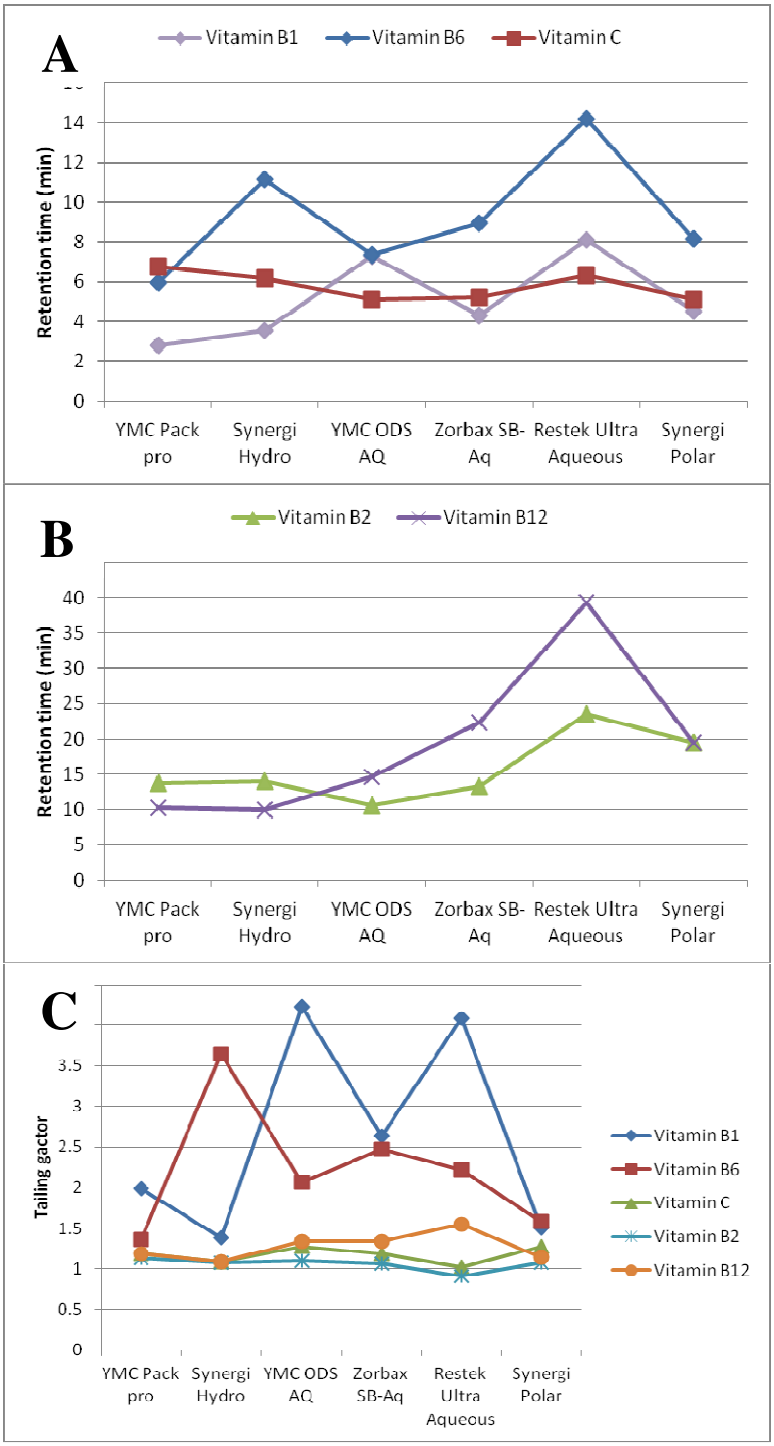


Figure 2.10 Retention time and tailing factor of B1, B6, C, B2 and B12 on different aqueous-phase compatible columns

Notes: Mobile phase (A) 0.1% formic acid and (B) Acetonitrile. Group 1 (B1, B6 and C) were separated under 100% A while group 2 (B2 and B12) were separated under 85%A:15%B. Flow rate at 0.8 mL/min

Some generalization can be made about the chromatography of all the analytes in all columns tested from Figure 2.10A. Thiamine is the first to be eluted and pyridoxine in most cases comes out later than thiamine. Ascorbic acid seems to be retained equally in all columns as there is not much difference in the retention time across the columns. Apart from these trends, it is hard to generalize about the order of elution among the analytes in different columns. Within each group, there is no uniformity in the order of elution across the tested columns. The elution order depends on the nature of the packing materials in the columns tested.

Physical parameters such as particle size (μm), pore size (Å), total carbon content (%), surface area (m^2/g), etc, are provided by the column manufacturers. While they are useful for the purpose of quality control *(55)*, these parameters tell little about chromatographic performance of the bonded phases *(56, 57)*. On the other hand, many manufacturers are not always willing to reveal the information on the bonded phases, endcapping groups and/or embedded groups, the nature of which is important to the selectivity of the columns towards the analytes of interest *(55)*.

The H-S model was applied to explain chromatographic behaviors of the analytes in this study. For the purpose of column selectivity comparison, besides the column information provided by the manufacturers, column parameters obtained from H-S model are also conservatively used to interpret the stationary phase/analyte interactions when appropriate. The absolute column parameters shown in Table 2.6 were graphically

presented as relative measurements in Figure 2.11 for the convenience of column comparison.

Table 2.6 Column parameters obtained from H-S model
Notes: H: Hydrophobicity, S*: Steric resistance, A: Hydrogen bond acidity, B: Hydrogen bond basicity, C(2.8): Cation exchange at pH 2.8

Columns	H	S*	A	B	C(2.8)
Ypro	1.015	0.014	-0.12	-0.007	-0.155
SyHy	1.022	-0.006	0.169	-0.042	-0.077
YAq	0.965	-0.036	-0.135	0.004	-0.068
ZoAq	0.593	-0.12	-0.083	0.038	-0.136
UlAq	0.808	-0.128	0.378	0.013	0.229
SyPo	0.654	-0.148	-0.257	-0.007	0.057

Source: **http://www.usp.org/app/USPNF/columnsDB.html**

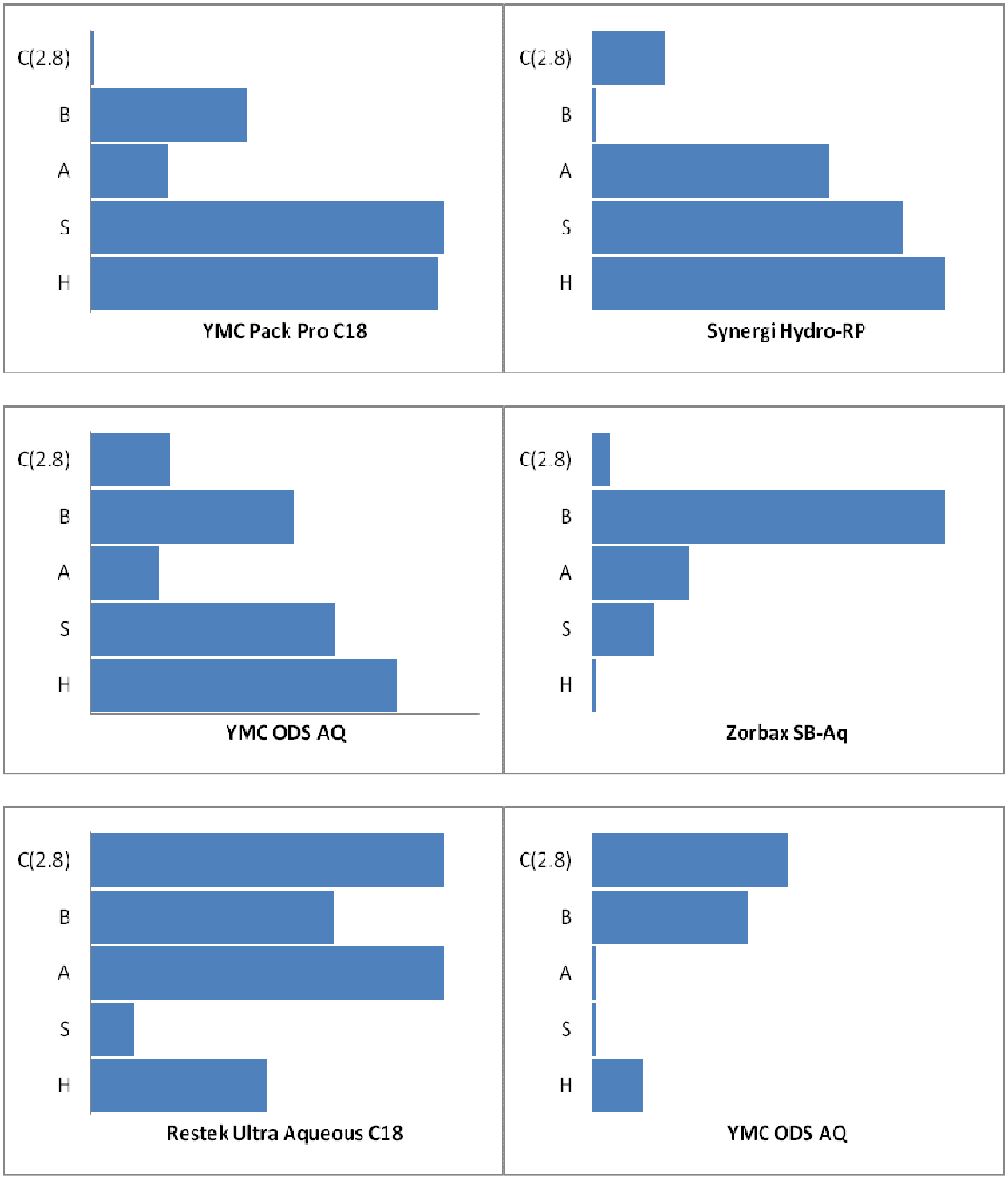


Figure 2.11 Relative measurement of column parameters by H-S model

2.5.3.1 Thiamine and pyridoxine

Thiamine and pyridoxine occur predominantly in ionized forms under the mobile phase pH 2.8. As charged molecules can be considered as an extreme case of polar analytes, the role of hydrophobicity of the column on their retention is minimal. Therefore, it is the ionic interaction of the charges with the ionized silanol groups of the stationary phases that is of major importance to their retention on RP columns *(31)*. Because these silanol groups are located on the silica surface, this interaction is dependent on steric resistance (or shape selectivity), the S* parameter in the H-S model. Because pyridoxine is smaller than thiamine, steric resistance has less impact on its retention than it does on thiamine. As a result, the interaction of pyridoxine with the ionized silanol groups is stronger, leading to better retention than thiamine in nearly all columns tested. It is noticed that for all columns tested, thiamine always comes out earlier than pyridoxine, even before the void volume in the case of YMC pack Pro.

Among the six columns tested, YMC Pro and Restek Ultra Aqueous have the lowest and highest retention, respectively, for all analytes, especially these two protonated vitamins. Further examination of their parameters revealed that the YMC Pro column has the lowest C and highest S* while Restek Ultra Aqueous had the highest C and second lowest S*. This observation agrees with the aforementioned speculation that the retention of these two vitamins is mainly dependent on the column cation-exchange capacity C and steric resistance S*. YMC is a typical type B alkyl bonded phase column while Restek Ultra Aqueous is a non-endcapped, polar-embedded column, which possibly explains their lowest and highest C values respectively. Polar-embedded groups

are believed to attract water to the silica surface, producing an immobilized layer that reduces the cationic interaction between bases and residual silanols *(58)*. If that is the case, then both protonated thiamine and pyridoxine should have been weakly retained on Ultra Aqueous. However, the data showed they were both very well retained on Ultra Aqueous. Because the chemistry of the embedded groups is unknown, benefit of the doubt is given to their potential cationic interaction with the analytes, resulting in longer retention.

Synergi Hydro and YMC ODS-Aq columns are both polar endcapped and have similar H values, which are also close to that of YMC Pack Pro. It has been reported that polar-endcapped columns have similar hydrophobicity to non-endcapped or traditionally endcapped columns (type B alkyl phases) *(59)*. In a study by Dolan and co-workers, it was found that these columns also behave similarly to and do not offer more enhanced selectivity than type B alkyl phases *(28)*. However, that does not seem to be the case concerning the retention of thiamine and pyridoxine in this study. These two columns retained the two vitamins better than YMC Pack Pro, a typical type B alkyl phase column, even though their selectivity towards the two vitamins is very different. Synergi Hydro is more selective towards pyridoxine and less towards thiamine than YMC ODS-Aq even though both C and S* values of the column are approximately equal to those of Synergi Hydro. It is interesting to note that both compounds elute nearly at the same time on the YMC-ODS AQ column. Retention of the thiamine increases while that of the pyridoxine decreases in comparison to their retention on Synergi Hydro. Just like the other two previous columns, endcapping means that the free silanol groups on the surface

of YMC ODS AQ are significantly decreased. There are only limited ionized silanol sites to bind with and retain certain number of positively charged species at one time. When the steric resistance is low enough to allow both compounds to have secondary interaction with ionized silanols, thiamine with more positive charges have better chance to bind the limited number of cationic-exchange site. Moreover, the different chemistry of the the polar end-capping group used, which is not specified by the manufacturer, may also be the reason for the differential selectivity in this case.

Zorbax SB-Aq and Synergi Polar RP have similar elution order of the three vitamins in group I. The two columns have approximately equal H and S* but very different C values. It appears that they have different mechanisms of reducing the cationic interaction between bases and residual silanols, leading to the difference in C. Among the six columns tested for this section, Zorbax SB-Aq is neither classified as polar-endcapped nor polar-embedded. According to the supplier, the siloxane bond to the silica surface is protected from acid hydrolysis by silanes with bulky diisopropyl side groups. This column is non-endacapped but these bulky side groups are believed to sterically shield silanols and diminish ionic interactions between protonated bases and residual silanols. The bonded phase of Synergi Polar RP, on the other hand, is phenyl embedded with a polar ether link which can form an aqueous layer masking silanols and reducing the ionic interactions. Somehow, the difference in C values does not result in significant difference in retention of the two compounds. In fact they have nearly the same retention in the two columns, even including vitamin C. This result may come from

the interactions with the bonded phase of the former and the polar groups of the latter, the nature of which is undisclosed by the manufacturers.

2.5.3.2 Ascorbic acid

Existing predominantly in the undissociated form under the mobile phase pH, ascorbic acid (pKa 4.3) is mainly retained due to the interaction with the hydrophobic ligand of the column. It either elutes before, in between or after thiamine and pyridoxine depending on column characteristic. In general, there is no significant difference in the retention of ascorbic among the columns tested. It is speculated that the minor difference in selectivity may come from the potential hydrogen bonding between the four hydroxyl groups of ascorbic acid with the water sorbed onto the bonded phase according to H-S model. Kiridena and Poole found that the hydrogen-bond basicity of polar-endcapped columns was lower than that of classical C18 counterparts and much lower than that of a polar-embedded phase suggesting the incorporation of less water into the polar-endcapped phase *(60)*. However, the random distribution of B values by H-S model and the arbitrary analyte retention ranking regardless of column types does not seem to confirm Kiridena's finding. Besides column hydrophobicity and hydrogen-bond basicity, it is thought that the unspecified chemistry of the polar-embedded or polar-endcapped groups of the columns may complicate the retention of ascorbic, contributing the difference in column selectivity towards this analyte.

2.5.3.3 Riboflavin and cyanocobalamin

Cyanocobalamin is a weak base that occurs as a positively charged species charged in acidic environment. Considering its structure, the positive charge is possibly due to the deprotonation of various nitrogenous moieties in the molecule. Partly-ionized bases are believed to be retained mainly as neutral species *(23)*. That seems to coincide with the symmetrical peak shape of cyanocobalamin, indicating little to no trace of secondary interaction with residual silanols on the stationary surface. Its bulky molecular structure may be held responsible for limiting this ionic interaction.

Riboflavin is predicted to be non-ionized and its chromatographic behavior does not depend on the pH of the buffer within the pH range of 2.0-7.0. Riboflavin is retained quite well on C18 column and it elutes together with cyanocobalamin within the same range of organic mobile phase (15% ACN in this study). Because there is no positive charge involved in the residual silanol intractions, riboflavin peak shows no tailing like cyanocabalamin.

Both of these analytes, as discussed above, are mainly retained as neutral species, which explains why they are retained longer on RP columns than the other three vitamin analytes. Besides hydrophobicity, steric resistance (or shape selectivity) is the second most important factor that affects a compound's retention. Vitamin B12 eluted before vitamin B2 on both YMC Pro and Synergi Hydro column. The other way around occurred to the remaining columns. The H-S model can be satisfactorily applied to explain the retention of riboflavin and cyanocobalamin. Their elution order in a way can be explained using an analogy of retention by size-exclusion chromatography (SEC) *(23)*.

In SEC, the solute retention is dependent on the accessibility of the solutes to the particle pores. Bigger, longer molecules have a larger hydrodynamic or Stokes diameter that challenges their entering the particle pores, leading to reduced retention in comparison to other molecules of smaller sized *(20)*. The same thing may have happened to the case of riboflavin and cyanocobalamin. The steric resistance of YMC Pro and Synergi Hydro are the highest among the columns tested, indicating the highest level of difficulty against penetration towards the analytes. Cyanocobalamin is much bulkier than riboflavin, therefore its chance of interaction with the alkyl ligands bonded phase is also much more reduced than that of riboflavin, leading to its earlier elution. On the other hand, when the steric resistance is the same towards the two molecules then stronger retention favors bulkier one. This explains the trend of elution of these two vitamin analytes in the case of YMC-ODS Aq, Zorbax SB-Aq and Ultra Aqueous C18. Synergi Polar RP is a special case in that it has the same selectivity towards both riboflavin and cyanocobalamin even though its S* value is the lowest. The difference in its bonded phase (ether linked phenyl) is possibly the cause of this chromatographic behavior.

2.5.3.4 Peak tailing

Taking a look at Figure 2.10C gives us an idea about the asymmetry of the peak shapes of all five analytes. While ascorbic, riboflavin and cyanocobalamin have quite stable tailing factors and all stay within the range of 0.9 to 1.6, the other two vitamins have much more random tailing patterns. As explained from above, the main retention force for these two compounds is ionic interaction between the protonated amine groups

with the ionized silanol residues on the silica surface. It is this interaction that gives rise to the tailing issues *(28)*.

According to Giddings and others, tailing may result from slow adsorption-desorption kinetics of retained solute between the few strong sites of high adsorption energy and a large number of sites of low adsorption energy *(61, 62)*. The strong sites in the case of RP-LC involve the interactions between the protonated bases and the small number of ionized silanol residues on the silica surface that can be easily saturated by small amount of solutes while the weaker sites refer to the hydrophobic interactions between solute and alkyl ligands. The initial adsorption of positively charged molecules onto the hydrophobic surface of the stationary phase hampers further sorption of molecules with the same charge. This mutual ionic repulsion effect may also contribute to overloading (besides merely the saturation of rather than of small number of cation-exchange sites on columns) *(63)*. Buckenmaier proposed this ionic repulsion as the major factor contributing to peak tailing mechanism of protonated bases on type-B alkyl-silica columns at low-pH conditions without ion-pairing mobile phases *(63)*. In comparison to Type A columns, these aqueous compatible columns cause much less tailing. Higher purity of silica and endcapping which were discussed above in section 2.5.2.2 are the two reasons for their enhanced performance of these columns *(39)*. Moreover, when further examining each column in this section on an individual basis, each seems to have different mechanism towards reduced silanol interaction, leading to less tailing. These mechanisms are the same as the ones discussed earlier in section 2.5.3.1 that affects the

retention of the two protonated basic compounds. In general, as the protonated analyte is retained longer, the tailing is increased.

2.5.4 H-S model fitting to predict the chromatographic behavior of analytes

The theory behind the H-S model was quite useful for explaining the general retention mechanism of the five analytes. However, when it comes to making relative comparison of column performance, only conservative interpretation of the column parameters should be made when appropriate as the correlation between column parameters and analyte retention is not clear and consistent. At least this observation is true for the linear relationship that was tested in this study (Table 2.7). In general, the correlation was found to be quite random. Statistically significant correlation coefficient was found between t_R and S for cyanocobalamin, t_R and A for pyridoxine and t_R and C2.8 (Column cation exchange parameters at pH 2.8) for all except ascorbic acid. Among these, the correlation between t_R and S makes sense for the case of cyanocobalamin. As this vitamin is quite bulky, shape selectivity plays an important role in its retention. The slope is negative (-116.65) indicating that as the steric resistance of the stationary phase increases, the retention time decreases accordingly.

Table 2.7 Values of r^2 and slope (a) for correlation between analyte retention and column parameters for the six aqueous compatible stationary phases

Vitamin	tR=f(H) r^2 (a)	tR=f(S) r^2 (a)	tR=f(A) r^2 (a)	tR=f(B) r^2 (a)	tR=f(C2.8) r^2 (a)
Thiamine	0.010	0.170	0.183	0.135	0.496
	-1.12	-12.59	3.87	29.63	10.35
Pyridoxine	0.016	0.155	0.820	4×10^{-5}	0.561
	-1.98	-16.63	11.33	-0.73	15.22
Ascorbic	0.357	0.271	0.307	0.135	0.005
	2.32	5.46	1.72	-10.15	0.36
Riboflavin	0.135	0.416	0.261	0.006	0.815
	-9.40	-44.37	10.40	13.51	29.84
Cyanocobalamin	0.261	0.551	0.333	0.312	0.697
	-29.83	-116.65	26.85	231.35	63.02

Satisfactory correlation between t_R and C2.8 for thiamine and pyridoxine is reasonable due to their positively charged condition. The cation exchange interaction with the residual silanols is confirmed. It is unexpected that the correlation coefficient for tR=f(C2.8) for riboflavin and cyanocobalamin are that high (0.815 and 0.697 respectively). This result is quite random as mentioned above, as under the mobile phase condition, these two compounds stay undissociated. The highest correlation coefficient is obtained for tR= f(A) for pyridoxine (0.820). Theoretically, the only basic group on pyridoxine is protonated and favors cationic exchange interaction with inonized residual silanols instead.

The original purpose of this relationship study is to learn if linear modeling can explain some of the trends in the retention process and can be used to predict the retention of the same analytes on other columns if the H-S parameters of the new

columns are known. However, considering the correlation coefficients, it is not possible to use this to predict their chromatographic behaviors using linear model for single parameters. The retention mechanism is not a straight-forward simple process that involves a single interaction. Instead, it is the result of a more complicated combination of other secondary interactions besides the primary hydrophobic one. Multilinear model or different correlation model may work in this case but it is worth mentioning that the contribution from different interactions is not equal depending on the nature of the analyes in the conditions tested. These contribution cofactors can only be determined empirically from a bigger data set.

2.5.5 Improving the retention of weakly retained vitamins (thiamine, ascorbic and pyridoxine)

2.5.5.1 Acid modifiers and pH adjustment

The effects of different acid modifiers on the retention and tailing of the three vitamins are demonstrated in Table 2.8 and Figure 2.12. Thiamine occurs in equilibrium between the singly-charged and double-charged species under acidic condition. As the mobile phase pH gets close to the first pKa of thiamine (4.8), there is a significant shift in the equilibrium towards doubly-charged thiamine molecules, leading to a decrease in thiamine retention. This trend is observed for phosphoric, formic and acetic acid when their concentrations in the mobile phase increase. Substantial changes in both retention and tailing factor are noticed for acetic acid. As a weak acid, the pH range induced by acetic acid in the study (pH 3.06-3.73) falls close to the first pKa of thiamine in

comparison to the other two acid modifiers. As a result, every change in pH of the mobile phase by acetic acid causes a significant shift in the equilibrium between the two ionized species of thiamine. When ionized thiamine is retained longer on the column, its interaction with residual silanol is also increased, which consequently leads to serious tailing. Another reason for significant tailing issues is that at pH higher than 3, residual silanol activity is enhanced. The Zorbax-SB Aq column is not endcapped, which means there is a high number of residual silanol groups on the surface available for interaction with basic compounds, leading to tailing. This explains the wide range of tailing factors (2.4-8.2) when acetic acid is used.

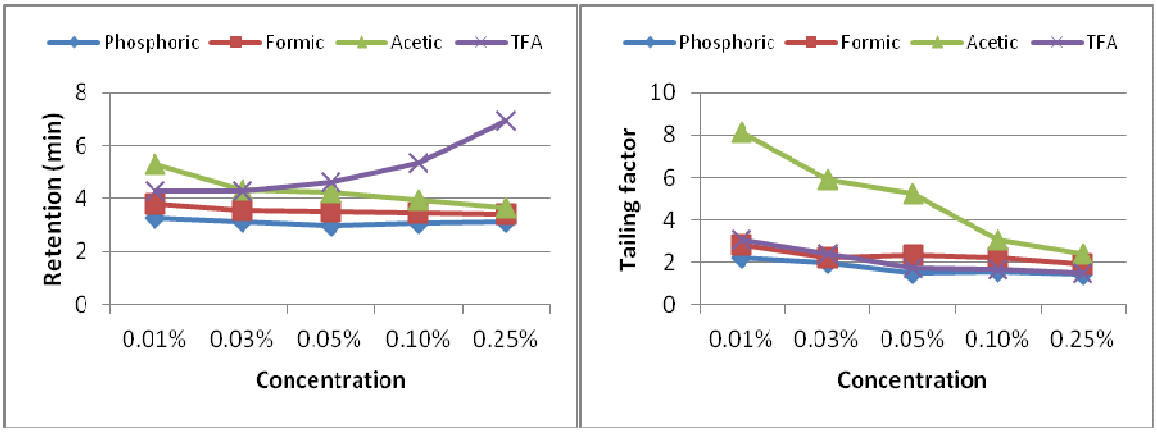
Pyridoxine has two possible ionizable sites, one at the pyridinium N and the other at 3-hydroxyl group with pKa=8 and 5.0, respectively. There is an equilibrium of 3-OH dissociation, which regulates the concentration of zwiterrionic pyridoxine and cationic pyridoxine. Because the mobile phase induced by the acid modifiers in this section has pH close to the lower pka of pyridoxine, the relative concentration of the two species of pyridoxine in the mobile phase may cause the difference in the interaction with the column, which in turn leads to the difference in the retention when different acid concentration is used. Retention is inversely related to the total charges on the analyte. When the pH of the mobile phase decreases with higher acid concentration, there is an increase in the amount of singly positively charged species, which are better retained on RP column than its zwitterionic counterpart. As to tailing, it is noticed that the tailing factor increases up to around pH 3.0-3.5 and then decreases. It is possibly due to the

repulsion effects of zwitterionic pyridoxine against the ionized silanols at increased pH, leading to less peak tailing.

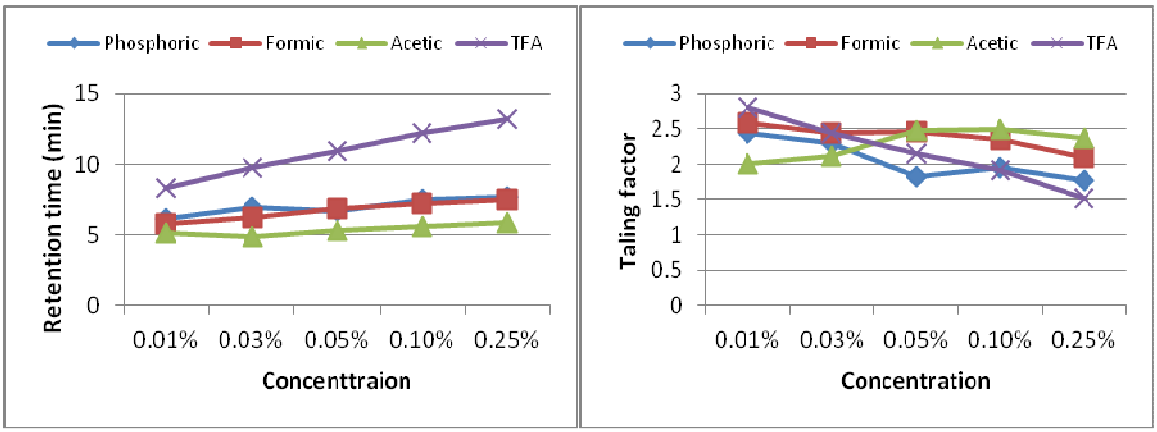
The retention time and tailing factor of ascorbic acid stays consistent across the pH gradient and does not depend on the kind of acid modifiers. Though it is an ionizable compound with a pKa of 4.04, no significant changes in these two chromatographic parameters are noticed even when the pH of the mobile phase gets close to its pKa.

Table 2.8 Retention time (t_R) and tailing factor (T_f) of B1, B6 and C on Zorbax SB-Aq column under different acid modifiers
Notes: 100% aqueous mobile phase containing various acid modifiers at five concentration levels (0.01%, 0.025%, 0.05%, 0.1%, 0.25%). Flow rate 1.0 ml/min.

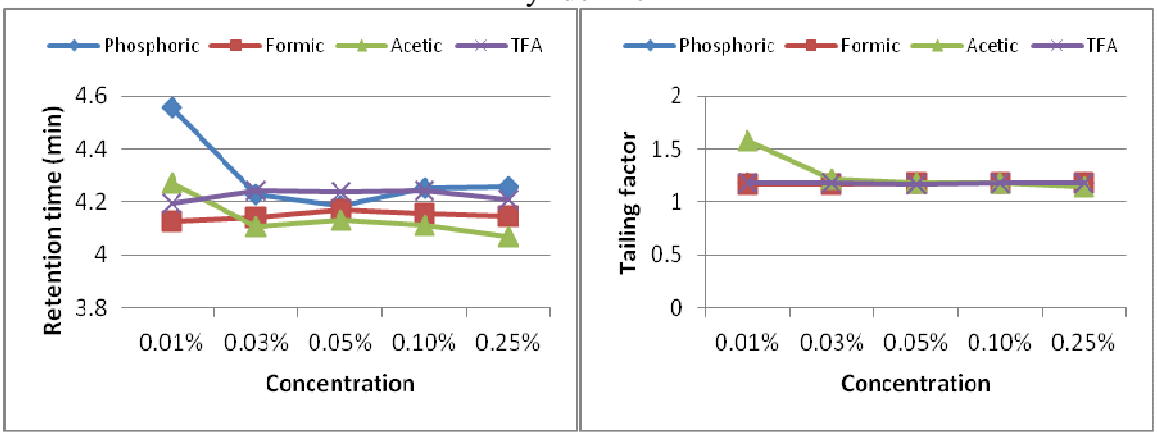
Modifiers	pH	B1		B6		C	
		t_R (min)	T_f	t_R (min)	T_f	t_R (min)	T_f
Acetic acid							
0.01% acetic	3.73	5.286	8.165	5.095	2.01	4.274	1.585
0.025% acetic	3.58	4.329	5.879	4.86	2.111	4.108	1.216
0.05% acetic	3.43	4.25	5.244	5.289	2.472	4.134	1.18
0.1% acetic	3.28	3.928	3.046	5.557	2.501	4.111	1.185
0.25% acetic	3.06	3.681	2.402	5.844	2.368	4.067	1.15
Formic acid							
0.01% formic	3.24	3.824	2.812	5.755	2.584	4.127	1.17
0.025% formic	3.04	3.558	2.227	6.196	2.435	4.141	1.168
0.05% formic	2.87	3.527	2.356	6.832	2.466	4.172	1.175
0.1% formic	2.72	3.443	2.19	7.179	2.349	4.158	1.176
0.25% formic	2.52	3.412	1.927	7.516	2.099	4.145	1.181
Phosphoric acid							
0.01% phosphoric	3.05	3.275	2.244	6.09	2.446	4.558	1.181
0.025% phosphoric	2.75	3.118	1.955	6.918	2.292	4.23	1.179
0.05% phosphoric	2.5	2.985	1.485	6.644	1.834	4.187	1.181
0.1% phosphoric	2.3	3.07	1.536	7.536	1.959	4.251	1.181
0.25% phosphoric	2.05	3.104	1.411	7.694	1.768	4.256	1.181
Trifluoroacetic acid (TFA)							
0.01% TFA	2.94	4.278	3.034	8.335	2.802	4.193	1.178
0.025% TFA	2.61	4.312	2.398	9.807	2.439	4.243	1.175
0.05% TFA	2.3	4.638	1.754	10.949	2.145	4.239	1.174
0.1% TFA	2.05	5.371	1.649	12.185	1.912	4.241	1.182
0.25% TFA	1.76	6.931	1.469	13.201	1.515	4.208	1.182



Thiamine



Pyridoxine



Ascorbic acid

Figure 2.12 Retention time and tailing factors for thiamine, pyridoxine and ascorbic with different acid modifiers on Zorbax SB-Aq column

Notes: 100% aqueous mobile phase containing various acid modifiers at five concentration levels (0.01%, 0.025%, 0.05%, 0.1%, 0.25%). Flow rate 1.0 ml/min.

2.5.5.2 Perfluorinated carboxylic acid and ion-pairing effects

The overall discussion on the effects of acid modifiers on the chromatographic behaviors of the analytes above can be better summarized in another graph that focuses only on the final pH of the mobile phase instead of the modifiers added. Examination of Figure 2.13 reveals that it is the pH, not the modifiers themselves that cause changes in both analyte retention and peak tailing. However, TFA is a special case as it exerts a substantial effect on the retention of protonated vitamins (i.e., thiamine and pyridoxine) while causing only a small change, if any, to their peak tailing.

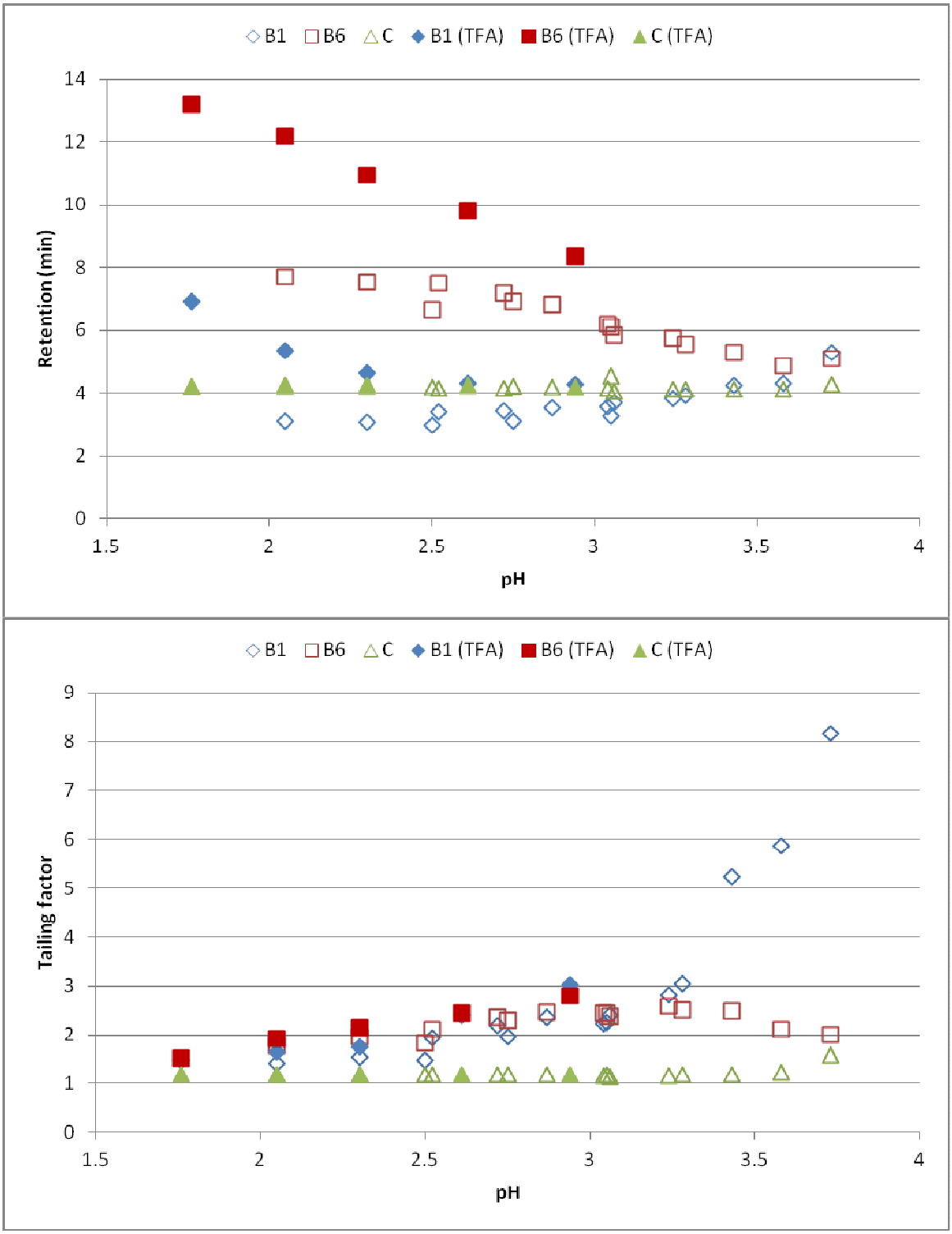


Figure 2.13 Retention and tailing factors of thiamine, pyridoxine and ascorbic v.s final pH of mobile phase (regardless of modifiers used)

TFA belongs to the group of perfluorinated acid modifiers that are believed to enhance protonated analytes through a process of dualistic nature: (a) ion pairing and (b) chaotropic effects. The latter phenomenon occurs when the counterions introduce chaos to the ionic solvated analyte, disrupting the structured solvation shell which causes an increase in its apparent hydrophobicity and retention. These ions are hence named chaotropic ions *(64, 65)*. They are arranged into the Hofmeister series based on their ranking of salvation shell disruption ability as follows *(66-71)*:

$H_2PO_4^-$< $HCOO^-$< $CH_3SO_3^-$<CH_3COO^-< Cl^-< NO_3^-< CF_3COO^-< BF_4^-<ClO_4^-< PF_6^-

The increased chaotropicity related to the ions' charge delocalization and polarizability is ranked from left to right, with a simultaneous increase in the symmetry. According to this series, TFA ions have a stronger chaotropic effects than phosphate, formate and acetate counterparts. Moreover, the ion-pairing capacity of TFA was found to be stronger than that of acetate, formate and phosphate. The ion-pairing of chaotropic ions is also similar in mechanism to classical amphiphilic ions discussed above with dualistic nature *(72)*: (a) the formation of the neutral ion pairs that are then retained on the hydrophobic bonded phase; (b) the adsorption of the counterions on the stationary phase surface introduces the ionic interaction to the analytes. Trifluoromethyl CF_3- has a significant electronegativity, making it a strong electron withdrawing group. Electron density from a conjugated π system of the carboxylate ion is removed via resonance or inductive electron withdrawal, thus making the π system less nucleophilic. The formation of ion pairs with protonated basic analytes may be slower than in the case of other counterions. However, CF_3^- is

more hydrophobic, which seems to be a determinant factor for the enhanced retention of thiamine and pyridoxine observed in this study.

Other perfluorinated carboxylic acids include pentafluoropropionic acid (PFPA), heptafluorobutanoic acid (HFBA), nonafluoropentanoic acid (NFPA), tridecafluoroheptanoic acid (TDFHA) and pentadecafluorooctanoic acid (PDFOA). Elongation of the carbon chain of these modifiers results in an increasing retention of protonated analytes *(67)*. Moreover, the influence of these additives on the column is reversible and equilibration only requires minimal time *(33)*. It should be noted that besides all of the mentioned effects, perfluorinated carboxylic acids also adjust the pH of the mobile phase, affecting the analyte chromatographic behaviors as well. The most prominent consequence of the low pH induced by TFA in the study is the suppression of silanol ionization, thereby reducing peak tailing.

Within the scope of this study, HFBA was also tested under the same chromatographic conditions used for this section (0% aqueous phase on Zorbax-SB Aq, 250x4.6mm). Figure 2.14 shows the chromatogram obtained with isocratic run using HFBA as an additive. Mobile phase containing 0.1% HFBA (~7.7mM) was found to significantly increase the retention of both thiamine and pyridoxine in comparison to TFA. Higher percentage of organic solvent (15% acetonitrile) was used to elute these two compounds. Moreover, the order of elution was even inversed with pyridoxine eluting earlier than thiamine. With two positive sites available for the binding of ion-pairing reagent, thiamine–ion complex is more hydrophobic, hence retained more strongly. Ascorbic acid is not a basic compound; therefore its retention was not affected by the

addition of HFBA and remained the same even when the organic phase percentage (acetonitrile) increased to 2%, 5% and 10%. The adjustment of phase B percentage was made to obtain the optimal condition for the separation of thiamine, pyridoxine and ascorbic acid which is shown in chapter 3.

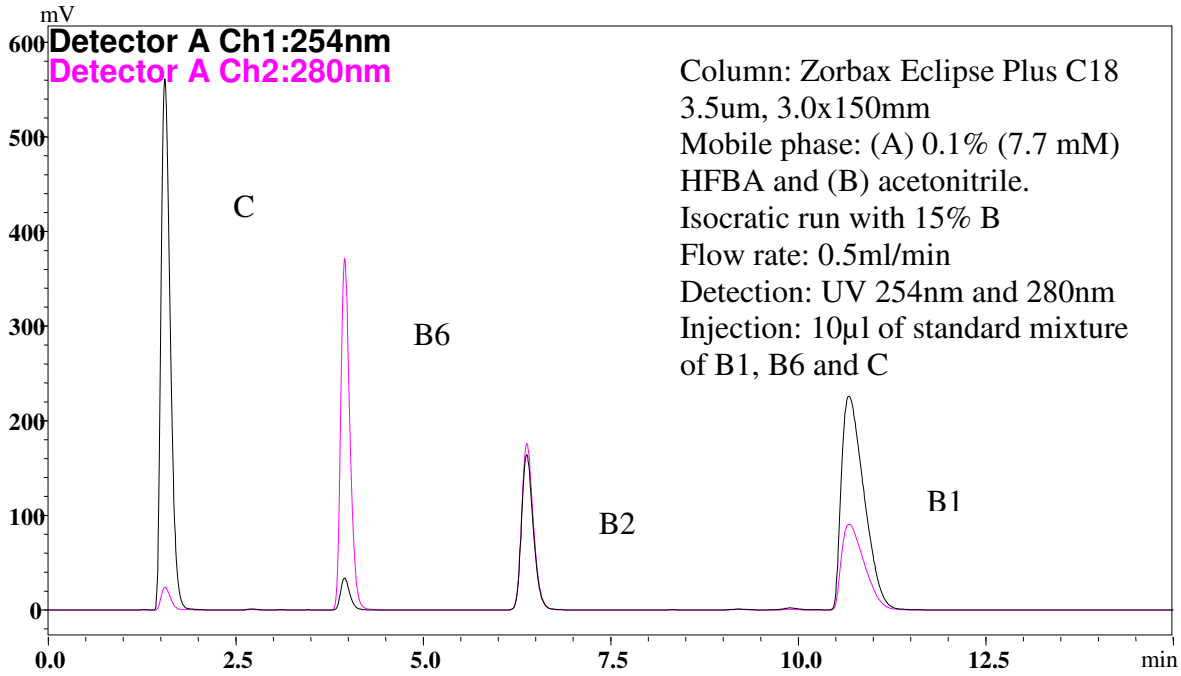


Figure 2.14 HPLC method using HFBA as an additive in the mobile phase

2.5.5.3 Buffered mobile phases

Low retention of thiamine and pyridoxine under acidic condition are due to the positive charges they carry. It is logical to think that removing these positive charges helps enhance their retention on RP columns. This goal can be achieved by raising the mobile phase pH with buffers. As these two compounds are all ionizable, the choice of which pH to use is of major importance. As discussed in section 2.4.1 on mobile phase

choice, it is recommended that the final pH should be about 2 pH units away from the pKa of the analytes to ensure reproducibility for the chromatographic method.

Thiamine and pyridoxine have the highest pKa at ~9.0; therefore, the positive charge can be removed if the mobile phase is raised to a pH higher than 9.0, which falls out of the normal working pH range of most columns. There are some manufacturers nowadays offering columns with special design (either silica-based or polymer-based) that can withstand such a high pH condition. However, such a high pH buffer may not be necessary as buffers with pH lower than 8.0, which are within the recommended operating range of normal silica-based columns, appears to have a sufficient effect on the retention time of these two compounds. There have been many methods developed using phosphate buffer pH 5.0-7.0 as the aqueous mobile phase for the HPLC analysis of thiamine and pyridoxine. Within this range, phosphoric acid and phosphate salt with low UV cutoffs (below 200 nm) are favorable additives used in UV-Vis detection. However, HPLC methods using phosphate buffers are not transferable to LCMS system due to these salts' non-volatility. Ammonium acetate (pka~4.8) is the most versatile buffer between pH 5.0 and 7.0 for LCMS that can be used in this case. Figure 2.15 shows two chromatograms obtained by using the mobile phase buffered with this volatile salt. At pH higher than its first pKa (4.8), thiamine loses one positive charge. Thought it is still a cation, the effect of removing one charge from the molecule is quite significant to its retention on RP columns. As to pyridoxine, within the pH range of 5.0 to 7.0, it occurs in zwiterrionic form (or there is an equilibrium shift towards zwitterions). With one more negative charge, it was predicted that the retention of pyridoxine would decrease.

However, it happened the other way around, which is unexpected. It came out of the column at 8% of the organic phase (acetonitrile). The zwitterionic form somehow had a higher apparent hydrophobicity, hence a stronger interaction with the non-polar stationary phase. Or possibly, the zwitterions interacted more strongly with each other, resulting in an increase in their apparent hydrophobicity.

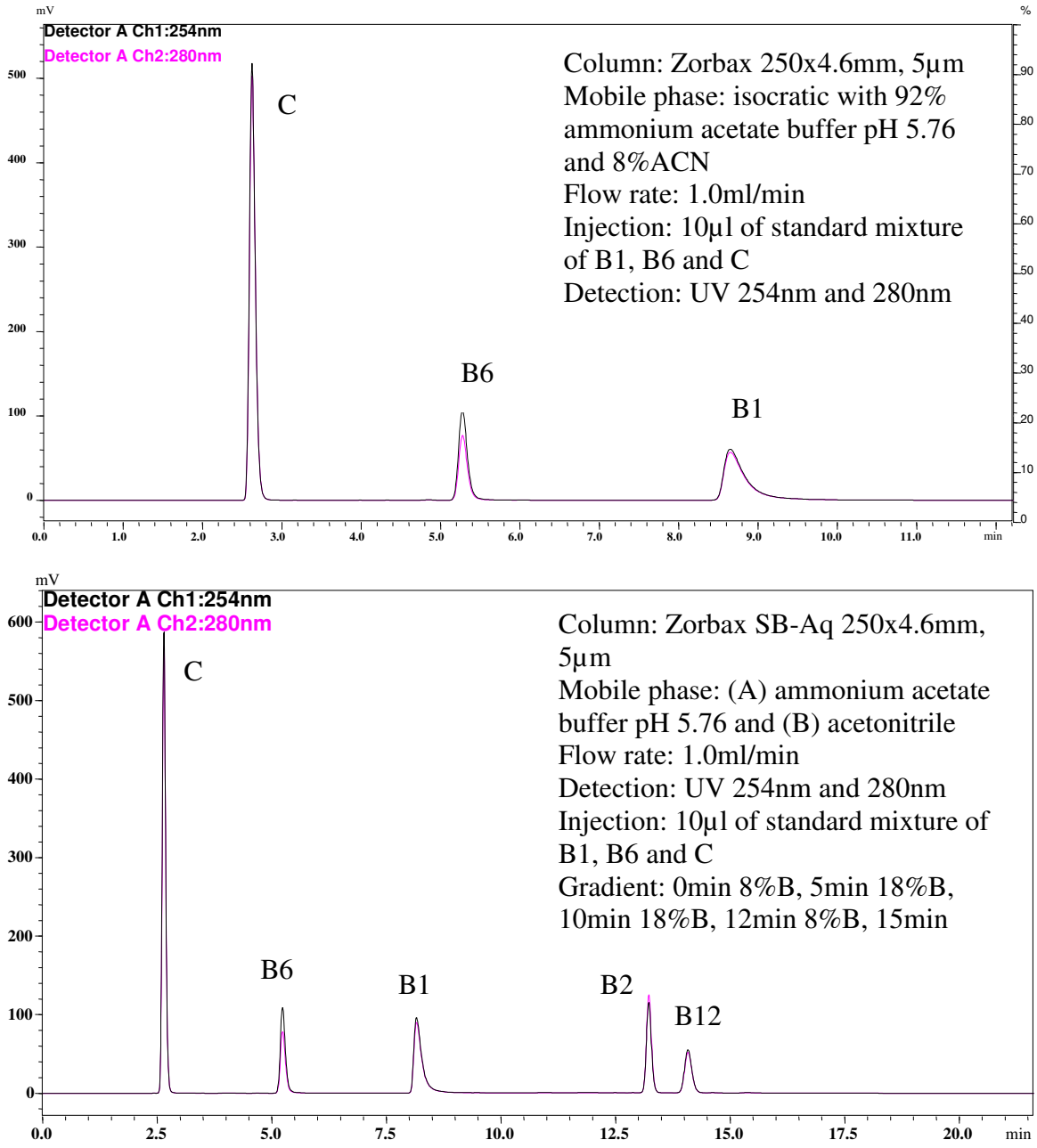


Figure 2.15 HPLC methods using buffered mobile phase to enhance retention of thiamine (B1) and pyridoxine (B6).

2.5.5.4 HILIC column

As a variation of normal phase chromatography, the combination of polar stationary phases with aqueous mobile phases has been around since the 1970s *(73, 74)*. However, the popular term HILIC (Hydrophilic interaction liquid chromatography) referring to this technique was not coined until 1990 by Alpert *(75, 76)*. Attracting great attention in the last decade, HILIC has been widely recognized as a distinct chromatographic mode useful for the retention and separation of polar compounds. HILIC utilizes a polar stationary phase such as bare silica, cyano, amino, phenyl, pentafluorophenyl (PFPP) or diol and a relatively non-polar mobile phase to facilitate resolution of polar anlaytes *(77)*. Typical components of HILIC mobile phase include a high percentage of organic solvent with water and buffer as the modifier. Offering selectivity complementary to reversed-phase chromatography, HILIC can also be referred to as "reverse reversed-phase" or "aqueous normal phase".

Even though HILIC has garnered extensive attention from HPLC application chemists and theoreticians for the past decade, its retention mechanism is still in controversy today *(77, 78)*. The most common explanation for HILIC mechanism is based on partitioning theory *(79)*. It is proposed that the aqueous portion in the mobile phase is preferentially adsorbed onto the polar stationary phase, establishing a water-enriched layer. This semi-immobilized polar layer is sandwiched between the stationary phase surface and the organic-solvent rich mobile phase *(80)*. It is the partitioning of the analytes between these two layers that result in the retention and separation in HILIC. More polar solutes tend to be distributed more in the aqueous layer, thus be retained

longer than their less polar counterparts. However, the partitioning mechanism is not the sole component responsible for the analyte retention in HILIC. Several studies suggest that HILIC retention mechanism is more of a multimodal process involving hydrogen bonding, dipole-dipole interaction and ion-exchange between the analytes with the water layer and the stationary phase surface *(79)*.

HILIC provides many advantages over traditional reversed-phase chromatography, especially when being coupled with LCMS. Not only does it offer enhanced retention to highly polar compounds that would otherwise be unretained on RP columns, HILIC also gives good peak shape to basic analytes. Due to the high volatility of the mobile phase, this technique potentially improves the sensitivity in MS and ELSD detection. Moreover, the high organic solvent content in the mobile phase has low viscosity, which allows higher flow rates for reduced analysis run time. In terms of sample preparation procedures, high organic solid phase extraction (SPE) eluents can be directly injected into the HPLC without further evaporation and re-constitution.

Demonstration for the performance of HILIC column is given in Figure 2.16. In the first chromatogram, isocratic condition with 98% acetonitrile was used to elute thiamine. It was retained longer than pyridoxine and even riboflavin, which is an inversion of retention order in reversed-phase.

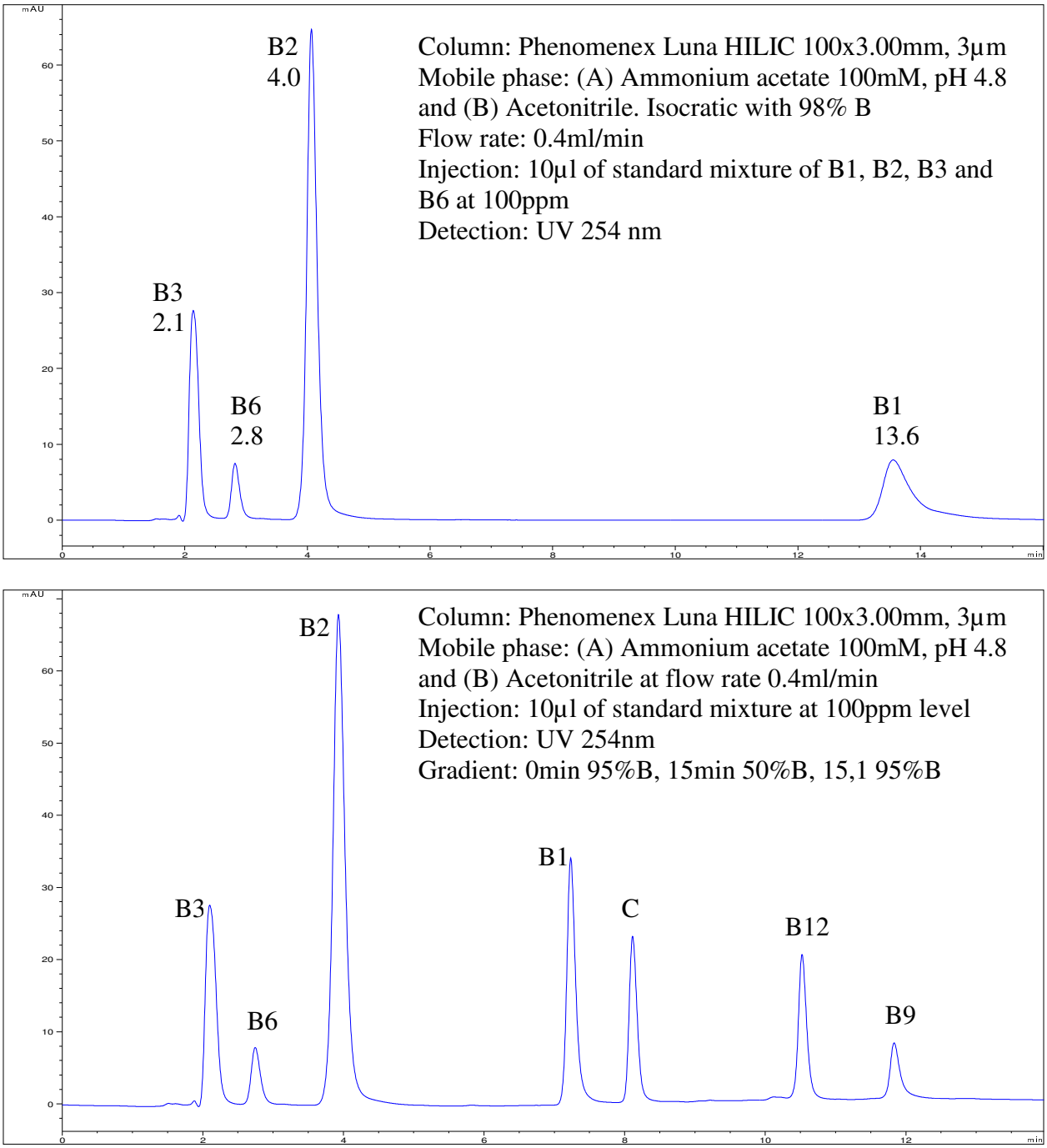


Figure 2.16 HPLC methods using HILIC column

2.5.6 Consideration for method transferring

2.5.6.1. Mobile phase

Among all the HPLC detectors used in this study, ELSD and MS require mobile phases devoid of nonvolatile salts and modifiers. When used with ELSD, such additives may collect inside the drift tube, damage the nebulizer, foul the optical cell and cause an excessively noisy baseline *(81, 82)*. In the case of MS, they can pollute the mass spectrometer, resulting in source blockages *(37)*. The common consequence for the use of nonvolatile components in both detectors is the downgrade of the system integrity and a decrease in detection sensitivity, compromising the analysis accuracy. Commonly used volatile additives for MS and ELSD are formic acid, acetic acid, triethyl amine, ammonium hydroxide, ammonium formate and ammonium acetate *(37)*. Perfulorinated acids are also acceptable for both detectors but their use in MS may lead to significant ion suppression in positive ion mode *(83, 84)*. Moreover, these acid modifiers have high surface tension which can potentially prevent efficient spray formation. Detection sensitivity may significantly decrease as a consequence.

UV-Vis detector, on the other hand, has no strict requirements about mobile phase additives. The choice of mobile phase components is dependent on the detection wavelength of the method. UV cutoffs for acetonitrile and methanol, the two most commonly used organic solvents in HPLC are 190nm and 205 nm, respectively *(72, 82)*. Considering the low wavelength of absorbance (below 210nm) by pantothenic (vitamin B5) and biotin (vitamin B7), if the target of multi-vitamin analysis with UV-Vis detector includes these two compounds, acetonitrile is a better choice than methanol. As to acid

modifiers, TFA, formic and acetic all have UV cutoffs at 210nm *(72)*. Depending on the final concentration of these acids in mobile phase, excessive baseline drift and noisy background interference can be observed at wavelengths below 240nm, which dramatically affects the detection sensitivity *(72, 82)*. The same behavior is expected for volatile buffer salts commonly used in ELSD and MS such as ammonium acetate, ammonium formate and ammonium bicarbonate. Phosphate buffers with low UV cutoffs (below 200nm) are more suitable for the UV-Vis analysis of non-chromphoric compounds. Table 2.9 displays the properties of commonly used additives for RP chromatography.

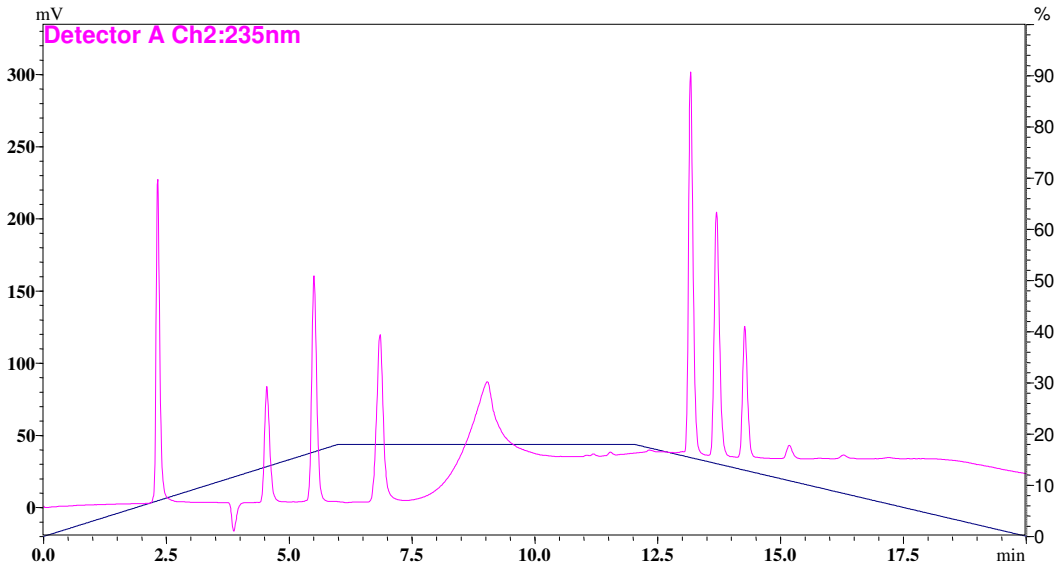
Table 2.9 UV cutoffs of common additives

Additives	UV cutoffs
TFA	210 nm (0.05% v/v)
Phosphates	<200 nm (10nM)
Formic	210 nm (10mM)
Acetic	210 nm (10mM)
Phosphoric	<200 nm
Ammonium acetate	210 nm (10 mM)
Ammonium formate	210 nm
Ammonium bicarbonate	<200 nm
Ammonium hydroxide	<200 nm

2.5.7.2. Dwell volume (gradient delay volume)

Dwell volume is defined as the volume between the solvent mixing point and the beginning of the columns *(85)*. While this volume is of no significance to isocratic runs, it plays an important role in gradient separations. The difference in dwell volume between different HPLC systems explains why gradient conditions developed in one

chromatography method do not necessarily transfer to another. Dwell volume in this case can be thought of as a de facto isocratic hold time at the beginning of the gradient *(85)*. This gradient delay time, or dwell time is equal to the dwell volume divided by the flow rate. If the dwell volume of a system is too large, it is not feasible for the use of narrow-bore columns. Demonstration of the dwell volume/dwell time concept is displayed in Figure 2.17. The gradient was scheduled to start right after sample injection. However, based on the baseline drift in chromatogram, the gradient actually did not start until after 6 minutes into the run. This delay in signal response includes the dwell time and column void time. Empirical determination of dwell volume can be found in the Appendix B.



Column: YMC Pack pro C18 5um, 250x4.6mm
Mobile phase: (A) 0.025% TFA and (B) acetonitrile at flow rate of 1.0 ml/min
Injection: 10ul of nine water-soluble vitamin mixture at 100ppm
Gradient: 0min 0%B, 6min, 18%B, 12min 18%B, 20min 0%B

Figure 2.17 Demonstration of dwell volume/dwell time

2.5.7.3. Time efficiency

Refer to the following equation in Appendix B

$$R_s = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k+1)}$$

When the values of k and α are optimized to achieve the desirable resolution (at least more than 1.5) for the critical band pair (the pair with the smallest Rs in the chromatogram), then if the separation has more resolution than required (Rs>>1.5), this excess resolution can be traded for a shorter run time, which is done by reducing the column length and/or increasing flow rate *(32)*. Demonstration of this adjustment is shown in Figure 2.18. Further reduction in run time can be achieved by using column with smaller particle size.

Columns with sub-3µm particles require very small pore frits (0.5 µm) to hold the particles in place; therefore they are easy to get plugged, which potentially reduces the column lifetime. On the contrary, the use of 3.5 µm packings seems to be a good compromise between the high performance of smaller particles and column life time *(86)*. In comparison to the common 5 µm particles, these 3.5 µm packings reduce the run time by one-half for the same column length while providing equivalent resolution *(87)*. The pressure for columns of 3.5um particles size also conveniently falls within the operating pressure range for normal HPLC system (max 400bar or ~6000psi). However, for narrow-bore columns or those with sub 3um particle size, in order to take advantage of their high efficiency with high flow rate, many factors need to be considered, including the back pressure. In those cases, UHPLC is needed.

Nowadays, in light of improvement in modern technology, new generations of columns offer more solutions to the rising demand of fast HPLC analysis. Superficially porous particle columns which were first developed in the 1970s have reemerged for the past few years *(88)*. These particles contain a solid core and an external porous silica layer. In comparison to fully porous particles, they have more uniform particle size distribution which allows them to be packed with large porosity frits. Therefore they are more resistant against clogging. Moreover, the narrower particle size distribution creates a more consistent packed bed, which greatly reduces analyte diffusion through the column. This is the A term in the Van Deemter equation. Moreover, the short diffusion paths of the analytes between the mobile phase and the thin porous crust minimize resistance to mass transfer, which is the C term in the Van Deemter equation. Superficially porous particle columns have a greatly reduced back pressure, allowing the analysis to be performed at a high flow rate.

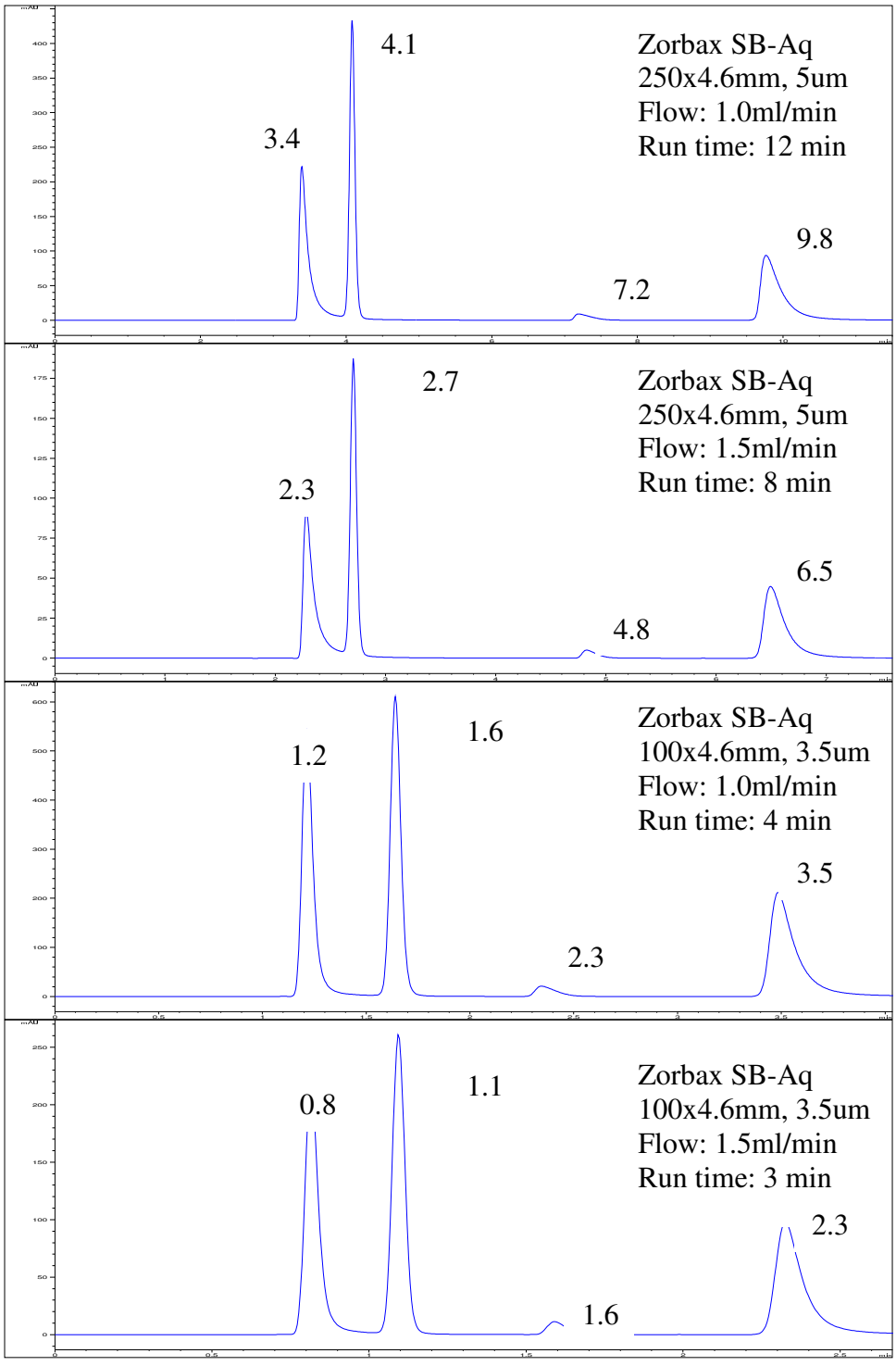


Figure 2.18 Shortened run time with flow rate adjustment or shorter column with smaller particle size

2.6 Summary on the optimization for the analysis of thiamine, riboflavin, pyridoxine, cyanocobalamin and ascorbic acid

2.6.1 Objective

The ultimate goal of the analytical methods in this study was to quantify the vitamin content in a variety of pharmaceuticals and fortified food products. In order to ensure accuracy for quantification and reproducibility for the methods, it is recommended that

- Resolution R between the peak of interest and the closest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) should be more than 2.0
- Tailing factor (T_f) which characterizes peak shape and peak symmetry should be less than 2.0
- Retention factor (k) should be more than 2.0

These requirements are stated in the Reviewer Guidance on Validation of Chromatographic Methods by FDA and should be considered during the method development and optimization phase *(89)*. However, they are not necessarily hard-and-fast rules that must be met for the analysis purpose. In reality, it is sometimes challenging to accomplish all of these requirements, especially when the analysis involves different compounds with diverse chromatographic behaviors. A more lenient requirement allowing resolution of at least 1.5 and retention factor k of more than 1.0 is acceptable for practice *(32)*. Further details on the concepts of resolution, tailing factor and retention factor are included in the Appendix B.

2.6.2 Column consideration

Due to high level of metal impurities and residual silanols, Type A columns cause serious peak tailing for basic analytes and therefore should be avoided. On the other hand, Type B columns with conventional bonded reversed-phase have dewetting issues when used under highly aqueous mobile phase. In order to avoid this inconvenience, Type B columns with novel stationary phase that is compatible with 100% aqueous mobile phase are recommended. The column classification can be looked up on the USP Column Equivalency Application Database, the website address of which was previously provided in chapter 2. Moreover, this database can serve as a useful reference source for column replacement if a specific column cited for a to-be-used method is not available. The list of equivalent columns to those used in this study is provided in the Appendix C.

2.6.2 Mobile phase consideration

Acidic mobile phase with pH of 2.0-3.0 is favored as it is easily prepared with only one single component of acid modifier. At such a low pH, the ionization of the residual silanol groups are mostly suppressed, resulting in minimal cation-exchange interactions between, hence minimizing peak tailing for basic analytes. Another advantage of acidic mobile phase is that it is consistent in preparation, at least more than pH-based salt buffers at higher pH levels. Other considerations about buffered mobile phase are discussed in section 2.5.6.3 and “Method transferring” section above.

Thiamine, pyridoxine and ascorbic acid are all ionizable compounds; therefore, pH of the mobile phase is of major importance for optimal separation. With pKa of 4.3,

ascorbic acid stays unionized at low pH, which gives its maximum retention in RP columns. Pyridoxine, on the other hand, is positively charged under acidic conditions. These two compounds have quite adequate retention in RP columns under 100% aqueous acidic mobile phase, with retention factor k bigger than 1.0 at least in all Type B columns tested in this study. Thiamine is the least retained among the three ionizable vitamins. In many tested columns, the retention was between 0 and 1. In conventional RP columns, it even elutes before the void time. Thiamine and pyridoxine are basic compounds; therefore a simple way to enhance their retention is to increase the pH of the mobile phase using buffer salts with pH of 5.0-7.0. Another way is to use perfluorinated acid modifiers. TFA enhances the retention of the two compounds a little bit, but not as significantly as HFBA and possibly other higher-chain acids in the series. HILIC columns, which provide complementary retention to RP columns, can also be used as another alternative for better retention of all these three analytes. A chromatographic method using HILIC column for the analysis of vitamin B1 in dry-cured sausages was reported *(90)*.

For riboflavin and cyanocobalamin, there are no issues with early elution or peak tailing at least within the working pH range of silica-based columns and for all the columns tested in this study. They are both well-retained in RP columns and can be conveniently eluted with about 15% of organic solvent in the mobile phase.

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CHAPTER THREE

SIMULTANEOUS ANALYSIS OF WATER-SOLUBLE VITAMINS IN PHARMACEUTICALS AND FORTIFIED FOOD PRODUCTS

3.1 Introduction

Ever since liquid chromatography was first utilized for the analysis of vitamins, chromatographers have made efforts to develop methods targeting at simultaneous determination of more than one vitamin. Implementation of such multi-analyte analysis for routine analysis results in time and cost efficiency. Due to their diverse chemical properties, it is challenging to resolve all water-soluble vitamins in one chromatographic run without compromising the analytical sensitivity, accuracy and precision when compared to single-analyte methods. Selected analytical methods for multi- and single-vitamin analysis that are available in chromatography literature are presented in Table 3.1 below and Tables A10 to A14 in the Appendix D. Based on the review of these reported procedures and the chromatographic behavior study of the five water-soluble vitamins presented in chapter 2, two methods for the simultaneous analysis of nine water-soluble vitamins in pharmaceuticals and fortified food products using 1) DAD-ELSD and 2) LCMS were developed and are presented in depth.

Table 3.1 Selected HPLC methods for multi-analyte analysis of water-soluble vitamins

Sample matrix/Analyte	Extraction/Clean up	Column/Mobile phase	Detection
Fortified cereals/ thiamine, riboflavin, pyridoxine *(1)*	Hydrolyze 2g sample with 35 mL 0.1N H_2SO_4 in boiling water bath for 30min. Add clara amylase, incubate at 55°C for 1 hour. Centrifuge and filter.	µBondapak C18 (10µm, 250 x 4.6mm) Mobile phase: 30:69:1 methanol/water/acetic acid mobile phase containing 0.005 M hexanesulfonate	Post-column thiochrome reaction for B1 Ex λ= 288 nm and Em λ= 418 nm
Soybeans, tofu/ thiamine, riboflavin *(2)*	Heat hydrated sample at 90°C for 30 min. Adjust to pH 2 with 5N HCl and autoclave for 15 min. Adjust to pH 4.5, centrifuge, filter, dilute. Thiochrome derivatization for B1 analysis	Ultrasphere (Beckman) 5µm, 150x4.6mm Mobile phase: acetonitrile and 0.01M acetate buffer (13:87), pH 5.5 at flow rate of 1.2 ml/min	Fluorescense B2: Ex λ= 436 nm and Em λ= 535 nm; Thiochrome: Ex λ= 364 nm and Em λ= 436 nm
Meat, liver/ thiamine, riboflavin *(3)*	Autoclave homogenized sample in 0.01M HCl at 121°C for 30 min. Cool, adjust pH to 4.5, add takadiastase and incubate at 37°C for 16-18hr. Filter, adjust to pH 6.5 then dilute.	Nucleosil ODS (3 µm, 150 x 4.6mm) kept at 45°C Mobile phase: 0.01 KH_2PO_4 (pH 3.0)-acetonitrile (84:16) containing 5mM hexanesulfonate	UV 254 nm
Pharmaceutical preparations/ nine water-soluble vitamins *(4)*	Dissolve ground sample or dilute liquid sample with water. Clean-up with Lichrolut RP-18 SPE	Lichrosorb RP-18 (5µm, 250 x 4.0 mm) Mobile phase: gradient with methanol and 0.05M ammonium acetate at the flow rate of 1 ml/min	UV 270 nm and 290 nm
Infant formula/ B1, B2, B3, B6, B9, B12	Mix 8g infant powder milk with 10ml water. For	Tracer Spherisorb ODS 2 C18, (5µm, 250 x 4.6mm) Mobile phase: isocratic	PDA at different wavelengths

(5)	liquid milk, leave as it is. Ad 1g TCA to 10.5g liquid preparation, stir for 10min. Centrifuge and add 3ml TCA 4%, extract again. Dilute pooled extracts to 10ml with TCA 4% and filter.	with water:methanol (85:15) containing 0.5% triethylamine, 2.4% glacial acetic acid and 5mM octanesulfonic acid (pH 3.6) at flow rate—1 mL/min	
Pet foods, animal feedingstuffs/ thiamine, riboflavin *(6)*	Acid hydrolysis sample in 0.1M HCl in boiling water bath for 1hr. Cool, adjust to pH 4.3-4.7, add claradiastase and incubate at 37^{o}C for 16-17hr. Add TCA 50% and heat in water bath for 10min. Cool, filter. Thichrome derivatization	For B1: Spherisorb NH_2 5µm, 250x4.6mm isocratic at 2ml/min flow rate with chloroform-methanol (90:10) For B2: Spherisorb ODS1 (5µm, 250 x 4.6mm), isocratic at 1mL/min flow rate with methanol-water (50:50)	Fluorescense: B1 (Ex λ =365 nm, Em λ =435 nm) and B2 (Ex λ =450 nm, Em λ =510 nm)
Various foodstuffs/ thiamine, riboflavin, pyridoxine *(7)*	Acid and enzyme hydrolysis with various modifying changes to test the efficiency of the extraction procedure	Lichrospher 100RP 18 endcapped, (5µm, 250 x 4mm)	Fluorescence detection
Pharmaceuticals/ thiamine, pyridoxine , niacinamide, riboflavin, cyanocobalamine *(8)*	Simple dissolution of sample and SPE clean-up with C18 AR	Nova-Pack C18, (4µm, 150 x 3.9mm) Mobile phase: methanol and 0.05M ammonium acetate	UV 270 nm and 362 nm
Mushroom/ thiamine, riboflavin *(9)*	Hydrolyze 2g of sample homogenate in 60ml 0.1M HCl in a water bath (95-100^{o}C) for 30 min. Cool, adjust pH to	Spherisorb ODS-2 C18, (5µm, 250 x 4.6mm) Mobile phase: 0.04M H_2SO_4 in water; 0.2M acetate buffer containing 0.005 M octanosulfonic	Fluorescense B2: Ex λ= 422 nm and Em λ= 515 nm; Thiochrome: Ex λ= 360 nm and

	4-4.5 then add takadiastase. Incubate for 3hr at 45-50^{o}C. Add 2ml TCA 50% (w/v) then heat at 100^{o}C for 5 min. Filter, dilute. Thiochrome derivatization for thiamine.	acid in water, with acetonitrile at different ratios; methanol/water (50:50, v/v) and acetonitrile/ water (50:50, v/v). Flow rate of 1mL/min	Em λ= 425 nm
Pharmaceutical preparations/ nine water-soluble vitamins *(10)*	Dissolve tablets in 50 ml of 0.1M phosphate buffer (pH 7.0). Dilute 100 times with phosphate buffer and filter	µBondapak C18 (10µm, 300 x 3.9 mm) Mobile phase: gradient with methanol and 0.1M KH_2PO_4 buffer (pH 7.0) at flow rate of 1.5 mL/min	PDA multiple wavelengths
Infant formula/ B1, B2,B3, B6 *(11)*	Mix 6g milk powder with 30ml warm water. Add 30ml 0.6M TCA to the solution, shake for 15min and filter	Luna Prodigy ODS 3, (5µm, 150 x 4.6mm) Mobile phase: isocratic with methanol:water:formic acid (25:74:1) containing 0.1% sodium dioctylsulfosuccinate, pH 2.8 at flow rate of 2 mL/min	Fluorescence Ex λ = 290nm Em λ = 390nm for B6 Ex λ = 450nm Em λ = 510nm for B2 UV 258 nm for niacin
Supplemented infant formulas and baby foods/ vitamins B1, B2, B3, B6, B9 and B12 *(12)*	Acid hydrolysis: 10g sample in 25ml 0.1M HCl, stand in water bath at 90°C for 30 min. Cool, adjust pH to 4. Enzyme hydrolysis: Add 0.1 g Takadiastase, incubate for 2 h. Add 1ml 50% TCA, heat at 90°C (in water-bath) for10 min. Cool, adjust pH to 6, dilute to 50 mL with 10mM KH_2PO_4 (pH 6),	Supelco RP-Amide C16, (5µm, 150 x 4.6mm) Mobile phase: gradient with 10mM KH_2PO_4 (pH 6) and acetonitrile at flow rate of 1mL/min	PDA multiwavelength

	centrifuge and filter		
Multivitamin tablets/ thiamine, riboflavin, pantothenic and pyridoxine *(13)*	Dissolve samples in water, dilute at different levels	C18 Microsorb (5µm, 250 x 4.6mm) Mobile phase: gradient with 0.01 M ammonium Acetate (A) and methanol (B)	FTIR; UV 210 nm and 254 nm
Pharmaceutical formulations/ thiamine, pyridoxine, cyanocobalamin *(14)*	Dissolve ground sample with 100ml water, mix and stand in dark for 10 min. Filter.	Supelco C18 (5µm, 250 x 4.6mm) Mobile phase: isocratic with 0.05M sodium phosphate dibasic, heptahydrate, 10% methanol (v/v) and 0.018M trimethylamine adjusted to pH 3.55 with 85% phosphoric acid. Flow rate of 1ml/min	Coulometric electrochemical and UV detection
Italian pasta/ B1, B2, B3, B5, B6, B9 *(15)*	Three extraction procedures for three groups (group 1 with B1, B2, B3 and B6), group 2 with B5 and group 3 with B9).	Discovery RP-Amide C16 5µm, 150x4.6 mm, Mobile phase: gradient with ammonium formate buffer 20 mM, pH 3.75 and methanol.	LC-MS/MS ESI+
Turkish food/ C, B1, B2, B3, B5, B6, B9 *(16)*	Mix 5g sample with 20ml water, homogenize and centrifuge. Clean-up with Sep-Pak C18 (500 mg)	Discovery C18, (5µm, 150 x 4.6mm) Mobile phase: isocratic with methanol:0.1M KH_2PO_4 (pH 7.0) (10:90) at flow rate of 0.7ml/min	UV 290 nm and Visible 550 nm
Polyvitaminated premixes/ B1, B2, B3, B6, B9, B12, C *(17)*	Mix 2g sample with 40ml water then add 4ml NaOH 2M. Add 50ml phosphate buffer 1M, pH 5.5, dilute to 100 ml with water. Sonicate for 10 min and filter	YMC-Pack Pro C18, (5µm, 250 x 4.6 mm) Mobile phase: gradient with 0.025% TFA (pH 2.6) and acetonitrile at flow rate of 0.8ml/min	UV 210 nm and 275 nm
Supplemented foods/eight water-soluble vitamins *(18)*	Mix 0.5g sample with 4.5 mL warm water (40°C), homogenize for 1	Spherisorb ODS-2, (3µm, 250 x 4.6 mm) Mobile phase: gradient with methanol-phosphate	Fluorescence and UV detection with multiwavelength

	min, sonicate for 5 min. Let stand in the dark for 60 min. Centrifuge and filter the supernatant.	buffer (pH 2.95) at flow rate of 1ml/min	
Multivitamin tab-lets/ B1, B2, B3, B5, B6, B7, B9, C *(19)*	Mix ground sample with water containing 0.024% ammonia, shake for 1 min. Sonicate for 30 min at 60°C. Cool down and adjust pH to 7 with formic acid. Filter supernatant	Spherigel C18, 5µm, 250x4.6 mm Mobile phase—gradient with 5mM HFBA and methanol at flow rate of 1 ml/min, split 0.2ml/min into MS	MS-ESI+/-
Multi-vitamin,-mineral supplements/ (B1, B2, B3, B6, biotin, pantothenic and folic *(20)*	Weigh portions slightly more than $1/10^{th}$ of an average tablet (0.1527 g) into red-colored 50-ml volumetric flasks, and bring to volume with 10 mM phosphate buffer (pH 2.5).	Hydro-RP C18, (4µm, 250 x 2.0mm) Mobile phase: (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile	MS ESI+ with MRM UV at different wavelengths
Enriched Brazilian Dairy Products/ B1, B2, B3, B6 *(21)*	Weigh 5-8g sample in a 100 mL flask, add 45 mL 0.1N H_2SO_4. Sonicate for 1hr. Transfer the solution to a 100ml volumetric flask, fill with methanol and store at -18°C for one hour. Mix and filter.	Spherisorb ODS-2 C18, (5µm, 150 x 4.6 mm) Mobile phase: acetonitrile, aqueous phase (5 mmol/L hexanesulfonic acid, 0.15% TEA, adjusted with 10% H_2SO_4 to pH 2.8) and methanol	UV 254 nm and 278 nm
Fortified foods/ ascorbic, riboflavin, niacinamide, pyridoxine, folic *(22)*	Weigh homogenized samples in 50-mL graded polyethylene centrifuge tubes, add 25 mL with 2% (m/v) metaphosphoric	Waters XTerra MS C18, (5µm, 250 x 4.6 mm) Mobile phase: gradient with (A) 0.02% (m/v) TFA in water and (B) 0.02% TFA 90:10 methanol/water (v/v%)	UV 266 nm

	acid, shake for 10min. Centrifuge and filter. Add 5 ml of 1% (m/v) L-cysteine solution and asjust pH to 7.0 with ammonia or metaphosphoric solution.		
Multivitamin syrup/ ascorbic, thiamine, FMN, pyridoxine, niacinnamide, d(+)-panthenol *(23)*	Dilute 5ml syrup in a 25-ml flask with 0.1% (m/v) o-phosphoric acid	Zorbax SB-Aq C18, 5µm, 250x4.6 mm Mobile phase: gradient with (A) 0.0125 M hexane-1-sulfonic acid sodium salt in 0.1% (m/v) o-phosphoric acid, pH 2.4–2.5 and (B) acetonitrile at the flow rate of 1 mL/min .	UV 210 nm for vitamins C, B6 and B5; 254 nm for vitamins C, B3, B2, B1
Various foods/ fourteen water-soluble vitamins *(24)*	Add BHT to 2g homogenized sample. Clean-up with C18 sorbent.	Alltima C18, (5µm, 250 x 4.6mm) Mobile phase: 5 mM formic in water and acetonitrile	LC-MS/MS ESI+ with MRM
Infant formula/ B1, B2, B3, B5, B6 *(25)*	Mix sample with 5mM HCl 20% methanol extraction solvent. Sonicate, adjust pH to 4.5-5.5. Filter	Zorbax XDB C18, 3.5µm, 100x2.1mm Mobile phase: 0.1% acetic in water and 0.1% acetic in methanol	LC-MS/MS ESI+
Multivitamin tablets/ eight water-soluble vitamins *(26)*	Weigh portions slightly more than $1/10^{th}$ of an average tablet (0.1527 g) into red-colored 50-ml volumetric flasks, and bring to volume with 10 mM phosphate buffer (pH 2.0).	Hydro-RP C18, (4µm, 250 x 2.0 mm) Mobile phase: gradient with (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile	MS ESI+ with MRM UV at different wavelengths
Infant/Adult nutritional formula powder/ nine water-soluble vitamins	N/A	Hydro-RP C18, (4 µm, 250 x 2.0mm). Mobile phase: (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile	MS ESI+ with MRM

(27)		SeQuant ZIC-HILIC 3.5µm, 150x2.0 mm. Mobile phase: (A) 20mM pH3.7 formic acid/ammonium formate and (B) 0.1% formic acid in acetonitrile	
Fortified foods/ B1, B2, B3, B6, B9 and C *(28)*	Weigh homogenized samples in 50-ml graded polyethylene centrifuge tubes, add 25 ml with 2% (m/v) metaphosphoric acid, shake for 10min. Centrifuge and filter. Add 5 ml of 1% (m/v) L-cysteine solution and asjust pH to 7.0 with ammonia or metaphosphoric solution.	Restek Ultra Aqueous C18 (5µm, 150 x 3.2 mm) Mobile phase: (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol	UV 266 nm and 290 nm
Supplemented foods/ nine water-soluble vitamins *(29)*	Place 0.5 g of sample in a 15 mL plastic tube with 2.0 mL of water. Homogenize for 3min at room temperature, add 6.0 ml of acetonitrile. Centrifuge, evaporate to dryness and reconstitute with water.	Develosil RPAQUEOUS C30 (5µm, 250 x 4.6mm) Mobile phase: 20 mM phosphate buffer (pH 3.0) and acetonitrile	UV 205nm and 275nm

3.2 Materials and methods

3.2.1. Standards and reagents

Vitamin standards were purchased from different suppliers/manufacturers: thiamine hydrochloride, pyridoxine hydrochloride and cyanocobalamin from Enzo Life Sciences (Farmingdale, NY); riboflavin from Eastman Kodak Co. (Rochester, NY), ascorbic acid from Fisher Scientific (New Jersey, USA), pantothenic from Sigma-Aldrich (St. Louis, MO, USA) , niacinamide and biotin from Acros Organics (New Jersey, USA), folic acid from ICN Nutritional Biochemicals (Cleveland, O., USA). All reagents are of analytical grade.

HPLC grade acetonitrile was purchased from Fisher Scientific (New Jersey, USA). Trifluoroacetic acid 99% (TFA) and formic acid 99% were obtained from Acros Organics (New Jersey, USA). Water was purified using a Millipore Synergy UV system (Millipore Billerica, MA, USA). Mobile phase pH was measured using UB-10 pH meter from Denver Instrument (New York, USA).

3.2.2. Standard preparation

Stock individual solutions of thiamine, pyridoxine, cyanocobalamin and ascorbic acid were prepared monthly at concentration of 1000 ppm (1 mg/mL) in Millipore-purified water. Riboflavin, biotin and folic acid were also prepared at 1000 ppm by dissolving 10 mg of the components into 10 mL of 0.5% sodium hydroxide. These stock solutions were kept in 1.5 mL Eppendorf tubes and stored at -80^{o}C to avoid degradation. Working solutions of vitamin standards were prepared daily by mixing and diluting

individual stock solutions in water to desired concentrations. Preparation steps were performed in the subdued light condition using glasswares covered with foil to prevent vitamins from degradation, especially vitamin B2, B6 and B12.

3.2.3 Stability study

The nine analytes were divided into two groups: group 1 includes thiamine, pyridoxine, niacinamide and ascorbic acid; group 2 includes pantothenic acid, folic acid, cyanocobalamin, riboflavin and biotin. In each group, mixtures were adjusted to three different pH ranges (acidic 2.0-3.0, neutral 6.0-7.0 and basic 9.0-10.0) with either 0.1% formic acid or 0.05% NaOH. Chromatographic conditions for monitoring the stability of all compounds in two groups are provided in Table 3.2. Each group was tested separately on two different days. Stability graphs were obtained by plotting peak areas of thiamine, niacinamide and ascorbic acid at 254 nm, pyridoxine and riboflavin at 280 nm and pantothenic acid, folic acid, cyanocobalamin and biotin at 210 nm against time.

Table 3.2 Chromatographic conditions for stability study

LC systems	Shimadzu SIL-20A HT auto-sampler, Shimadzu LC-20AT liquid chromatograph, Shimadzu DGU-20A5 degasser and Shimadzu SPD-20A UV-Vis detector.
Column	YMC Pack Pro C18 3.5µm, 150x4.6mm
Column temperature	Ambient
Mobile phase A	0.1% formic acid in water
Mobile phase B	Acetonitrile
Flow rate	1.0 ml/min
Detection	UV 254 mn and 280 nm for Group I UV 210 nm and 280 nm for Group II
Run time	10 min for Group I and 15min for Group II

3.2.4 Vitamin analysis by DAD-ELSD and LCMS

3.2.4.1 Sample preparation

Three brands of multivitamin tablets, two brands of fortified cereals and one brand of infant formula were purchased from the local grocery stores.

For multivitamin tablets, a composite of ten counts of each brand was ground into fine powder and a portion equivalent to one tablet was weighed into a 100 ml volumetric flask covered with foil. About 50 mL of NaOH 0.05% was added into the flask and the mixture was vigorously shaken and sonicated in the dark for 10 min. The pH of the mixture was then adjusted with 1% formic acid before the final solution was brought to the mark with deionized water. The extract was run through a 0.45 µm nylon membrane filter before the injection.

For fortified cereals, 1.0 gram of each brand was weighed into 15 mL plastic centrifuge tube and mixed with 10 mL deionized water. The mixture was vortexed to mix thoroughly, sonicated for 10 min in the dark with intermittent shaking and centrifuged at 5000 rpm for 10 min at 4^{o}C. The supernatant was filtered through a 0.45µm nylon membrane, then ready for the analysis.

For infant formula, about 10.0 gram of the powder was mixed with 20 mL of deionized water in a 50 mL plastic centrifuge tube. The mixture was vortexed and sonicated for 10 min then 200 µL formic acid was added to precipitate protein in the sample. Following vigorous shaking, the mixture was centrifuged at 5000rpm for 10min at 4^{o}C. The supernatant was filtered through a 0.45 µm nylon membrane, then ready for the analysis.

3.2.4.2 Chromatographic conditions

Table 3.3 Chromatographic conditions for DAD-ELSD

LC systems	Shimadzu LC-20AT Liquid chromatograph, Shimadzu DGU-20A5 Degasser, Shimadzu CTO-20A Column oven, Shimadzu CBM-20A Communication bus module, Shimadzu SPD-M20A Diode array detector, ELSD-LTII Low-temperature evaporative light scattering detector
Column	YMC Pack Pro C18 (3.5µm, 150 x 4.6mm)
Column temperature	Ambient
Mobile phase A	0.025% TFA in water
Mobile phase B	Acetonitrile
Flow rate	1.0 mL/min
Injection volume	20 µL
DAD	190 to 400 nm
ELSD	Nebulizer temperature 40C, Gas pressure 350KPa, Gain 8
Gradient	Time %A %B 0min 100 0 1min 100 0 4min 84 16 9min 84 16 9.1min 100 0 16min Stop

Table 3.4 Chromatographic conditions for LCMS

LC systems	Agilent Technologies 1200 Series LC system consisted of G1379B Degasser, G1312A Binary pump, G1329A Autosampler, G1316A Thermostatted column compartment and G1314B Variable wavelength detector
Column	Agilent Zorbax SB-Aq, (3.5 µm, 4.6 x 100 mm)
Column temperature	Ambient
Mobile phase A	0.1% formic acid in water
Mobile phase B	Acetonitrile
Flow rate	0.6 mL/min
Injection volume	10 µL
UV Detection	254nm from 0 min to 11.5 min, 210 nm from 11.5 min to 18.5 min, 254 nm from 118.5 min till stop
MS Condition	API-ES+, drying gas flow 12.0 L/min, drying gas temperature 350^{o}C, nebulizer pressure 45 psig, capillary voltage 4000V
Gradient	Time %A %B 0min 100 0 5min 100 0 6min 85 15 15min 85 15 15.1min 20 80 17min 20 80 17.1min 100 0 25min Stop

3.3 Results and discussion

3.3.1 Stability study

3.3.1.1 Vitamin stability

Originally, this project intended to focus on the analysis of the five water-soluble vitamins introduced in chapter 1, including thiamine, pyridoxine, ascorbic, riboflavin and cyanocobalamin only. However, when optimizing the HPLC methods for the analysis of those five vitamins in the samples, the interferences from other vitamins in the samples must be resolved from them. The optimized method ended up separating all water-soluble

vitamins in the sample. Consequently, the project was expanded to the analysis of all nine water-soluble vitamins in pharmaceuticals and functional food products. This stability study serves as a preliminary screening for the optimization of standard storage and sample extraction procedures. Stability of all the water-soluble vitamins was tested in three different pH conditions (acidic, basic and neutral) over a 24-hour period in HPC vials at ambient temperature.

Pyridoxine and niacinamide were the most stable at all pH conditions. All the analytes were stored in amber HPLC vials at ambient temperature in the autosampler. It is the limited light exposure that protected pyridoxine from degradation. Thiamine was stable in acidic and neutral pH but steadily degraded over a one-day period. Ascorbic acid is the least stable analyte of all with degradation being observed under all three pH conditions. It degraded extremely fast under basic pH with only less than 0.5% left after 5 hours. Steady degradation was observed for the neutral condition while it is the most stable in acidic pH.

It is hard to explain the unexpectedly increasing trend of pantothenic at acidic pH and biotin at basic pH. The former underwent a steady degradation of more than 20% by the end of the testing period under the basic condition while appearing stable under the neutral condition. The latter showed high stability at both acidic and neutral pH.

Cyanocobalamin and riboflavin are susceptible to degradation in basic solution while being more stable in the other two pH ranges. Folic acid has no sign of degradation under neutral and basic condition but it shows a dramatic decrease in detection response under acidic pH. Folic acid is notorious for its solubility dependence on solution pH;

therefore this behavior needs to be examined more carefully to determine if a degradation or solubility issue or both are to be responsible.

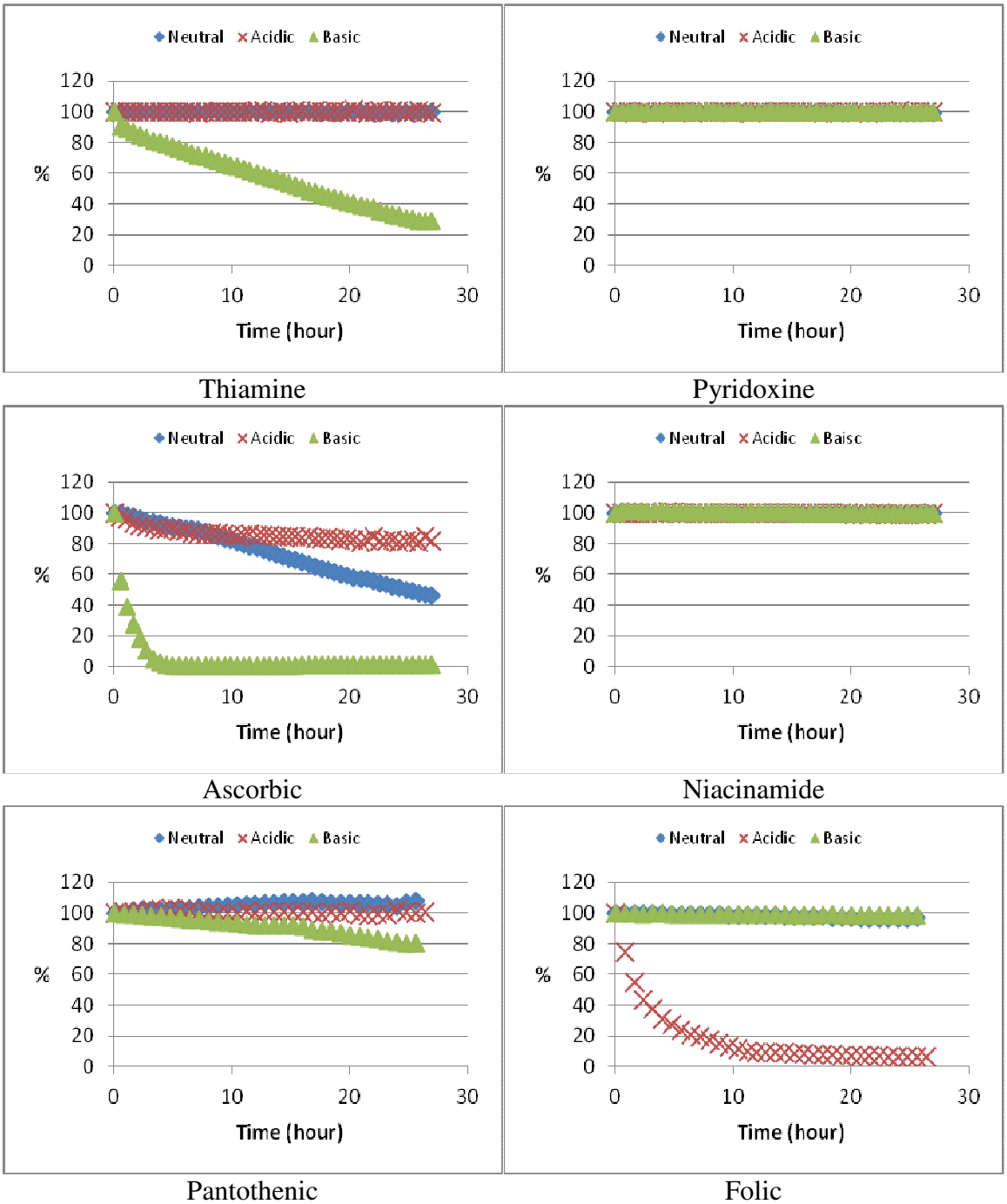
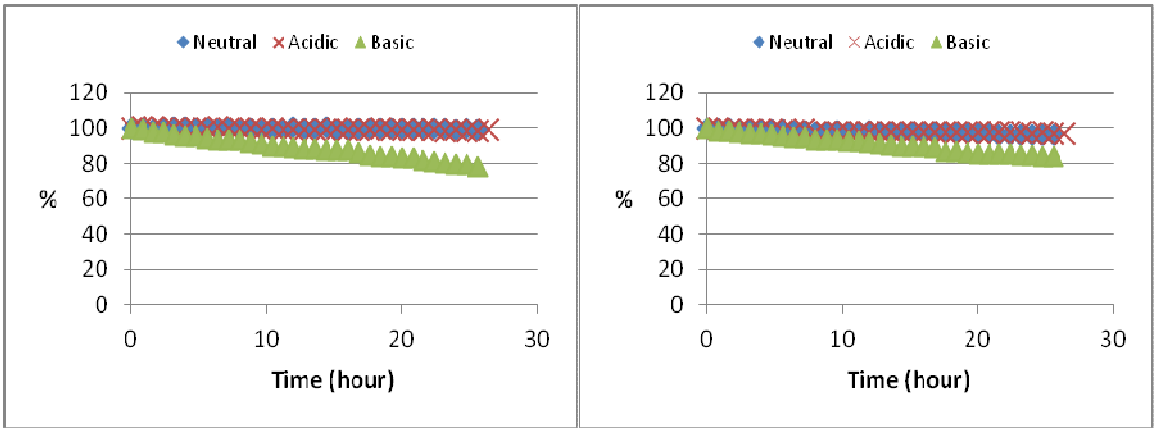
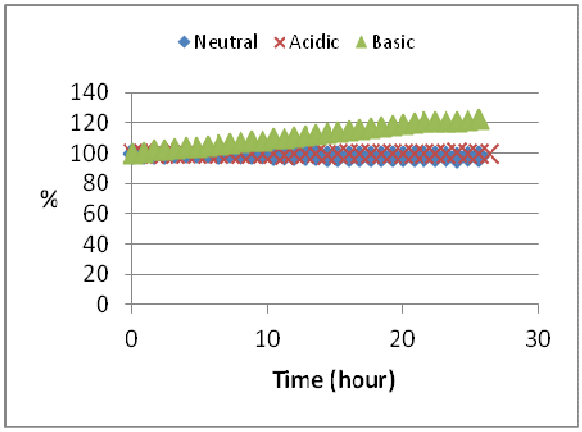


Figure 3.1 Stability of nine water-soluble vitamins in neutral, acidic and basic solutions within one day period



Cyanocobalamin

Riboflavin



Biotin

Figure 3.1 (continued)

3.3.1.2 Sample extraction procedure

Solubility and stability of the vitamin analytes present a real challenge for designing an optimal extraction procedure. Riboflavin, biotin and folic acid are the three compounds with the lowest solubility in the water-soluble vitamin group. Riboflavin is limitedly soluble in distilled water and slightly more soluble in an acidic condition. Literature cited the solubility of riboflavin in water as up to 0.013 g per 100 mL, equal to about 130 ppm *(30)*. However, this optimal solubility can only be reached with heating

and agitation for a prolonged period of time *(31)*. Considering the stability issue of vitamins, high temperature and long preparation time should be avoided. On the other hand, a basic solution is an alternative as riboflavin is dissolved much more easily at high pH condition. Stock solution of riboflavin was prepared in 0.05% NaOH. Though riboflavin is slightly unstable in such a high pH condition, the storage of its stock solution at -80^{o}C managed to avoid possible degradation. When a working solution is needed, the adjustment of pH is maybe needed if the solution is planned to be used for an extensive amount of time.

Biotin and folic acid are also prepared and stored as riboflavin due to their higher solubility in basic solution *(30)*. However, as the sample extraction may involve the adjustment of pH, it is questionable if the analyte may fall out of solution when brought from high pH to neutral or acidic pH. When evaluating the peak areas for stability, it was noticed that the peak area of biotin and riboflavin were about the same at all three different pH conditions, which means they did not fall out of the solution after being diluted from their basic stock solution. However, the result for folic acid showed otherwise when its stock was diluted with acidic solution.

In order to ensure that that these three low-soluble vitamins were completely extracted from the multivitamin tablet, basic condition was used first before the solution was adjusted back to the neutral pH. The extraction procedures were modified a little for fortified food samples. Because the three vitamins with low solubility occur in small amount in these samples, high pH was not required for their optimal extraction. Fortified cereals were only extracted in deionized water. Infant formula, on the other hand,

requires acidic treatment with 0.1% formic acid (pH~2.7) for the removal of its protein content. However, the amount of folic acid added in infant formula is so small that it is within the solubility limit of this compound at pH 2.7 of the extractant *(32)*.

3.3.2 Multivitamin analysis using DAD-ELSD

3.3.2.1 Chromatographic conditions

The chromatography of all nine water-soluble vitamins was performed on YMC Pack Pro C18 column (150 x 4.6mm, 3.5 µm). TFA was used in the aqueous mobile phase at the concentration of 0.025%. This small amount of TFA is sufficient to maintain the low pH of 2.6 for the mobile phase while significantly enhancing the retention of thiamine and does not cause much of baseline shift at low wavelength, where pantothenic and biotin are detected. Moreover, this volatile acid modifier is suitable for ELS detector which is run in tandem with DAD.

DAD detector was set to collect signals within the range of 190-400 nm. Due to their diverse molecular structures and different spectroscopic properties, it is impossible to choose a single wavelength for the detection of all nine water-soluble vitamins. The maximum absorbance is normally chosen. However, the interference of impurities at certain wavelengths around the maxima is also an important factor to be considered. The selected wavelengths should produce a high absorbance signals for the analytes of interest without much background interference. In the case of biotin and pantothenic, due to their lack of chromophores, the wavelength choice is quite limited. For the purpose of quantification, three following single wavelengths were used: 210 nm for pantothenic

acid, cyanocobalamin and biotin; 254 nm for thiamine, ascorbic acid, niacinamide and riboflavin; 280 nm for pyridoxine and folic acid. Chromatograms at these wavelengths were displayed in Figure 3.2.

UV-Vis detector is moderately selective because their response to analytes is dependent on the analytes' light absorbing property at certain wavelengths *(33)*. However, the detector may partially become universal used at low wavelengths (195 nm to 210 nm) because most organic compounds respond more or less within this range *(34)*. This characteristic is taken advantage for the detection of pantothenic acid and biotin, both of which lack chromophores, at 210 nm for this HPLC method. One drawback of detection at such a wavelength is the shifted baseline and noisy background, which may significantly reduce the detection sensitivity *(35)*.

ELSD measures the amount of light scattered by particles in the eluent after the mobile phase has evaporated. In that sense, ELSD responds to all compounds that do not evaporate or decompose, hence being considered as a nearly universal detector *(36-38)*. The chromatogram in Figure 3.3 shows the separation of all nine water-soluble vitamins, including the two non-chromaphoric pantothenic and biotin. The peak size of the analytes (except for niancinamide) at the same concentration in ELSD is more uniform than that in DAD. This uniformity in response factor is due to the fact that the output given by ELSD reflects the quantity of total analyte in the sample that causes light scattering *(33)*.

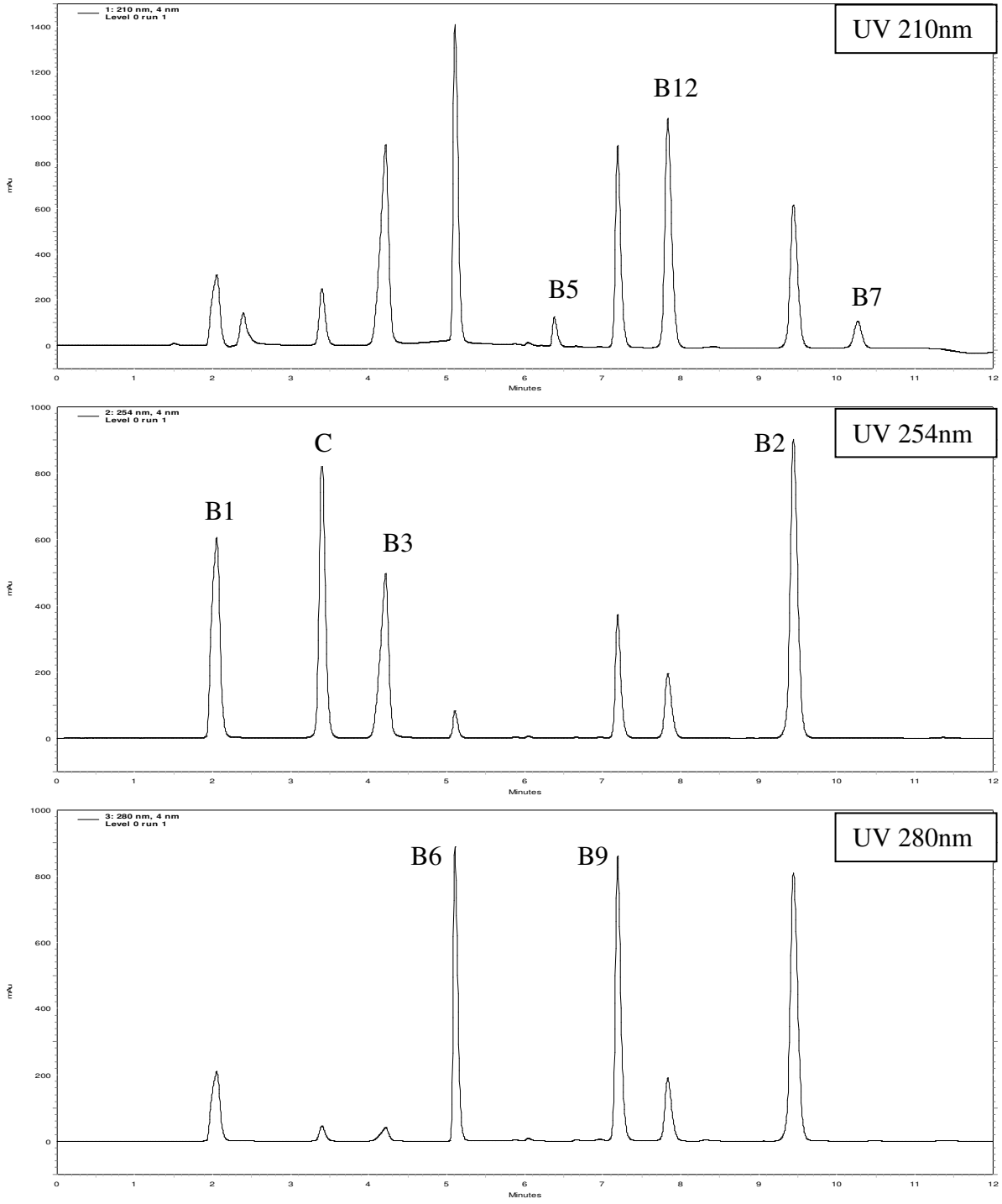


Figure 3.2 HPLC-DAD chromatograms of nine water-soluble vitamin standards

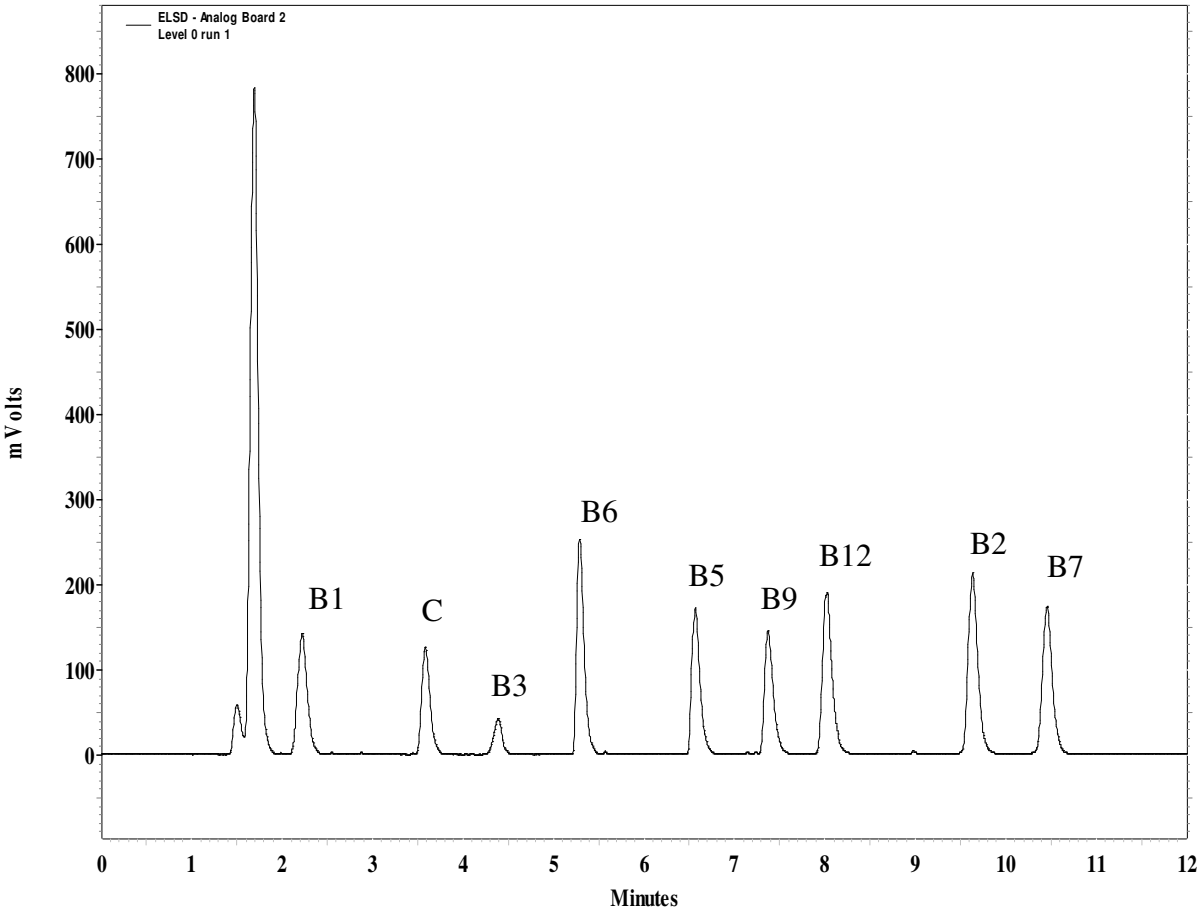


Figure 3.3 HPLC-ELSD chromatograms of nine water-soluble vitamin standards

3.3.2.2 Calibration range, reproducibility, LOD-LOQ

Before the sample analysis, method precision was evaluated with RSDs of retention time and peak area calculated for 7 replicate injections. Calibration range varies depending on the amount of vitamins contained in the multivitamin tablet samples. Three replicates of the standard mixtures at five or six concentration levels were obtained for the standard curve. LOD and LOQ were determined by the analyte concentration which produce signals of peak height three times and ten times of the background noise,

respectively (S/N=3 for LOD and S/N=10 for LOQ). RSDs, linearity range, LOD, LOQ and correlation coefficient are reported in Table 3.5 for DAD and Table 3.6 for ELSD.

Because DAD and ELSD were run in tandem, their retention repeatability was similar and quite high, which is demonstrated by the low RSDs. However, the same does not go for the signal produced by the two detectors. As noticed from the comparison of the two detectors, the area repeatability of ELSD is lower than DAD, shown through its high RSDs for all analytes. In general, DAD has a LOQ about 100 times lower than ELSD, which means the former has a much higher sensitivity than the latter. Though this result is expected, it is open to question if ELSD sensitivity level can be improved. For ELSD detection in this method, the gain number was empirically determined to give the highest possible S/N ratio for the analytes tested. However, signal optimization was not performed for the nebulizer temperature, which may have been the culprit for the low sensitivity of the detection. It is worth mentioning that the mobile phase containing a significantly high percentage of water may have required a higher nebulizer temperature to completely vaporize. At the temperature set up for this method (40^{o}C), it is possible that the eluent did not evaporate completely, leading to signal suppression of the analyte and a noise baseline *(39, 40)*. In order to ensure adequate eluent evaporation, either a lower mobile phase flow rate or a higher nebulizer temperature is needed. However, when the nebulizer temperature adjustment approach is taken, possible thermal degradation of the analytes needs to be taken into consideration.

Table 3.5 Linear dynamic range, correlation coefficients (r^2), limits of detection (LOD), limits of quantitation (LOQ) and precision of the DAD detector for the determination of nine water-soluble vitamins

Analyte	Detection wavelength (nm)	Retention time (tR)	Correlation coefficient (r^2)	Range (ppm)	LOD (ppb)	LOQ (ppb)	Retention time precision (%RSD)	Peak area precision (%RSD)
B1	254	2.02	0.9998	0.39-100	4.86	16.20	0.26	0.21
C	254	3.35	0.9998	0.39-100	10.25	34.17	0.44	1.41
B3	254	4.07	0.9999	0.39-100	6.86	22.85	0.40	0.27
B6	280	5.03	0.9995	0.39-250	1.51	5.04	0.43	0.68
B5	210	6.36	0.9998	0.39-250	7.71	25.70	0.07	0.77
B9	280	7.14	0.9959	0.19-12.5	2.65	8.85	0.12	0.68
B12	210	7.77	0.9998	0.19-6.25	8.01	26.69	0.15	0.58
B2	210	9.36	0.9996	0.39-100	2.95	9.82	0.21	0.43
B7	210	10.12	0.9997	0.19-6.25	42.36	141.19	0.22	0.37

Table 3.6 Linear dynamic range, correlation coefficients (r^2), limits of detection (LOD), limits of quantitation (LOQ) and precision of the ELSD detector for the determination of nine water-soluble vitamins

Analyte	Retention time (tR)	Correlation coefficient (r2)	Range (ppm)	LOD (ppm)	LOQ (ppm)	Retention time precision (%RSD)	Peak area precision (%RSD)
B1	2.20	0.9981	3.125-100	0.71	2.36	0.31	3.47
C	3.54	0.9967	3.125-100	1.71	5.70	0.40	3.21
B3	4.25	0.9956	3.125-100	0.62	2.06	0.24	4.05
B6	5.22	0.9987	3.125-100	0.57	1.91	0.33	2.99
B5	6.54	0.9980	3.125-100	0.84	2.78	0.08	3.01
B9	7.33	0.9832	3.125-100	1.43	4.75	0.10	3.44
B12	7.95	0.9975	3.125-100	1.28	4.27	0.14	2.43
B2	9.54	0.9982	3.125-100	0.63	2.10	0.14	2.36
B7	10.32	0.9988	3.125-100	0.71	2.36	0.16	3.77

With higher LOQs for all analytes, ELSD has a much narrower calibration range than DAD. The response produced by ELSD do not follow the normal linearity but rather

an exponential relationship $y= a.m^b$ *(37, 41)*. According to this function, the observed peak area (A) and the sample mass on-column (m) are related to each other through the relationship described by the exponent b and the response factor a. These two coefficients a and b are dependent on a variety of factors including droplet size, concentration, solute nature, gas and liquid flow rates, nebulizer temperature, etc. The calibration curve for thiamine response using ELSD is given in Figure 3.4 as a demonstration.

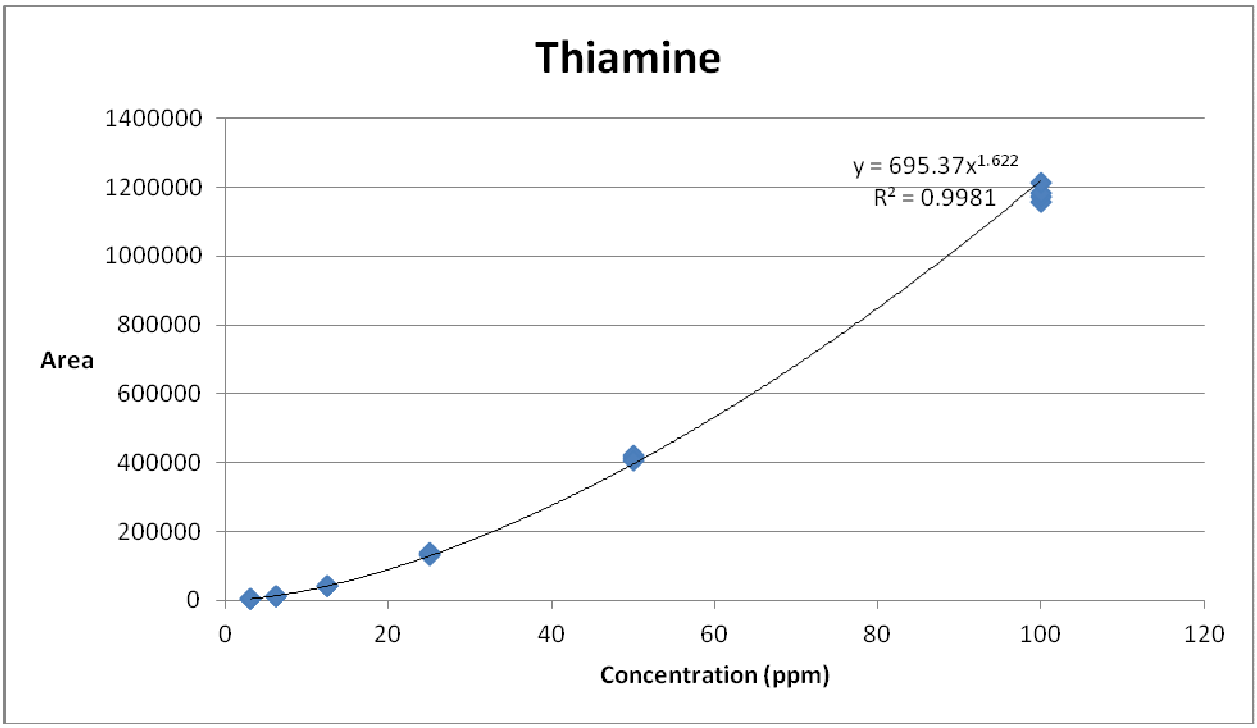


Figure 3.4 Correlation between ELSD response and concentration of thiamine standard

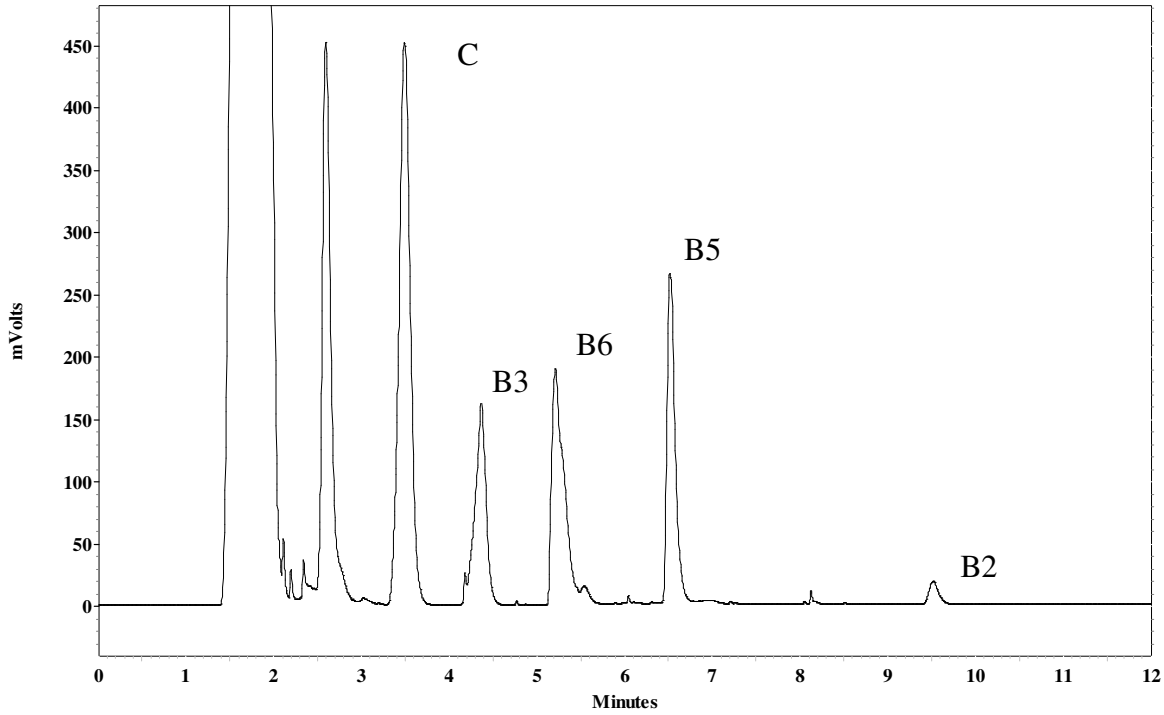


Figure 3.5 ELSD chromatogram of Brand A tablet sample

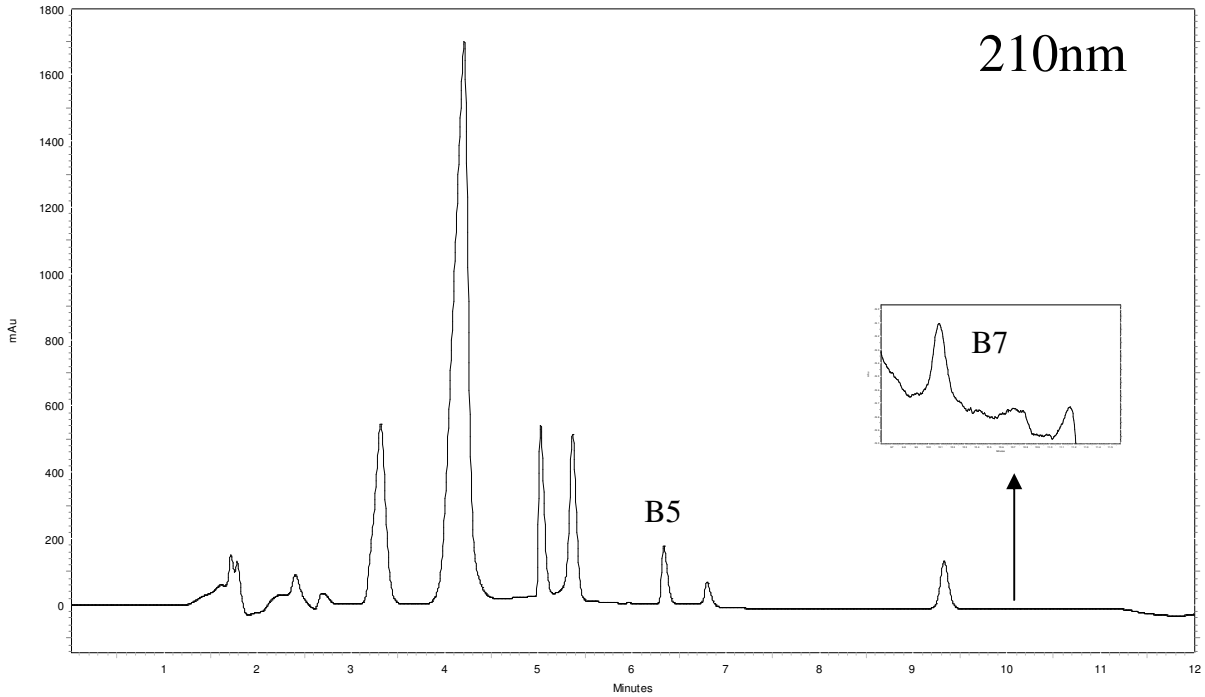


Figure 3.6 DAD chromatogram of brand A tablet sample

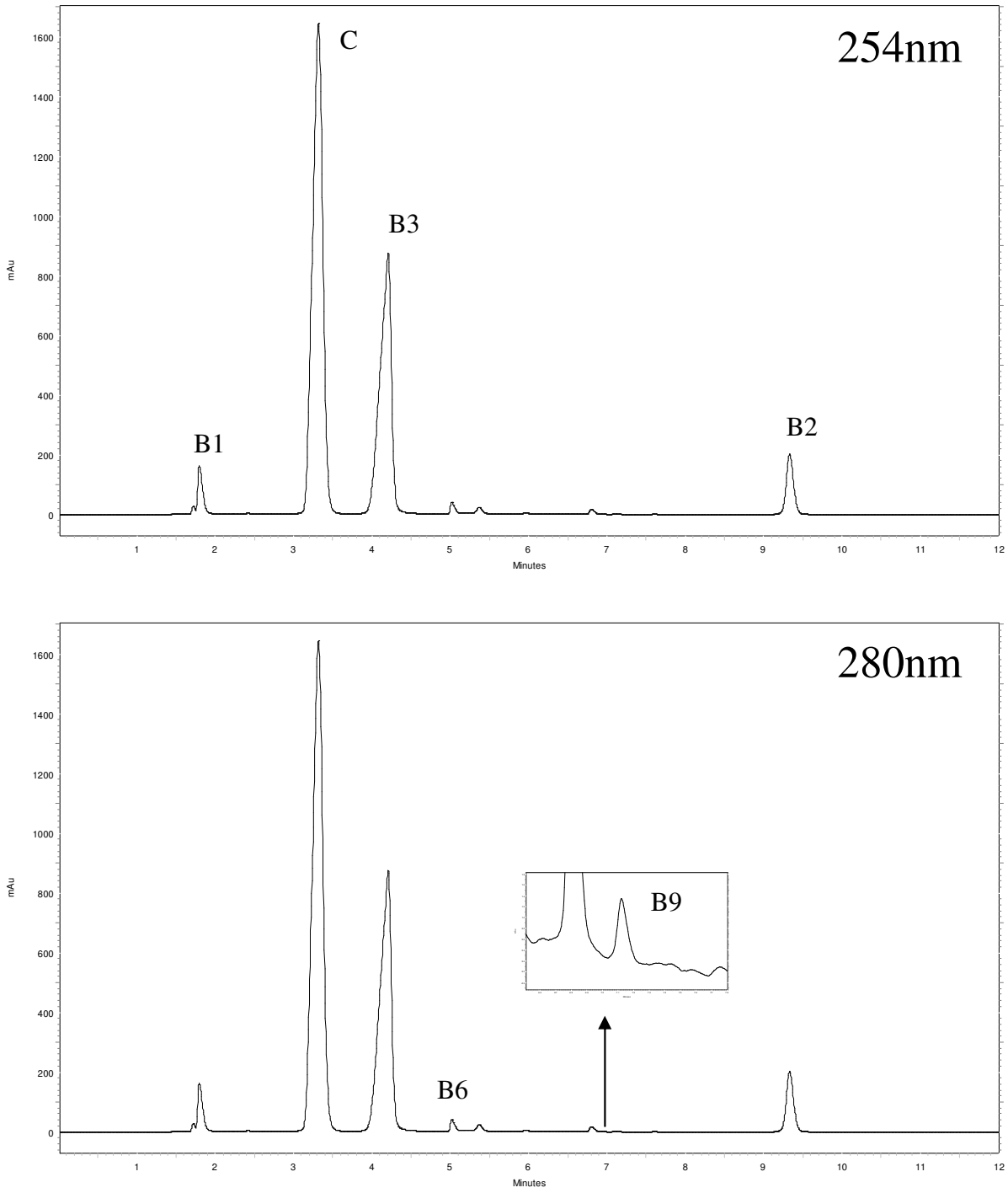


Figure 3.6 DAD chromatogram of brand A tablet sample (Continued)

Table 3.7 Analysis results of multivitamins tablets by DAD

Analyte	Retention time (tR)	Retention time precision (%RSD)	Peak area precision (%RSD)	Amount found (per tablet)	Amount labeled (per tablet)
Brand A					
B1	2.02	0.31	0.47	1.80 mg	1.5 mg
C	3.32	0.09	8.73	38.86 mg	60 mg
B3	4.11	0.07	0.53	22.48 mg	20 mg
B6	5.03	0.08	0.39	2.64 mg	2 mg
B5	6.34	0.04	0.23	13.12 mg	10 mg
B9	7.12	0.04	2.64	105.82 µg	400 µg
B12	7.73	0.55	4.99	ND	6 µg
B2	9.34	0.05	0.90	2.13 mg	1.7 mg
B7	10.09	0.03	2.21	45.19 µg	30 µg
Brand B					
B1	1.97	0.77	0.52	11.20 mg	10 mg
C	3.29	0.44	0.42	259.98 mg	300 mg
B3	4.10	0.29	0.77	98.01 mg	100 mg
B6	5.03	0.24	0.44	11.74 mg	10 mg
B5	6.34	0.06	0.71	53.41 mg	50 mg
B9	7.13	0.06	3.08	143.69 µg	400 µg
B12	7.75	0.14	0.94	62.05 µg	75 µg
B2	9.32	0.09	0.88	9.53 mg	10 mg
B7	10.09	0.10	2.20	32.10 µg	30 µg
Brand C					
B1	2.02	0.52	0.38	166.45 mg	125 mg
C	3.34	0.24	0.46	94.76 mg	125 mg
B3	4.04	0.15	0.20	126.91 mg	125 mg
B6	5.03	0.12	0.73	159.28 mg	125 mg
B5	6.35	0.07	0.14	145.50 mg	125 mg
B9	7.13	0.03	1.80	383.29 µg	400 µg
B12	7.75	0.03	1.74	159.67 µg	125 µg
B2	9.35	0.07	0.73	136.32 mg	125 mg
B7	10.35	0.25	1.55	256.46 µg	125 µg

Table 3.8 Analysis results of multivitamin tablets by ELSD
/: Not available; ND: Not detectable

Analyte	Retention time (tR)	Retention time precision (%RSD)	Peak area precision (%RSD)	Amount found (per tablet)	Amount labeled (per tablet)
Brand A					
B1	/	/	/	ND	1.5 mg
C	3.50	0.18	11.73	36.96 mg	60 mg
B3	4.37	0.37	2.47	23.02 mg	20 mg
B6	5.21	0.13	4.61	2.69 mg	2 mg
B5	6.52	0.10	2.62	12.95 mg	10 mg
B9	/	/	/	ND	400 µg
B12	/	/	/	ND	6 µg
B2	9.52	0.09	4.14	2.21 mg	1.7 mg
B7	/	/	/	ND	30 µg
Brand B					
B1	/	/	/	ND	10 mg
C	3.52	0.26	2.14	226.17 mg	300 mg
B3	4.26	0.24	1.14	99.22 mg	100 mg
B6	5.22	0.21	7.16	12.44 mg	10 mg
B5	6.54	0.03	1.69	52.35 mg	50 mg
B9	/	/	/	ND	400 µg
B12	/	/	/	ND	75 µg
B2	9.51	0.08	2.07	11.68 mg	10 mg
B7	/	/	/	ND	30 µg
Brand C					
B1	2.19	0.69	2.91	166.57 mg	125 mg
C	3.52	0.33	1.77	92.32 mg	125 mg
B3	4.22	0.30	2.00	128.40 mg	125 mg
B6	5.22	0.07	2.94	156.99 mg	125 mg
B5	6.53	0.06	1.62	125.57 mg	125 mg
B9	/	/	/	ND	400 µg
B12	/	/	/	ND	125 µg
B2	9.54	0.02	1.83	132.41 mg	125 mg
B7	/	/	/	ND	125 µg

Table 3.9 Paired T-test statistical analysis of sample results by DAD and ELSD

Brand	Analyte	Labeled amount	DAD mean	ELSD mean	DAD v.s ELSD	
					P-value	Significance level
A	B1	1.5 mg	1.80 mg	ND	NA	NA
	C	60 mg	38.86 mg	36.96 mg	0.0915	-
	B3	20 mg	22.48 mg	23.02 mg	0.0899	-
	B6	2 mg	2.64 mg	2.69 mg	0.6172	-
	B5	10 mg	13.12 mg	12.95 mg	0.2572	-
	B9	400 µg	105.82 µg	ND	NA	NA
	B12	6 µg	ND	ND	NA	NA
	B2	1.7 mg	2.13 mg	2.21 mg	0.4452	-
	B7	30 ug	45.19 µg	ND	NA	NA
B	B1	10 mg	11.20 mg	ND	NA	NA
	C	300 mg	259.98 mg	226.17 mg	0.0039	**
	B3	100 mg	98.01 mg	99.22 mg	0.0425	*
	B6	10 mg	11.74 mg	12.44 mg	0.0951	-
	B5	50 mg	53.41 mg	52.35 mg	0.1028	-
	B9	400 µg	143.69 µg	ND	NA	NA
	B12	75 µg	62.05 µg	ND	NA	NA
	B2	10 mg	9.53 mg	11.68 mg	0.0017	**
	B7	30 µg	32.10 µg	ND	NA	NA
C	B1	125 mg	166.45 mg	166.57 mg	0.9613	-
	C	125 mg	94.76 mg	92.32 mg	0.0928	-
	B3	125 mg	126.91 mg	128.40 mg	0.3008	-
	B6	125 mg	159.28 mg	156.99 mg	0.1649	-
	B5	125 mg	145.50 mg	125.57 mg	0.0008	**
	B9	400 µg	383.29 ug	ND	NA	NA
	B12	125 µg	159.67 ug	ND	NA	NA
	B2	125 mg	136.32 mg	132.41 mg	0.0360	*
	B7	125 µg	256.46 ug	ND	NA	NA

Significance is characterized as (*) α=0.05, (**) α=0.01, (-) = samples are not significantly different

3.3.2.3 Comparing DAD and ELSD results

Typical ELSD and DAD chromatograms of the multivitamin tablet samples are displayed in Figure 3.5 and 3.6. Analytical results of vitamin content determined by both detectors were provided in Table 3.7 and Table 3.8. Most of the water-soluble vitamins added into the supplements were detectable by DAD except for the too low amount of cyanocobalamin in brand A tablet. In contrast, due to much lower sensitivity, the number of analytes detected and quantified by ELSD is lower than that by DAD. The three vitamins added in the smallest amount biotin, folic acid and cyanocobalamin were below LOD in all tested samples. Thiamine co-eluted with other interferents at the beginning of the chromatogram in brand A and brand B extracts; therefore it was undetectable in those two samples. The remaining five vitamins (B3, B5, B6, B2 and C) were all detected and quantified in all three brands by ELSD.

A paired t-test was used to compare the mean amount of water-soluble vitamins per tablet found by DAD and ELSD for all three brands. The statistical analysis was performed by SAS 9.3 (SAS Institute Inc. Cary, NC, USA). Comparison was only made for those vitamins that were detected by both detectors, as shown in Table 3.9. The results found by the two detectors for the five vitamins in brand A are not significantly different at the significance level of 0.05 (α=0.05). For brand B, except for pantothenic (B5) and pyridoxine (B6), the other three were found to differ significantly between the results given by the two detectors. The mean amounts of ascorbic acid (C) and riboflavin (B2) are different at α=0.01 while those of niacinamide (B3) are different at α=0.05. Out of six paired comparison made for brand C, the amounts of thiamine (B1), ascorbic (C),

niacinamide (B3) and pyridoxine (B6) are significantly the same between the two detectors. Difference was found for pantothenic (B5) at α=0.01 and riboflavin (B2) at α=0.05.

3.3.3 Multivitamin analysis using LC-MS

3.3.3.1 Chromatographic conditions

The chromatography of all nine water-soluble vitamins was performed on Agilent Zorbax SB-Aq column (3.5 µm, 4.6x100mm). TFA was not chosen to be used as an additive in the mobile phase because of a concern about its possible adverse effect on MS detection. This perfulorinated acid has a high surface tension that prevents efficient spray formation and in the gas phase, its ions can ion pair with basic analytes, leading to analyte ionization suppression *(42, 43)*. As a result, formic acid 0.1% was used instead as the mobile phase for this method. In order to maximize the signal, low flow rate is recommended for highly aqueous mobile phase. Higher flow rate is possible for a faster run as long as the volume of effluent going to the MS detector is maintained adequately low. However, due to the lack of flow splitter, the flow rate for this method was set at 0.6 mL/min as a compromise between the analysis time and enhanced MS signal. The LC system was equipped with a variable wavelength UV detector connected ahead of the MS detector. Different wavelengths were set up for the nine analytes as stated in Table 3.4. However, the UV detector was not used for the purpose of identification and quantitation of the vitamin content in the fortified food samples due to its low specificity and sensitivity.

Table 3.10 LCMS parameters for the identification of water-soluble vitamins

Time (min)	Compound	Molecular weight	SIM ions	Fragmentor voltage
0.00	Thiamine	337	265.1	70
	Ascorbic	176	177.1	70
3.50	Pyridoxine	169	170.1	70
5.50	Niacinamide	122	123.1	100
9.00	Pantothenic	219	220.1	80
11.50	Folic	441	295.1	140
	Riboflavin	376	377.1	140
	Biotin	244	227.1	140
15.50	Cyanocobalamin	1356	678.4	140

Compounds with different structures respond differently to the fragmentor setting. The ideal fragmentation voltage for each vitamin analyte was determined through flow injection analysis which involves the injection of the standards multiple times without a column. The fragmentor was set up to change over time and both positive and negative ionization mode were tested for every single vitamin. Based on the mass signal of the standards under different fragmentor voltage, the optimal setting was decided as in Table 3.10. All nine analytes respond well in positive ionization mode while only folic and ascorbic respond in the negative ionization mode. Because the Agilent 6100 LCMS model cannot alternate the two ionization modes, it was decided that the positive mode was to be used for the analysis. Flow injection analysis mode was also used to select the ionization mode, the drying gas flow and temperature and the nebulizer pressure. The last two parameters are dependent on LC flow rate and mobile phase composition. For LC flow rate higher than 0.3 mL/min, it is recommended that the flow rate of nitrogen drying gas be fixed at 13 L/min. Moreover, due to the high percentage of aqueous components in

the mobile phase, the drying gas temperature of 350°C and the nebulizer pressure of 45 psi were used. Finally the capillary voltage was set at 4000 psi.

The mass spectra of almost all water-soluble vitamins display the protonated molecular ion $[M+H]^+$ as the base peak. Thiamine was detected as $[M]^+$ instead of $[M+H]^+$. Cyanocobalamin mass to charge ratio (m/z=678.4) was only half the value suggested in its empirical formula $C_{63}H_{88}CoN_{14}O_{14}P$. In fact, this compound was detected as a double charge species $[M+H]^+$ *(24, 44)*. Chromatograms of LCMS method for the determination of all water-soluble vitamins are included in Figure 3.7 and 3.8.

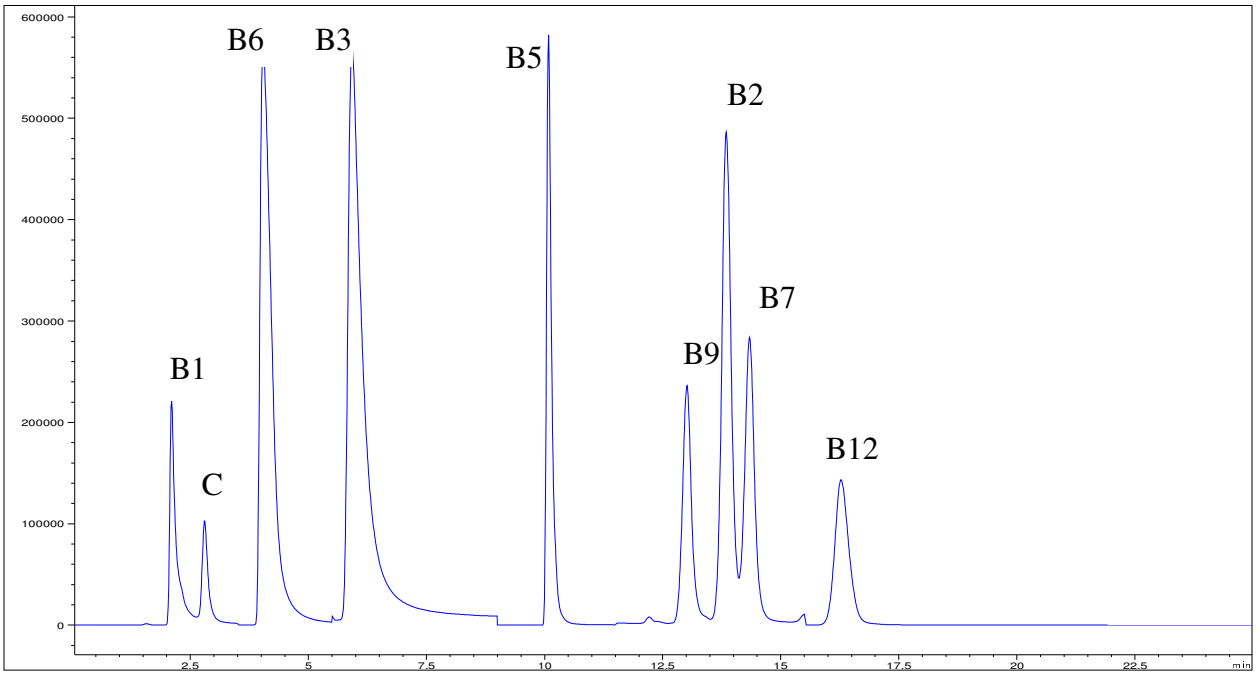


Figure 3.7 LCMS chromatogram of nine water-soluble vitamin standards

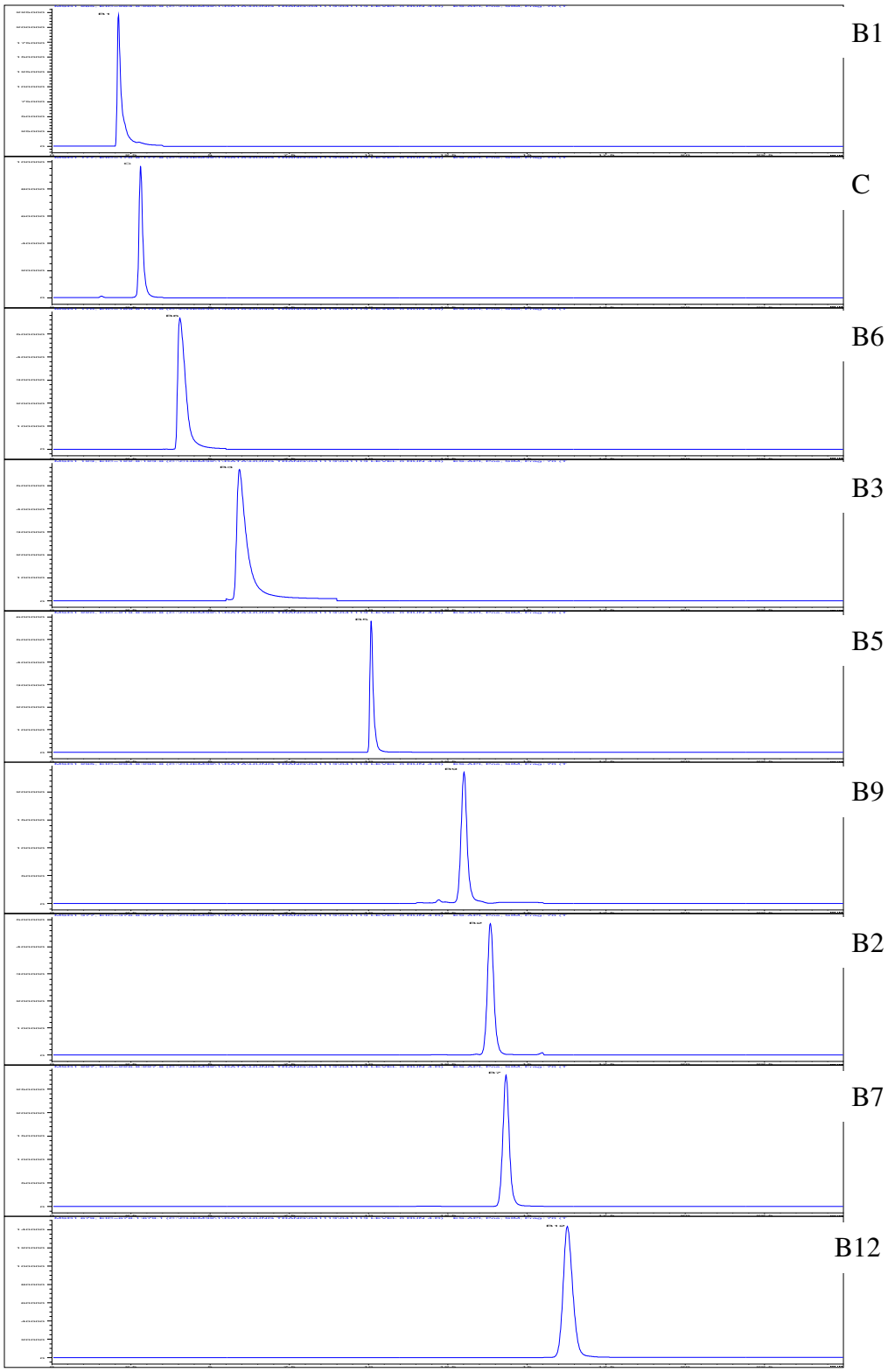


Figure 3.8 Extracted ion chromatograms of nine water-soluble vitamins

3.3.3.2 Method validation and analysis results

Method precision was evaluated with %RSDs of retention time and peak area calculated for 7 replicate injections. Calibration range varies depending on the quantity of vitamins contained in the fortified food samples. Three replicates of the standard mixtures at three or four concentration levels were obtained for the standard curve. LOD and LOQ were determined by the analyte concentration which produced signals of peak height three times and ten times the background noise respectively (S/N=3 for LOD and S/N=10 for LOQ). RSDs, linearity range, LOD, LOQ and correlation coefficient are reported in Table 3.11.

The chromatograms for the three samples were shown in Figure 3.9, 3.10 and 3.11. The results of the vitamin content determination with MS detection were presented in Table 3.12. Though pantothenic was not listed on the nutritional labels of the fortified cereals, it was actually detected at a quantifiable amount in both samples. Cyanocobalamin was added in fortified foods at such a small amount that it was only detectable in cereal brand B. Thiamine in infant formula did not show in one single peak but a group of non-baseline-separated peaks instead as demonstrated in Figure 3.11. There was a big shift in biotin retention time as it eluted much earlier than it did in the standard mixture solution (11.8 min in the infant formula sample v.s 14.3 min in standard mixture). The identity of the peak was confirmed by its mass spectrum. As a result, both thiamine and biotin were not quantified. These strange behaviors were possibly due to the matrix effects. In fact, complicated matrices of food samples may have caused a shift in retention time to other analytes in comparison to that of the standards, as shown in Table

3.13. The retention time shift of the water-soluble vitamins in fortified cereals is within 3% range of the standard retention time. The range is higher in the infant formula sample with biotin being the extreme case with 17.62% deviation in retention time. Though peak identification in LCMS method is not entirely dependent on retention time, a tighter retention window is highly recommended for accurate quantification of the analytes. In order to factor in the effects of the complicated matrix in food samples, inter standard approach should be considered for future work.

Table 3.11 Linear dynamic range, correlation coefficients (r^2), limits of detection (LOD), limits of quantitation (LOQ) and precision of the LCMS method for the determination of nine water-soluble vitamins

Analyte	SIM ions	Retention time (tR)	Correlation coefficient (r^2)	Range (ppm)	LOD (ppb)	LOQ (ppb)	Retention time precision (%RSD)	Peak area precision (%RSD)
B1	265.1	2.14	0.9988	0.78-3.12	0.24	0.80	0.78	2.69
C	177.1	2.80	0.9963	6.25-50	2.92	9.75	0.06	2.67
B6	170.1	4.21	0.9981	0.39-12.5	0.77	2.56	0.36	3.36
B3	123.1	6.13	0.9937	6.25-50	5.17	17.24	0.60	4.84
B5	220.1	10.10	0.9938	0.048-12.5	0.59	1.97	0.06	4.73
B9	295.1	12.89	0.9988	0.09-1.56	1.48	4.92	0.33	2.02
B2	377.1	13.77	0.9959	0.39-6.25	0.46	1.52	0.16	1.68
B7	227.1	14.30	0.9996	0.024-0.097	1.37	4.55	0.24	2.49
B12	678.4	16.10	0.9997	0.012-3.12	1.63	5.44	0.44	1.84

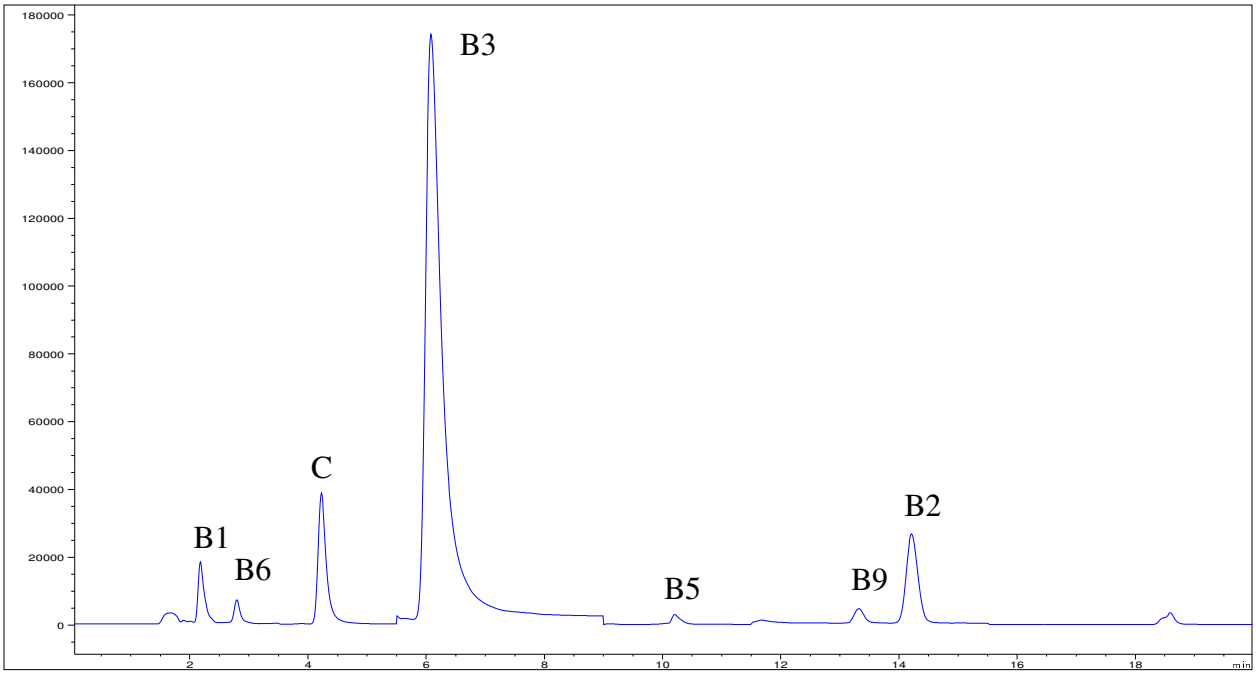


Figure 3.9 LCMS chromatogram of brand A cereal sample

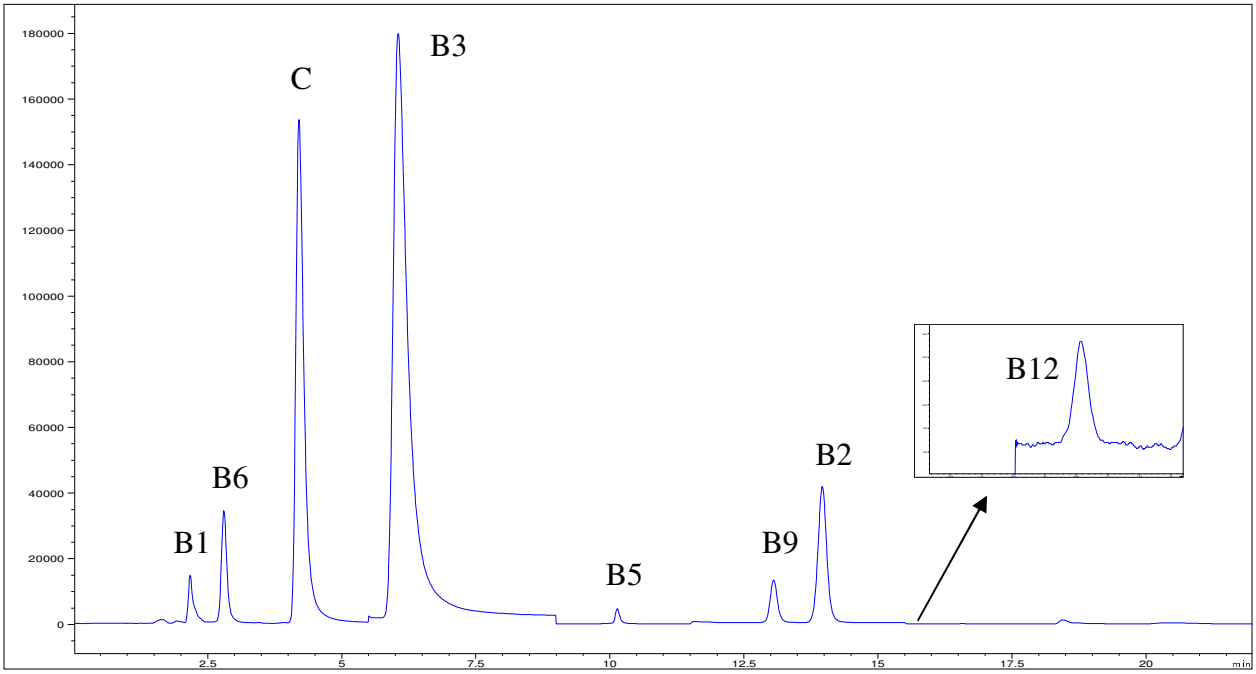


Figure 3.10 LCMS chromatogram of brand B cereal sample

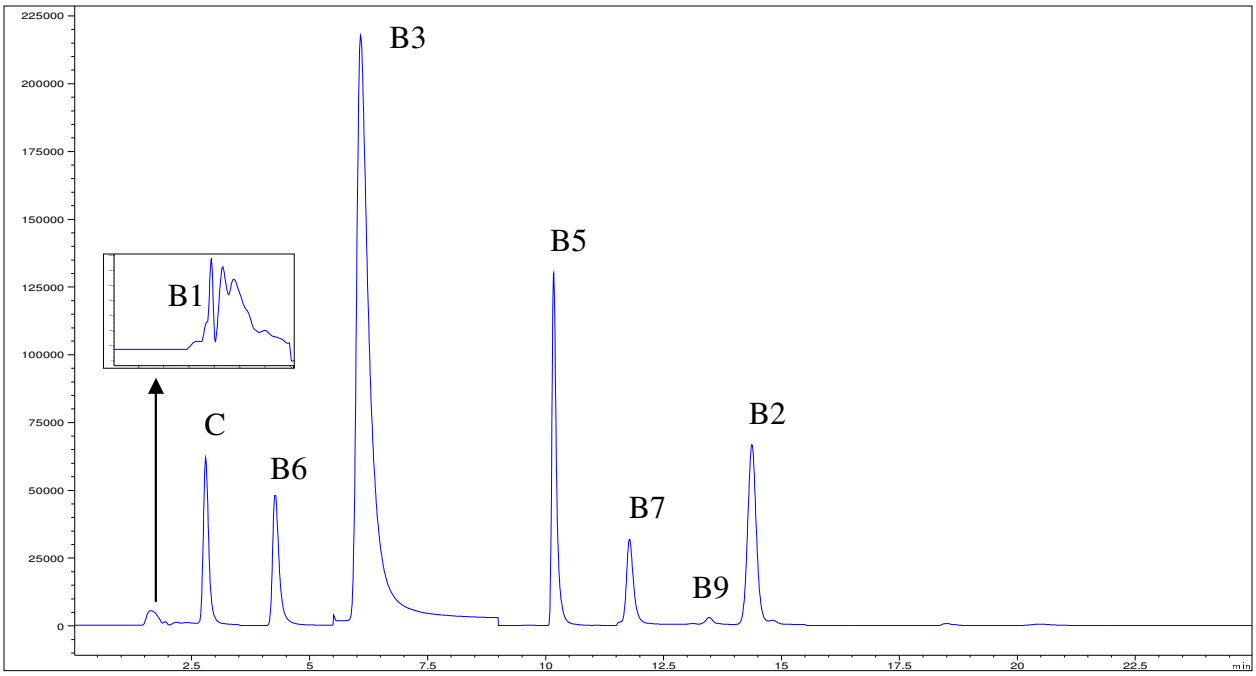


Figure 3.11 LCMS chromatogram of infant formula sample

Table 3.12 Analysis results of fortified foods by LCMS
/: Not available; ND: Not detectable

Analyte	Retention time (tR)	Retention time precision (%RSD)	Peak area precision (%RSD)	Amount found (per 100 gr)	Amount labeled (per 100 gr)
Cereal Brand A					
B1	2.18	0.07	2.50	2.31 mg	1.34 mg
C	2.79	0.04	20.66	1.63 mg	21.43 mg
B6	4.23	0.05	0.37	1.95 mg	1.79 mg
B3	6.06	0.38	0.26	17.49 mg	17.86 mg
B5	10.15	0.49	2.09	0.15 mg	/
B9	13.20	0.78	0.38	0.35 mg	0.71 mg
B2	14.10	0.72	0.58	1.58 mg	1.52 mg
B7	ND	/	/	ND	/
B12	ND	/	/	ND	5.36 ug
Cereal Brand B					
B1	2.16	0.28	0.71	1.95 mg	1.69 mg
C	2.80	0.07	2.81	17.33 mg	67.74 mg
B6	4.19	0.24	0.57	10.53 mg	6.45 mg
B3	6.03	0.27	0.47	18.22 mg	22.58 mg
B5	10.14	0.06	3.04	0.14 mg	/
B9	13.07	0.32	0.79	0.86 mg	1.29 mg
B2	13.96	0.18	1.21	4.62 mg	1.92 mg
B7	/	/	/	ND	/
B12	16.58	0.18	2.42	23.88 µg	19.36 µg
Infant formula					
B1	ND	/	/	ND	0.37 mg
C	2.79	/	/	8.86 mg	55.81 mg
B6	4.26	0.11	1.33	0.49 mg	0.28 mg
B3	6.07	0.19	1.77	4.65 mg	4.65 mg
B5	10.18	0.17	1.60	1.94 mg	2.33 mg
B9	13.45	0.17	2.35	0.035 mg	0.074 mg
B2	14.34	0.36	1.42	0.87 mg	0.65 mg
B7	14.78	/	/	/	0.014 mg
B12	/	/	/	ND	0.0014 mg

Table 3.13 Comparison of retention time in standard solution and in samples
/: Not available; ND: Not detectable

Analyte	Standard retention time (min)	Cereal Brand A		Cereal Brand B		Infant formula	
		Retention time (min)	Deviation (%)	Retention time (min)	Deviation (%)	Retention time (min)	Deviation (%)
B1	2.14	2.18	1.86	2.16	0.92	ND	/
C	2.80	2.79	0.42	2.80	0.06	2.79	0.30
B6	4.21	4.23	0.46	4.19	0.49	4.26	1.24
B3	6.13	6.06	1.14	6.03	1.63	6.07	1.02
B5	10.10	10.15	0.54	10.14	0.44	10.18	0.80
B9	12.89	13.20	2.39	13.07	1.38	13.45	4.35
B2	13.77	14.10	2.42	13.96	1.40	14.34	4.15
B7	14.30	ND	/	ND	/	11.78	17.62
B12	16.10	ND	/	16.58	2.96	ND	/

3.3.4 Sample extraction limits

Except for infant formula, the final pH of the extract in all samples was neutral (6.0-7.0), which is not the best condition to maintain optimal stability for ascorbic acid based on results of the stability study. Considering its high amount in the samples, it had been expected that ascorbic acid degradation would not be a concern as long as the analysis was performed right after the extraction. However, the analysis results proved otherwise. The peak area %RSD of this vitamin was extremely high in brand A tablet (8.73%) and brand B fortified cereals (20.66%). In comparison to brand B and brand C tablets, the amount of ascorbic acid found in brand A was much lower than the labeled amount (38.86 mg for DAD and 36.96 mg for ELSD v.s 60 mg labeled). It was possibly due to the fact that brand A tablets also contain multi minerals which potentially catalyzes the reduction of ascorbic acid. The same situation must have been applied to

fortified food samples, considering their complicated matrices. A side study on the degradation speed of ascorbic in brand A tablet in acidic and neutral conditions was conducted to gain more insight into how to improve this compound's stability in samples high in metal content. According to Figure 3.12, acidic condition was once again confirmed to be optimal for its stability. Separate extraction of ascorbic acid under low pH condition should be considered for accurate analysis of this vitamin in future work. Another alternative is to use antioxidants or metal-chelating agents to slow down ascorbic acid's degradation.

Folic acid was also found at a much lower amount than being labeled in all samples tested. Further examination may be needed to know if this compound was efficiently extracted under the proposed procedures. Folic acid is notorious for its low solubility in water, the degree of which greatly depends on the solution pH. Considering the low pH of the mobile phase used, it is also open to question if folic acid may have fallen out of solution during the separation process inside the column.

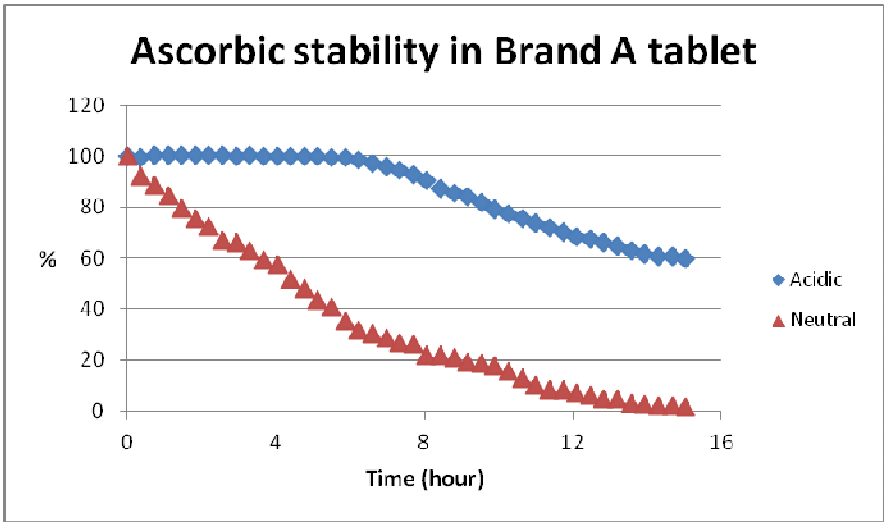


Figure 3.12 Stability of ascorbic acid in Brand A tablet sample

3.4 Conclusion

Simultaneous determination of water-soluble vitamins is complicated by many factors. First of all, due to their diverse chemical properties, it is difficult to separate all of them in one chromatographic run. On the other hand, their difference in solubility and stability presents another challenge with the optimization of sample preparation procedures. Furthermore, these vitamins are added into pharmaceuticals and fortified food products at different amounts and respond unequally to different modes of detection. Among the three detectors used in this study, ELSD is the least feasible for routine analysis. In spite of universal response to all vitamins, its sensitivity is too low to even allow the detection of those vitamins occurring at high level in pharmaceuticals. DAD, on the other hand, provides sufficient specificity and sensitivity to the analysis of pharmaceuticals. However, detection at low wavelengths (e.g., 210 nm) required for non-chromophoric vitamins like pantothenic and biotin is possibly subject to background interferences and noisy baseline. LCMS is both universal and highly sensitive, which is suitable for the analysis of complicated food matrices like fortified cereals and infant formula powder.

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APPENDICES

APPENDIX A

VITAMIN NAMES AND PROPERTIES

Table A.1 Common names and scientific names of all water-soluble vitamins in the study

Scientific names	Common names
Thiamine	Vitamin B1
Riboflavin	Vitamin B2
Niacinamide	Vitamin B3
Pantothenic acid	Vitamin B5
Pyridoxine	Vitamin B6
Biotin	Vitamin B7
Folic acid	Vitamin B9
Cyanocobalamin	Vitamin B12
Ascorbic acid	Vitamin C

Table A.2 Physiochemical properties of thiamine and related compounds

Compound name/CAS number	Molecular Formula	Molar mass	Physical properties	Absorbance	
				λ_{max} (nm)	$\varepsilon \times 10^{-3}$
Thiamine Hydrochloride CAS No. 67-03-8	$C_{12}H_{17}ClN_4OS \cdot HCl$	337.26	- White crystalline powder; melting point: 246°C-250°C (decomposition) - Solubility: Water-1.0g/ml, Ethanol (95%)-1.0g /100 ml, Glycerol-1.0 g/18 ml. Insoluble in ether, benzene, hexane and chloroform - The pH of a 1% (w/v) solution in water is 3.13, and of a 0.1% in water is 3.58.	246[a] 234[b] 264[b]	14.3 11.6 8.6
Thiamine mononitrate CAS No. 532-43-4	$C_{12}H_{17}N_5O_4S$	327.37	- White to yellow crystals - Melting point: 374°C-392°C - Density: 0.35 g/cm^3 - Solubility: 2.7g/100ml		
Thiamine monophosphate CAS No. 532-40-1	$C_{12}H_{17}N_4O_4PS$	344.33			
Thiamine pyrophosphate CAS No. 154-87-0	$C_{12}H_{18}N_4O_7P_2S$	424.31	- Melting point: 240°C-244°C (decomposition) - Solubility: 220 g/l at 25°C		
Thiamine triphosphate CAS No. 3475-65-8	$C_{12}H_{19}N_4O_{10}P_3S$	504.29			

[a]: In 0.1M phosphate buffer, pH 2.9 [b]: In 0.1M phosphate buffer, pH 5.5

Sources: The Merck Index, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001; Eitenmiller, R.R.; Ye, L.; Landen, W.O. *Vitamin analysis for the health and food sciences.* CRC Press: Boca Raton, 2008; Kawasaki, T.; Egi, Y. Thiamine, In *Modern chromatographic analysis of vitamins,* Third Edition, Revised and Expanded ed.; Leenheer, A.P.d., Lambert, W.E., Van Bocxlaer, J.F. and NetLibrary, I., Eds.; Marcel Dekker: New York, 2000, Vol.84. pp. 375; Bates, C.J. Thiamine, In *Handbook of vitamins,* Fourth ed.; Zempleni, J., Rucker, R.B. and McCormick D.B., S.J.W., Eds.; CRC Press: Boca Raton, 2007, pp. 253; Ball, G.F.M. *Vitamins in foods: analysis, bioavailability, and stability.* Taylor & Francis: Boca Raton, FL, 2006; Vol. 156

Table A.3 Physiochemical properties of riboflavin, FMN and FAD

Compound name/CAS number	Molecular Formula	Molar mass	Physical properties	Absorbance		Fluorescence	
				λ_{max} (nm)	$\varepsilon \times 10^{-3}$	Ex λ (nm)	Em λ (nm)
Riboflavin Vitamin B 2 CAS No. 83-88-5	$C_{17}H_{20}N_4O_6$	376.37	- Fine yellow-orange powder - Melting point : 278°C–282°C (decomposition) - Solubility: Slightly soluble in water (10–13 mg/100 ml at 25–27.5°C; 19 mg/100 ml at 40°C; 230 mg/100 ml at 100°C) ; Slightly soluble in ethanol (4.5 mg/100 ml at 27°C) and phenol; Insoluble in chloroform , acetone, benzene and ether. Solubility can be enhanced in acidic or alkaline conditions.	260 375 450	27.7 10.6 12.2	360[a] 465[a]	521[a]
Riboflavin-5′-phosphate CAS No. 146-17-8	$C_{17}H_{21}N_4O_9P$	456.35	- Fine, yellow-orange crystalline powder - Melting point: 280°C-290°C (decomposition) - Solubility: Soluble in water, 30 g/l (Na salt); Insoluble in acetone, benzene and ether	260 375 450	27.1 10.4 12.2	440[a] 500[a]	530[a]
Flavin-adenine dinucleotide FAD CAS No. 146-14-5	$C_{27}H_{33}N_9O_{15}P_2$	785.56	Soluble in water; Insoluble in chloroform , acetone, benzene and ether	260 375 450	37.0 9.3 11.3	440[b] 500[b]	530[b]

[a]: At pH 3.5-7.5 [b]: At pH 2.7-3.1

Sources: The Merck Index, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001; Eitenmiller, R.R.; Ye, L.; Landen, W.O. *Vitamin analysis for the health and food sciences.* CRC Press: Boca Raton, 2008; Ball, G.F.M. *Vitamins in foods: analysis, bioavailability, and stability.* Taylor & Francis: Boca Raton, FL, 2006, Vol. 156; Rivlin, R.S. Riboflavin (Vitamin B2), In *Handbook of vitamins,* Fourth ed.; Zempleni, J., Rucker, R.B. and McCormick D.B., S.J.W., Eds.; CRC Press: Boca Raton, 2007, pp. 233; Nielsen, P. Flavins, In *Modern chromatographic analysis of vitamins,* Third Edition, Revised and Expanded ed.; Leenheer, A.P.d., Lambert, W.E., Van Bocxlaer, J.F. and NetLibrary, I., Eds.; Marcel Dekker: New York, 2000; Vol.84. pp. 400.

Table A.4 Physiochemical properties of vitamin B6

Compound name/CAS number	Molecular Formula	Molar mass	Physical properties	Absorbance		Solvent	Fluorescence		
				λ_{max} (nm)	$\varepsilon \times 10^{-3}$		Ex λ (nm)	Em λ (nm)	pH range
Pyridoxal HCl 65-22-5	$C_8H_9NO_3 \cdot HCl$	203.63	- Melting point: 165°C (decomposition) - Solubility: Soluble in water, 95% ethanol	390 318	200 8128	Water	330 310	382 365	6.0 12.0
Pyridoxine HCl 58-56-0	$C_8H_{12}ClNO_3$	205.64	- White, odorless crystalline powder; Melting point: 206°C-208°C (Sublimes); Solubility: Readily soluble in water (1g/5ml). Sparingly soluble in alcohol (1g/100ml) and propylene glycol. Insoluble in ether, chloroform	292 290 253 325	7720 8400 3700 7100	Methanol 0.1 M HCl Phosphate pH7.0 -			
Pyridoxine 65-23-6	$C_8H_{11}NO_3$	169.18	- Melting point: 160°C - Solubility: Soluble in water; Weakly soluble in alcohol, acetone; Insoluble in ether, chloroform	324 254	7244 3891	pH 6.8 pH 6.8	332 320	400 380	6.5-7.5 12-14
Pyridoxamine Dihydrochloride 524-36-7	$C_8H_{12}N_2O_2 \cdot 2HCl$	241.12	- Melting point: 226°C-227°C (decomposition) - Solubility: Soluble in water, 95% alcohol	328 253	7763 4571	Water	337 320	400 370	4.0-5.5 14
Pyridoxal-5'-Phosphate 54-47-7	$C_8H_{10}NO_6P$	247.14	- Melting point: 330°C				365 360 330	423 430 410	2.5-4.8 8.7-13 6.0

Sources: The Merck Index, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001; Eitenmiller, R.R.; Ye, L.; Landen, W.O. *Vitamin analysis for the health and food sciences.* CRC Press: Boca Raton, 2008; Ball, G.F.M. *Vitamins in foods: analysis, bioavailability, and stability.* Taylor & Francis: Boca Raton, FL, 2006, Vol. 156; Ollilainen, V. HPLC analysis of vitamin B6 in foods. *Agric. Food Sci. Finland* **1999,** *8*; Dakshinamurti, S.; Dakshinamurti, K. Vitamin B6, In *Handbook of vitamins,* Fourth ed.; Zempleni, J., Rucker, R.B. and McCormick D.B., S.J.W., Eds.; CRC Press: Boca Raton, 2007; pp. 315; Ubbink, J.B. Vitamin B6, In *Modern chromatographic analysis of vitamins,* Third Edition, Revised and Expanded ed.; Leenheer, A.P.d., Lambert, W.E., Van Bocxlaer, J.F. and NetLibrary, I., Eds.; Marcel Dekker: New York, 2000; Vol.84. pp. 443.

Table A.5 Physiochemical properties of vitamin B12

Compound name/CAS number	Molecular Formula	Molar mass	Physical properties	Absorbance		Solvent
				λ_{max} (nm)	$\varepsilon \times 10^{-3}$	
Cyanocobalamin B 12 CAS No. 68-19-9	$C_{63}H_{88}CoN_{14}O_{14}P$	1355.38	- Dark red hygroscopic crystalline. Anhydrous form can take up to 12% moisture; Darkens at 210–220° C - Soluble in water (1.25g/100 ml). Aqueous solution is of neutral pH.	278 361 551	15.6 27.6 8.7	Water
Hydroxocobalamin B 12a CAS No. 13422-51-0	$C_{62}H_{89}CoN_{13}O_{15}P$	1346.37	- Dark red. Darkens at 200°C - Moderately soluble in water. Insoluble in acetone, ether, petroleum, ether and benzene	279 325 359	19.0 11.4 20.6	Water
Aquacobalamin B 12b CAS No. 13422-52-1	$C_{62}H_{90}CoN_{13}O_{15}POH$	1347.0		274 351 499	20.6 26.5 8.1	Water
Nitrocobalamin B 12c	$C_{62}H_{88}CoN_{14}O_{16}P$	1374.6	- Red crystalline solids	352 528 357	21.0 8.4 19.1	Water Water 0.01N NaOH
Sulfitocobalamin CAS No. 15671-27-9	$C_{62}H_{89}CoN_{13}O_{17}PS$	1409.5		275 365 418	46.2 18.3 6.9	Water
Adenosylcobalamin Cobamamide CAS No. 13870-90-1	$C_{72}H_{100}CoN_{18}O_{17}P$	1579.6	- Yellow-orange crystal - Soluble in ethanol, phenol. Insoluble in acetone, ether, dioxane	288 340 375 522	18.1 12.3 10.9 8.0	Water
Methylcobalamin CAS No. 13422-55-4	$C_{63}H_{91}CoN_{13}O_{14}P$	1344.4	Bright red	266 342 264 304	19.9 14.4 24.7 22.9	Water 0.1N HCl

Sources: The Merck Index, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001; Eitenmiller, R.R.; Ye, L.; Landen, W.O. *Vitamin analysis for the health and food sciences.* CRC Press: Boca Raton, 2008; Ball, G.F.M. *Vitamins in foods: analysis, bioavailability, and stability.* Taylor & Francis: Boca Raton, FL, 2006, Vol. 156;

Table A.6 Physiochemical properties of vitamin C

Compound name/CAS number	Molecular Formula	Molar mass	Physical properties	Absorbance		Solvent
				λ_{max} (nm)	$\varepsilon \times 10^{-3}$	
Ascorbic acid CAS No. 50-81-7 867	$C_6H_8O_6$	176.13	- Odorless, white or very pale yellow crystalline powder with a pleasant sharp taste; Melting point at 190-192 °C with decomposition; Stable in dry form at room temperature for a long time - Readily soluble in water (33 g/100 ml at 258C), less soluble in 95% ethanol (3.3 g/100 ml), absolute ethanol (2 g/100 ml), acetic acid (0.2 g/100 ml), and acetonitrile (0.05 g/100 ml); Insoluble in ether, $CHCl_3$, benzene, petroleum ether, oils, and fats - 5% aqueous solution has a pH of 2.2–2.5	245 265	12.2 16.6	Water, pH 2.0 Water, pH 4.0
Na ascorbate CAS No. 134-03-2 8723	$C_6H_7O_6Na$	198.11	- Highly soluble in water (90g/100 g) - Very slightly soluble in alcohol and insoluble in chloroform, ether - White to pale yellow crystalline powder form			
Ca ascorbate CAS No. 5743-27-1 1688	$(C_6H_7O_6)_2Ca$	390.31	- Much less soluble in water than ascorbic acid and Na ascorbate (5g/100 g) - Slightly soluble in alcohol and insoluble in ether			
Ascorbyl palmitate	$C_{22}H_{38}O_7$	414.54	- Slightly soluble in oils and freely soluble in alcohol (22 g/100 ml)			

Sources: The Merck Index, 13th ed., Merck and Company, Whitehouse Station, NJ, 2001; Eitenmiller, R.R.; Ye, L.; Landen, W.O. *Vitamin analysis for the health and food sciences.* CRC Press: Boca Raton, 2008; Ball, G.F.M. *Vitamins in foods: analysis, bioavailability, and stability.* Taylor & Francis: Boca Raton, FL, 2006, Vol. 156; Johnston, C.S.; Steinberg, F.M.; Rucker, R.B. Ascorbic acid, In *Handbook of vitamins,* Fourth ed.; Zempleni, J., Rucker, R.B. and McCormick D.B., S.J.W., Eds.; CRC Press: Boca Raton, 2007, pp. 489; Nyyssonen, K.; Salonen, J.T.; Parviainen, M.T. Ascorbic acid, In *Modern chromatographic analysis of vitamins,* Third Edition, Revised and Expanded ed.; Leenheer, A.P.d., Lambert, W.E., Van Bocxlaer, J.F. and NetLibrary, I., Eds.; Marcel Dekker: New York, 2000; Vol.84. pp. 282.

APPENDIX B

BASIC CONCEPTS

1. Retention factor

Formerly referred to as capacity factor, the retention factor (symbolized as k) measures the time that an analyte stays in a stationary phase relative to the time it resides in the mobile phase *(1)*. It is independent of column geometry or mobile phase flow rate. To put it simply, k value measures the analyte of interest elutes with regards to the void volume *(2)*. Retention factor can be calculated as follows:

$$k = \frac{(t_R - t_0)}{t_0}$$

where t_R is the retention time of the analyte peak and t_0 is the void time (or dead time).

If $t_R = t_0$, then k is 0, which means the analyte is not retained by the stationary at all and elute with the first column volume of the mobile phase. For k less than 1, chromatographers may encounter issues of less stable separation and chromatographic interferences, preventing accurate and reproducible analysis of the analytes *(1, 2)*. Many official agencies like USP and FDA recommend the minimal retention factor of 2 for HPLC methods. In fact, k values in range of 2 to 10 are favored because the analysts do not need to deal with issues of badly-retained compounds but enjoy a reasonable run time *(1)*. However, in reality, this ideal range may be not easily achieved, especially in the case of too polar compounds or when the separation involves analytes of wide polarity window *(1)*. In those cases, a more reasonable range for k is between 0.5 and 20 *(3)*.

2. Selectivity or separation factor α

Selectivity measures the relative distance between the two adjacent peaks, expressed by the ratio of their retention factor *(2)*.

$$\alpha = \frac{k_1}{k_2}$$

Selectivity can be modified by the adjustment of factors like mobile phase constituents, stationary phase or temperature.

3. Tailing factor

Peak tailing can be described by either tailing factor or asymmetry factor. These two values are typically similar for the same peak but they are not directly converted. The tailing factor is calculated as follows:

$$T_f = \frac{w_{0.05}}{2f_{0.05}}$$

where $w_{0.5}$ is the width of the peak and $f_{0.5}$ is the distance from the peak center line to the front slope, both measured at 5% of the maximum peak height *(2)*.

Asymmetry factor, on the other hand, is expressed differently as

$$A_f = \frac{A}{B}$$

where A and B are the distance between the center line of the peak to the back and the front slope respectively *(1)*. The measurements used to calculate the asymmetry factor are made at 10% of the maximum peak height.

Peaks of interest are expected to be symmetric with T_f or A_f ideally in the range of 0.9-1.5 *(1)*. Peaks with serious tailing are easily overlapped with adjacent peaks, leading

to reduced resolution *(3)*. Moreover, tailing also reduces detection sensitivity and causes difficulty to accurate peak integration. FDA guidance for validation of chromatographic methods suggests that T_f should be less than 2 for the purpose of quantitation *(4)*.

4. Resolution

Resolution describes how well two adjacent chromatographic peaks are separated from each other and is calculated as follows:

$$R_s = \frac{1.18(t_{R2} - t_{R1})}{w_{0.5,1} - w_{0.5,2}}$$

where t_{R1} and t_{R2} are the retention times of the two adjacent peaks, and $w_{0.5,1}$ and $w_{0.5,2}$ are their baseline widths at 50% maximum peak height *(2)*.

Baseline resolution for peaks of the similar size is achieved with R_s of 1.5 *(2)*. This minimum resolution is also recommended for quantitative analysis.

Resolution can also be described in three parameters N, α and k:

$$R_s = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k + 1)}$$

where N is the column plate number (or column efficiency), α is the selectivity and k is the average retention factor of the two peaks *(5)*.

The separation of any 2 peaks of interest can be optimized by modifying these terms experimentally.

5. Void volume

Void volume or dead volume (symbolized as V_M) is the total volume that the mobile phase has access to and is not taken up by packing materials *(6)*. In other words, V_M includes the interstitial volume and the pore volume that are accessible to the analyte molecules *(1, 5)*. The time required for this volume to pass through the column is called void time or dead time t_0, which is also equal to the elution time of an unretained compound *(5)*.

The determination of true void volume in LC columns is challenging and still in debate among scientists. This volume is usually approximated empirically by injecting a small and supposedly unretained species such as uracil and thiourea for reversed-phase columns. *(5)*. However, it is open to question if these species are essentially unretained as being assumed. It has been reported that they are all more or less retained on reversed-phase columns.

Another common way to estimate the void volume is to use the formula $V_M=0.5Ld_c^2$ with L and d_c being column length and column inner diameter in cm, respectively *(6)*. The estimated value is in the range of 10% error but it is acceptable for the method development purpose. This estimation of void volume is used in this study for the calculation of void time and retention factor.

6. Dwell volume

The concept and significance of dwell volume was already mentioned in section 2.5.7 in Chapter 2. This section presents how this volume is determined and includes the

dwell volume values for the HPLC systems used in the study. Procedures for measuring the system's dwell volume are listed as follows *(5)*:

1. Remove the column and connect the tubings with a short stainless union.
2. Prepare following mobile phase components: A-Water (UV-transparent), B-0.2% acetone in water (UV-maximal absorbance at 265nm).
3. Program the gradient profile from 0% to 100% phase B in 10 min.
4. Record, then print out the resulted chromatogram.
5. Locate the midpoint of the gradient and identify the time from the x-axis corresponding to this midpoint.
6. Subtract half the gradient time (5min) from this time

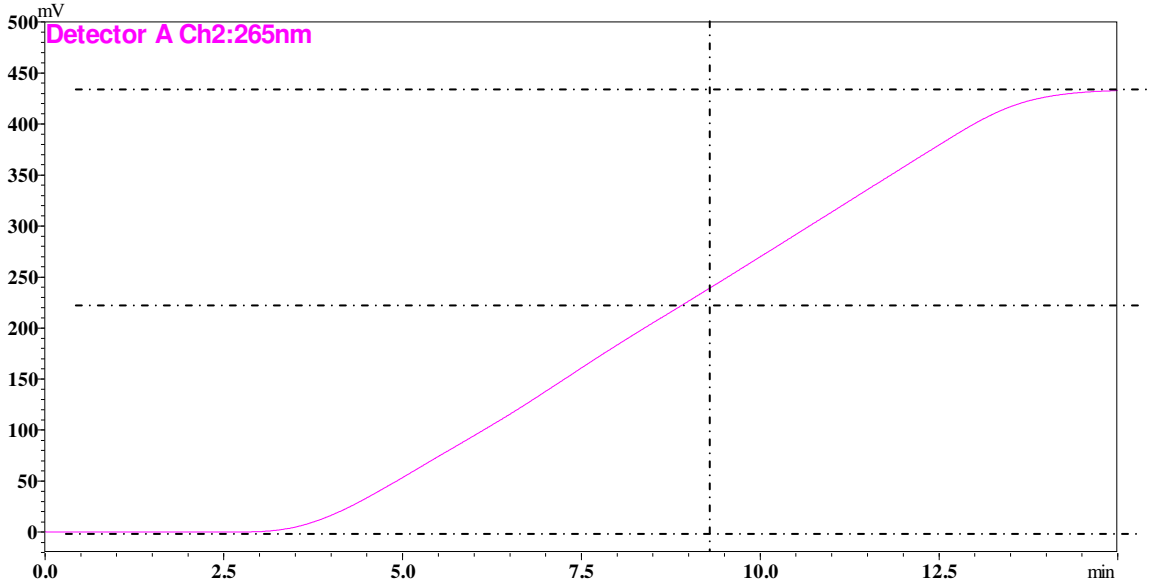

Figure A.1 Demonstration chromatogram for dwell volume determination

Above is the chromatograms obtained for the LC-UV/Vis system used in the study. The midpoint time was found to be 8.85min and the dwell time was 8.85min-5.00min=3.85min. As the flow rate was set up at 1.0 mL/min, the dwell volume of this

HPLC system was 3.85min x 1.0 mL/min=3.85 mL. Using the same method, the following are the dwell volume for all the HPLC systems used in this study.

Table A.7 Dwell volume for all the HPLC systems used for this study

System	Components	Dwell volume
Shimadzu LC-UV/Vis	Shimadzu SIL-20A HT auto-sampler, Shimadzu LC-20AT liquid chromatograph, Shimadzu DGU-20A5 degasser and Shimadzu SPD-20A UV-Vis detector.	3.85 mL
Shimadzu LC-DAD/ELSD	Shimadzu LC-20AT Liquid chromatograph, Shimadzu DGU-20A5 Degasser, Shimadzu CTO-20A Column oven, Shimadzu CBM-20A Communication bus module, Shimadzu SPD-M20A Diode array detector, ELSD-LTII Low-temperature evaporative light scattering detector	1.40 mL
Agilent LC-UV/MS	Agilent Technologies 1200 Series LC system consisted of G1379B Degasser, G1312A Binary pump, G1329A Autosampler, G1316A Thermostatted column compartment and G1314B Variable wavelength detector	1.20 mL

APPENDIX C

USP COLUMN DESIGNATION AND COLUMN EQUIVALENCY

Table A.8 USP Designation for columns used in the study

USP Designation	USP Packing Material
L1	Octadecyl silane chemically bonded to porous silica or ceramic micro-particles, 1.5 to 10 µm diameter, or a monolithic silica rod
L7	Octylsilane chemically bonded to totally porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod
L11	Phenyl groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter
L43	Pentafluorophenyl groups chemically bonded to silica particles by a propyl spacer, 5 to 10 µm in diameter
L56	Propyl silane chemically bonded to totally porous silica particles, 3 to 10 µm in diameter
L62	C30 silane bonded phase on a fully porous spherical silica, 3 to 15 µm in diameter

Table A.9 List of columns equivalent to those used in the study
Notes: This table was generated with PQRI database at **http://www.usp.org/app/USPNF/columnsDB.html**
F: Column comparison function, H: Hydrophobicity, S*: Steric resistance, A: Hydrogen bond acidity, B: Hydrogen bond basicity, C(2.8) and C(7.0): Cation exchange at pH 2.8 and 7.0 respectively

Rank	F	Column	H	S	A	B	C(2.8)	C(7.0)	Type	USP Designation	Manufacturer
YMC Pro C18											
0	0	YMC Pro C18	1.015	0.014	-0.12	-0.007	-0.155	-0.006	B	L1	YMC
1	0.24	SepaxGP-C18	1.014	0.014	-0.112	-0.019	0.103	0.096	B	L1	Sepax Technologies
2	0.56	ZodiacSil 120-5-C18SH	1.032	0.018	-0.109	-0.024	0.115	0.402	B	L1	Zodiac Life Sciences
3	0.56	ProntoSIL 120 C18 SH	1.031	0.018	-0.109	-0.024	0.113	0.402	B	L1	Bischoff
4	0.64	TSKgel ODS-100Z	1.032	0.018	-0.135	-0.031	-0.064	-0.161	B	L1	Tosoh Bioscience
5	0.74	ZodiacSil 120-5-C18H	1.006	0.008	-0.106	-0.004	0.127	0.156	B	L1	Zodiac Life Sciences
YMC ODS- AQ											
0	0	YMC ODS-AQ	0.965	-0.036	-0.135	0.004	-0.068	0.1	B	L1	YMC
1	0.45	Orosil C18	0.981	-0.032	-0.137	0.002	-0.048	0.155	B	L1	Orochem Technologies

2	0.66	Genesis AQ 120A	0.96	-0.036	-0.157	0.007	0.06	0.233	B	L1	Grace/Jones
3	0.85	TSKgel ODS-80Ts QA	0.94	-0.03	-0.118	0.005	0.004	0.361	B	L1	Tosoh Bioscience
4	0.9	Develosil ODS-MG-5	0.963	-0.036	-0.165	-0.003	-0.012	0.051	B	L1	Nomura
5	1.08	Fortis UniverSil C18	0.925	-0.027	-0.124	0.048	0.233	0.254	B	L1	Fortis Technologies
Zorbax SB-AQ											
0	0	Zorbax SB-AQ	0.593	-0.12	-0.083	0.038	-0.136	0.736	EP		Agilent
1	0.42	Zorbax StableBond 80A C3	0.601	-0.124	-0.081	0.038	-0.084	0.81	B	L56	Agilent
2	1.43	HALO PFP	0.702	-0.117	-0.073	-0.062	1.168	0.972	F	L43	Advanced Materials Technology
3	1.54	Halo 5 PFP	0.711	-0.122	-0.07	-0.052	1.159	1.847	F	L43	Advanced Materials Technology
4	1.74	Platinum EPS C8 300	0.584	-0.113	-0.136	0.089	0.481	0.961	EP		Grace/Alltech
5	2.3	TSKgel Super-Phenyl	0.58	-0.107	-0.146	0.016	0.085	0.672	phenyl	L11	Tosoh Bioscience
Synergi Polar-RP											
0	0	Synergi Polar-RP	0.654	-0.148	-0.257	-0.007	0.057	0.778	EP	L1	Phenomenex
1	1	MicroBondapak Phenyl	0.585	-0.152	-0.247	0.021	0.359	0.976	phenyl	L11	Waters

2	1.54	ACE 5 Phenyl	0.647	-0.138	-0.296	0.027	0.132	0.466	phenyl	L11	ACT
3	1.81	Nova-Pak Phenyl	0.704	-0.159	-0.3	0.015	0.767	0.812	phenyl	L11	Waters
4	1.83	Precision Phenyl	0.595	-0.136	-0.296	0.027	0.099	0.508	phenyl	L11	Mac-Mod Analytical
5	2.15	Microsorb 100-5 Phenyl	0.711	-0.14	-0.195	0.163	0.604	0.787	phenyl	L11	Agilent/Varian
Synergi Hydro-RP											
0	0	Synergi Hydro-RP	1.022	-0.006	0.169	-0.042	-0.077	0.26	EP	L1	Phenomenex
1	0.78	HyperClone PAH	0.98	-0.008	0.187	-0.024	1.169	1.116	other		Phenomenex
2	1.13	Ultrasphere ODS	1.085	-0.014	0.173	0.068	0.279	0.382	B	L1	Hichrom
3	1.17	GROM-SIL 120 ODS-5 ST	1.035	-0.001	0.134	-0.005	0.135	0.121	B	L1	Grace/Grom
4	1.49	ProntoSIL 60 C8 SH	0.929	-0.015	0.161	-0.017	-0.313	1.005	B	L7	Bischoff
5	1.57	LiChrospher 100 RP-18	1.006	-0.021	0.183	-0.036	0.646	0.896	A	L1	Merck KGaA (EMD Millipore)
Ultra Aqueous C18											
0	0	Ultra Aqueous C18	0.808	-0.128	0.378	0.013	0.229	1.255	B	L1	Restek
1	1.42	HSS C18 SB	0.73	-0.12	0.4	0.02	0.4	1.41	B	L1	Waters

2	2.2	Cogent Bidentate C8	0.681	-0.113	0.369	0	0.195	1.351	B	L7	MicroSolv
3	2.38	Resolve C18	0.968	-0.127	0.335	-0.046	1.921	2.144	A	L1	Waters
4	2.41	Vision C18 B	0.689	-0.111	0.35	0.031	0.39	1.41	A	L1	Grace/Alltech
5	2.89	ProntoSIL 300 C30	0.893	-0.107	0.322	0.03	0.401	1.547	A	L62	Bischoff

APPENDIX D

SELECTED HPLC METHODS FROM LITERATURE FOR THE

ANALYSIS OF VITAMIN B1, B2, B6, B12 AND C

Table A.10 Selected HPLC methods for vitamin B1 analysis

Sample matrix/ Analyte	Extraction/ Clean up	Column/ Mobile phase	Detection
Meat/Thiamine *(7)*	Digest with 0.1N HCl and takadiastase and papain. Precipitate proteins with TCA. Dilute and filter. Form and extract thiochrome with isobutanol	Spherisorb silica 20µm, 500x2.1 mm Mobile phase: 90% chloroform/10% methanol	Fluorescence 367/418 nm $K_3Fe(CN)_6$ pre-column
Multivitamin preparations/ Thiamine *(8)*	None (p-hydroxybenzoic acid as IS)	ODS C18 CH_3OH–water (25:75), 1% CH_3COOH, 3-8 mM hexanesulphonate	UV 254 nm
Foods Rice flour/ Thiamine *(9)*	Enzyme hydrolysis with takadiastase and centrifuge.	Nucleosil C18, 5µm, 150×4 mm Mobile phase-isocratic with 0.01M NaH_2PO_4 and 0.15 $NaClO_4$, pH 2.2 Flow rate 0.6 ml/min	Thiochrome Post-column derivatization Fluorescence E_x λ =375nm E_m λ =435nm
Various foods/ Thiamine, TMP, TPP, TTP *(10)*	Use amprolium as IS. Homogenize samples with 5% sulfosalicylic acid. Centrifuge, remove water layer. Clean-up: AG2-X8, anion-exchange	Perkin Elmer C18, 10µm, 300×3 mm Mobile phase-gradient with 0.1M Na_3PO_4, pH 5.5–0.1M Na_3PO_4, pH 2.6 Flow rate 1ml/min	Thiochrome Post-column Fluorescence E_x λ = 339nm

			E_m λ = 432nm
Multivitamin preparations/ Thiamine *(11)*	Extract three times with a total volume of 20 ml of methanol-water (8:2)	LiChrosorb RP-18, 5µm, 250 x 4mm CH_3OH–water-85% phosphoric acid (55:45:1) plus 65 mg of octane sulphonic acid Flow rate 1.5 ml/min Injection volume 5-25µl	UV 254 nm
Infant formula, milk, yogurt, eggs, salad dressing/non-phosphorylated thiamine *(12)*	Mix samples with water. Adjust pH to 1.7–2.0 with 6N HCl then to 4.0–4.2 with NaOH to precipitate proteins. Dilute and filter.	µBondapak C18, 10µm, 300×4.6 mm. Column temperature 50°C. Mobile phase-isocratic with Water:MeOH (80:20) containing 0.1% EDTA, 0.15% sodium hexane sulfonate and 0.75% HAC Flow rate 2.5 ml/min	UV 248nm
Legumes, pork, milk powder/ Thiamine *(13)*	Autoclave sample in 0.1 M HCl at121°C for 15 min. Adjust pH to 4.0–4.5 and hydrolyze with takadiastase at 48°C for 3h Clean-up: Amberlite CG-50, C18 Sep-Pak	µBondapak C18, 10µm, 300×3.9 mm Mobile phase: isocratic with Water:MeOH (69:31) containing 5mM sodium hexane sulfonate/ 5mM sodium heptanes sulfonate and 0.5% acetic acid Flow rate 1.0 ml/min	UV 254nm
Various foods/ Thiamine *(14)*	Autoclave samples in 0.25 N H_2SO_4 for 30 min. Adjust pH to 4.6 with acetate buffer and incubate with takadiastase at 40°C–45°C for 25 min. Digest with papain, 40°C–45°C for 2 h. Add TCA, heat at 50–60°C for 5 min to precipitate proteins. Centrifuge.	Mercksorb Si60, 10µm, 250×4.5 mm Mobile phase—isocratic with phosphate buffer (pH 5.6):EtOH (100:12) Flow rate 1 ml/min	Thiochrome Post-column Fluorescence E_x λ = 366nm E_m λ = 464nm
Whole grain/Thiamine *(15)*	Acid digestion with HCl Clean up with Sep-pak columns	C18 µBondapak 0.5mM Ammonium acetate/Methanol (72:28)	Fluorescence Post column derivatization
Cheese, corn flakes, pork,	Autoclave 5g sample in 65ml HCl 0.1M at 121°C for 30min. Cool,	Waters Novapak C18, 4µm, 150x3.9mm Mobile phase: 50mM phosphate buffer pH 7.0	Fluorescence E_x λ=366nm and

potato, flour/ thiamine *(16)*	adjust to pH 4.5, add β-amylase and takadiastase and incubate at 37°C overnight. Add TCA 50% to precipitate proteins, dilute and filter. Thiochrome derivatization. Solid-phase extraction clean-up.		E_m λ=435nm
Dried yeast/ Thiamine *(17)*	Acid hydrolysis with 10% HCl at 80–85°C, 30 min. Adjust pH to 4.5 with acetate buffer. Digest with diatase at 45–50°C for 3h. Clean–up: extract with isobutanol. Add chloroaniline as IS.	CAPCELL PAK C18, 6μm, 150×4.6 mm Mobile phase-isocratic with 50 mM acetate buffer (pH 3.5):MeCN (85:15) with 0.15% sodium 1-octanesulfonate	UV 254nm
Rodent feed/ Thiamine *(18)*	Acid hydrolysis with 0.1N HCl (20 ml) for 1g sample at 100°C for 30 min. Centrifuge and adjust to pH 4.0 with acetic acid.	SynChropak SCD-100, 250×4.6 mm Mobile phase-isocratic with MeOH:Water (40:60) containing 50 mM pentane sulfonate (adjusted to pH 4.0 with acetic acid) Flow rate 0.5 ml/min	Thiochrome Post-column Fluorescence E_x λ = 370nm E_m λ = 430nm
Multivitamins/ Thiamine *(19)*	Dissolve the tablet in 0.1M NaOH, homogenize for 15 min and centrifuge. Dilute with mobile phase	Polymeric RP Jones Chromatography, 5μm, 250×4.6 mm Mobile phase-isocratic with MeCN: phosphate buffer 20 mM, pH 11.0 (20:80) Flow rate 2 ml/min	EC
Various foods/ Thiamine *(20)*	Hydrolyze the samples with dilute HCl at100°C for 30 min. Adjust pH to 4–4.5, digest with takadiastase at 47°C for 3h. Clean-up: weak anion exchange column.	Lichrosphere 100 RP-18, 5μm, 125×4 mm Mobile phase-isocratic with H_3PO_4 –KH_2PO_4 (10mM, pH 3.5):MeOH (85:15) containing 5μM hexane sulfonic acid and 0.1% triethylamine	UV 254nm
Cooked sausages/ thiamine *(21)*	Autoclave 10g ground sample in 60 mL 0.1 N HCl at 120°C for 20 min. Adjust pH to 4.0–4.5 with 2.5M	Spherisorb C8, 5μm, 250×4 mm Mobile phase—Isocratic with phosphate buffer (5 mM, pH 7.0):MeCN (70:30)	Thiochrome Pre-column Fluorescence

	sodium acetate. Incubate with 5 mL (6%) Clara- diastase at 50°C for 3 h. Add 2 mL 50% TCA, heat at 90°C for 15 min to precipitate proteins. Dilute sample then filter. Pre-column derivatization to thiochrome. Clean-up: C 18 Sep-Pak cartridge	Flow rate 0.65 ml/min	Ex λ = 360nm Em λ = 430nm
Tablet, capsule, urine/thiamine, TMP, TPP *(22)*	Capsule/Tablet-dissolve sample with water and filter. Urine-filter sample	RP-Amide C16, 5µm, 150×4 mm Mobile phase-gradient with 25 mM KH_2PO_4 (pH 7):MeCN Flow rate 1 ml/min	UV 230nm
Beers and raw material/ thiamine, TMP, TPP *(23)*	Liquid sample-half dilute with 25 mM phosphate buffer (pH 7) then filter. Solid sample-mix with 5mL TCA, sonicate for 15 min, centrifuge. Extract twice w/2 mL TCA. Combine the supernatant and dilute with phosphate buffer. Filter.	RP-Amide C16, 5µm. Mobile phase- isocratic with 25 mM phosphate buffer (pH 7) Flow rate—1 ml/min	Thiochrome Post-column Fluorescence Ex λ = 375nm Em λ = 465nm
Dairy products/ thiamine *(24)*	Autoclave 5g homogenized sample in 35ml HCl 0.1N at 125°C for 15 min. Cool, adjust to pH 4.0-4.5, add claradiastase and incubate at 50°C for 3hr. Add 50% TCA, heat at 90°C for 15min. Cool, adjust to pH 3.5, dilute and filter. Thiochrome derivatization.	Nucleosil 100-5 C18 AB 125x4mm column Mobile phase: 35% methanol and 65% phosphate buffer (0.005 M, pH 7.0) at flow rate of 0.5 ml/min	Fluorescence Ex λ=360nm and Em λ=425nm
Pharmaceuticals/ thiamine *(25)*	Mix ground sample with water in 10-ml flask, sonicate and dilute to the mark. Dilute aliquots with 60%	TiO 2 Sachtopore-NP 3µm, 250x4.6 mm Mobile phase: 2mM phosphate (pH 6.5) and acetonitrile at flow rate of 1ml/min	UV 240nm

	acetonitrile (v/v) and filter (0.45µm)		
Dry-cured sausages/ thiamine *(26)*	Add 9ml HCL 0.1N into 1g homogenized sample. Heat the mixture at 100°C for 30 min in a water-bath. Cool, add 6 ml of 2.5 M sodium acetate and takadiastase. Incubate at 37°C Overnight. Centrifuge and dilute. SPE clean-up with Oasis WCX cartridge.	Luna HILIC 3µm, 150x3.0 mm Mobile phase: gradient with (A) acetonitrile: 50 mM ammonium acetate pH 5.8 (90:10 v/v) and (B) acetonitrile: 10 mM ammonium acetate pH 5.8 (50:50 v/v)	UV 270nm

Table A.11 Selected HPLC methods for vitamin B2 analysis

Sample matrix/Analyte	Extraction/Clean up	Column/Mobile phase	Detection
Pasta (enriched)/Riboflavin, lumichrome *(27)*	Ground sample mixed with 0.1 N HCl, autoclaved at 121°C for 30 min then cool down, centrifuge. Extract twice with 0.1 N HCl and dilute pooled supernatants to volume	mBondapak C 18 10µm 300x3.9mm Mobile phase: (1) Water/MeOH/acetic acid (56:43:1); (2) Water/MeOH/acetic acid (50:49:1)	Fluorescence (1) E_x λ=450 nm E_m λ= 510 nm (filters) (2) Ex λ= 300-350 nm Em λ=479 nm (filters)
Milk, dairy products / riboflavin *(28)*	Milk- clean up with Sep-Pak C18. Elute riboflavin with 50% 0.02 M acetate (pH 4.0): 50% MeOH Dairy products –homogenize in 0.02 M acetate buffer pH 4.0, cleanup Sep-Pak C18same as milk	Bio-Sil ODS-5S, 250×4 mm Mobile phase-isocratic with 0.1% acetic:methanol (65:35) Flow rate 1 ml/min	UV 270 nm
Fruit and Vegetables/ Riboflavin *(29)*	Hydrolyze sample in 0.1 N HCl at 100°C for 30 min then cool. Add mylase and incubate at 38°C overnight. Heat at 60°C with TCA 50% w/v for 5 min to remove proteins. Adjust pH to 4.0 then dilute and filter	Ultrasphere-ODS 5µm 250x4.6mm Mobile phase: MeOH/water (40:60) containing 5 mM sodium heptanesulfonate adjusted to pH 4.5 with phosphoric acid	Fluorescence Ex λ=450 nm, Em λ=530 nm
Cheese/ riboflavin *(30)*	Homogenize in water:MeOH (2:1). Acidify with HAC, mix and centrifuge. Extract three times with water:MeOH:acetic acid (65:25:10). Combine and dilute the extract then centrifuge	LiChrosorb RP 18, 5µm, 250×4.6 mm Mobile phase-isocratic with water:MeCN (80:20) Flow rate 1 ml/min	UV 446 nm
Baby foods,	Acid hydrolysis: autoclave	Hypersil-ODS 5 µm 250 x4.6 mm	Fluorescence

cereals, flours, dairy products/ Riboflavin *(31)*	homogenized sample with 0.1 N HCl at 121°C for 30 min. Cool, adjust pH to 4.5. Enzymatic hydrolysis: Add acid phosphatase and incubate at 45°C overnight. Proteins precipitated by heating at 100°C with TCA 50% w/v for 5 min. Adjust pH to 4.5 then dilute.	Mobile phase: Water/MeOH (3:2) adjusted to pH 4.5 with acetic acid	Ex λ=440 nm Em λ=520 nm
Flour, bread, beef, mush-rooms, milk, cereals/ Riboflavin, FMN *(32)*	Treat sample with hexane to remove fat. Autoclave homogenized sample with 0.1 N HCl at 121°C for 30 min. Adjust pH to 6.0, dilute with water then filter	LiChrosorb RP-8 (octyl) 10µm, 250 x4.0 mm Mobile phase: Water/MeOH (60:40) containing 5 mM sodium hexane sulfonate	Fluorescence Ex λ=440 nm (filter), Em λ=565 nm (filter)
Milk/ riboflavin *(33)*	Mix 20 ml sample with 2 ml of 10% lead acetate then filter	LiChrosorb C18, 10µm, 250×4.6 mm Mobile phase: isocratic with water:MeOH:HAC (50:49:1) Flow rate 1.5 ml/min –1	Fluorescence Filter fluorometer
Various foods (dairy, meat, fish, fruit, vegetables, flour, baked products, beer, coffee)/ riboflavin *(34)*	Autoclave homogenized sample in 0.2M H_2SO_4 for 20 min. Adjust pH and incubate with Claradiastase or Takadiastase at 45°C. Cool, dilute and filter Clean-up: Sep-Pak C 18	Spherisorb S5 ODS 2, 5µm, 250×4.6 mm Mobile phase-isocratic with water:MeOH (65:35) Flow rate 1 ml/min	Fluorescence Ex λ = 445 nm Em λ = 525 nm
Chick peas, green beans, milk powder /riboflavin *(35)*	Autoclave with 0.1N HCl for 15 min. Cool, sdjust pH and incubate with takadiastase at 48°C for 3 h. Filter and dilute.	µBondapak C18, 10µm, 300×3.9 mm Mobile phase-isocratic with (1) Water:MeOH:HAC (67:32:1) containing 5 mM Sodium hexsulfonate or (2) Water:MeOH:HAC (68:31:0.5) containing 5 mM	UV 254 nm

	Clean-up: Florisil followed by Sep-Pak C 18	Sodium heptanesulfonate and 5 mM hexanesulfonate (25:75)	
Dairy products (milk, milk powder, cheese)/ riboflavin, FMN, FAD *(36)*	Treat sample with 6% formic acid containing 2 M urea. Homogenize and centrifuge. Add sorboflavin as IS. Clean up: SPE C18. Elute with 10% formic/MeOH (4:1)	Supelco LC18, 3µm, 75×4.6 mm Mobile phase-isocratic with 100 mM KH_2PO_4:MeCN (86:14), pH 2.9 Flow rate 1 ml/min	Fluorescence Ex λ = 450nm Em λ = 530nm
Bovine milk/ Riboflavin, FAD, FMN *(37)*	Milk boiled to inactivate pyrophosphatase. Digested with buffered protease (pH 6.8) for 1h at 45°C then cooled and adjusted volume and pH with phosphate buffer.	Capcell Pak C18 5µm, 250x 4.6 mm, kept at 40°C Mobile phase: gradient with (A) 90% MeOH in water and (B) 0.01 M phosphate, pH 5.5.	Fluorescence Ex λ=462 nm Em λ=520 nm
Milk, nondairy imitation milk/ Riboflavin *(38)*	Precipitate proteins in samples with 10ml lead acetate (adjusted to pH 3.2) then filter	Spherisorb ODS 5µm, 150 ×3.9 mm Mobile phase: isocratic with 0.13% Acetic acid: MeOH (70:30) Flow rate 0.6 ml/min	UV 270nm
Various foods/ riboflavin, FMN, FAD *(39)*	Mix 0.5-4.0g sample with 9.0 ml MeOH and 10ml CH_2Cl_2. Add 7-ethyl-8-methyl-riboflavin as IS. Homogenize and add 0.1M citrate-phosphate buffer (pH 5.5) containing sodium azide. Mix, centrifuge, and filter	2 PLRP-S, 5µm, 100 Å, 250×4.6 mm and 150× 4.6 mm Mobile phase-gradient with MeCN and 0.1% sodium azide in 10 mM citrate- phosphate buffer, pH 5.5	Fluorescence Ex λ = 450nm Em λ = 522nm
Wines, beers, Fruit juices/ Riboflavin, FAD, FMN *(40)*	Filtered samples directly or after dilution with water (with 0.22 µm); injected for analysis	Hypersil C 18 5µm, 200 x2.1 mm Mobile phase: (A) 0.05 M phosphate buffer, pH 3.0 and (B) MeCN	Fluorescence Ex λ=265 nm Em λ=525 nm with a 500-nm cut-off filter
Sausages/	Acid hydrolysis: autoclave	Spherisorb ODS-2 5µm, 250x4mm kept at 35°C	Fluorescence

Riboflavin *(41)*	homogenized sample with 0.1 N HCl at 120^{o}C for 20 min. Cool, adjust pH to 4.0-4.5. Enzymatic hydrolysis: Add Claradiastase and incubate at 50^{o}C For 3 hours. Proteins precipitated by heating at 90^{o}C with TCA 50% w/v for 5 min. Dilute with water and filter.	Mobile phase: MeCN:water with 5 mM heptanesulfonic acid adjusted to pH 2.7 with phosphoric acid (75:25)	Ex λ=227 nm Em λ=520 nm
Foods/Riboflavin, FMN, FAD *(42)*	Suspend sample in mixture of MeOH-CH_2Cl_2 (9:10, v/v), homogenize for 1min. Add 0.1M ammonium hydrogen carbonate, pH 7.0 and mix for another min. Centrifuge	Symmetry C18 5µm, 150×3.9 mm Mobile phase-gradient with MeOH and 0.05M ammonium acetate pH 6.0	Fluorescence Ex λ = 450nn Em λ = 530nm
Foods/Riboflavin, FMN, FAD *(43)*	Homogenize sample in 10 ml MeCN for 10min. Add 10 ml 10 mM phosphate buffer pH 5 and mix. Centrifuge and dilute with phosphate buffer then filter	Discovery RP-Amide C16 5µm, 150×4.6 mm Mobile phase-isocratic with MeCN: KH_2PO_4 10mM pH 5 (10:90) Flow rate 1ml/min	Fluorescence Ex λ = 270nm Em λ = 516nm

Table A.12 Selected HPLC methods for vitamin B6 analysis

Sample matrix/ Analyte	Extraction/ Clean up	Column/ Mobile phase	Detection
Fortified cereals/PM, PL, PN *(44)*	Homogenize sample with 0.5 M potassium acetate (pH 4.5), centrifuge. Heating at 50°C with TCA 33.3% w/v to precipitate proteins, centrifuge to remove supernatant	µBondapak C18 10 µm, 300x3.9mm Mobile phase: isocratic with 0.033 M KH_2PO_4 buffer, pH 2.2	Fluorescence; Ex λ=295 nm, Em λ=405 nm
Plasma, various foods/PL, PM, PN, PLP, PMP, PNP, 4-PA *(45)*	3-hydroxypyridine was used as an internal standard. Homogenize 1 gram sample with 10 mL 5% sulfosalicylic acid, centrifuge at 4°C then filter. Evaporate off ¼ the volume under N_2. Add equivalent volume of hexane, vortex and remove the aqueous layer.	Two columns connected by a switching valve: Column 1-Aminex A-25, 55°C, 240x6mm Column 2-Aminex A-25, 18°C, 240x3 mm Mobile phase: 0.4 M NaCl: 0.01M glycine isocratic or gradient with flow rate of 1.2 ml/min	Fluorescence PM, PMP, PN, PNP Ex λ = 310nm Em λ = 380mn PL, PLP Ex λ = 280mn Em λ = 487mn
Beef, lima beans, wheat flour, nonfat dry milk /PMP, PM, PLP, PL, PN *(46)*	Remove fat with $CHCl_3$/petroleum ether (1:2). Air dry and store at -40°C overnight. Mix sample with water, adjust pH to 2.0. Incubate with pepsin at 37°C for 3h, adjust pH to 8.0. Digest with pancreatin at 37°C for 12h. Add TCA followed by MeOH, shake and centrifuge. Resuspend pellet in the mobile phase, centrifuge. Clean-up pooled supernatants with SPE C18 cartridge	µBondapak C18 10µm, 300x3.9mm Mobile phase: 0.075M KH_2PO_4 with monochloroacetic acid (1.5 g/l) adjusted to pH 2.75 with H_3PO_4	Post-column addition of sodium bisulfite in pH 7.5 phosphate buffer Fluorescence: Ex λ 310 nm Em λ 390 nm [69]
PLP, PMP, PN-glucoside, PL, PN, PM	Add 4-deoxypyridoxine as internal standard. Mix sample with 5% sulfosalicylic acid, centrifuge	Radial-PAK C18 4µm, 100x8mm Mobile phase: gradient with (A) 0.033 M H_3PO_4 containing 4mM octane- and heptanesulfonic acid	Post-column reaction with sodium bisulfite

(47)	Cleanup: remove sulfosalicylic acid with 40x8mm 100-120 mesh AG-X8 anion exchange column.	(pH 2.2)/2-propanol (97.5:2.5); (B) 0.33 M H_3PO_4 (pH 2.2)/2-propanol (82.5:17.5)	in pH 7.5 phosphate buffer Fluorescence Ex λ=338 nm Em λ=425 nm
Chicken/PL, PM, PLP, PMP *(48)*	Extract with 1M metaphosphoric acid	Biosil ODS-55, 250x4 mm Mobile phase: isocratic with 0.066 M KH_2PO_4, pH 3.0	Fluorescence Ex λ = 290nm Em λ = 395nm
Various foods/ PL, PM, PN, PLP, PMP, PNP, 4-PA *(49)*	Add 4-deoxypyridoxine as an internal standard. Extract with 0.1-0.5M cold perchloric acid, centrifuge and remove supernatant. Adjust to pH 7.5. Hydrolyze with alkaline phosphate at 25°C for 30min. Adjust to pH 4.0.	Lichrosphere RP-18, 5µm, 125x4 mm Mobile phase: gradient with (A) methanol and (B) 0.03 M KH_2PO_4, pH 2.7	Fluorescence Post-column derivatization sodium bisulfate Ex λ = 300nm Em λ = 375mn
Various foods/ PN *(50)*	Conversion of all B6 forms to PN: Add 25ml 0.05M sodium acetate pH4.5 + 1ml glyoxylic acid + 400 µL ferrous sulfate (2g/L) + 20mg acid phosphatase to 2.5 g sample. Incubate at 37°C overnight then dilute with water. Add To 4.5ml 0.1M sodium borohydride to 5 mL aliquot, shake then add 0.5 mL glacial acetic acid. Fillter	Lichrosorb 60 RP 5 µm, 250x5 mm Mobile phase: isocratic acetonitrile:0.05M KH_2PO_4 (4:96) containing 0.5mM sodium heptane sulfonate, pH 2.5	Fluorescence Ex λ = 290nm Em λ = 395nm
Various foods /PL, PN, PM *(51)*	Add 4-deoxypyridoxine as internal standard. Precipitate proteins with TCA 5% w/v TCA, centrifuge and filter. Dilute filtrate with 4M acetate buffer, pH 6.0. Add takadiastas and incubate at	Hypersil ODS 3µm, 125x4.6mm Mobile phase: 3% methanol and 1.25mM 1-octane-sulfonic acid in 0.1 M KH_2PO_4 adjusted to pH 2.15 with H_3PO_4	Post-column addition of 1M $K_2HPO_4.3H2O$ Fluorescence: Ex λ 333 nm Em λ 375 nm

	45°C for 3h. Cool, add TCA 16.7% w/v, centrifuge and filter.		
Wheat/PL, PM, PN, PMP, PN-glucoside *(52)*	Add 4′-deoxypyridoxine as internal standard. Homogenize samples in water. Precipitate proteins with metaphosphoric acid. Centrifuge and filter	Ultramex C18 5 µm, 150x4.6 mm Mobile phase: gradient with (A) 0.033 M KH_2PO_4 containing 0.008 M 1-octane sulfonic acid, pH 2.2 (B) 0.033 M phosphoric acid:10% MeCN, pH 2.2	Fluorescence Ex λ = 311nm Em λ = 360nm
Legumes/PM, PL, PN *(53)*	Precipitate proteins with TCA 5% w/v for 30 min, filter and dilute. Adjust an filtrate to pH 4.8, add acid phosphatase and incubate at 37°C for 5h. Add 20% TCA, cool down and filter (0.22 mm) Hydrolysis of glycosylated PN: adjust initial filtrate to pH 5.0, add b-glucosidase, incubate at 37°C for 5h. Add 20% TCA, cool down and filter (0.22 mm)	Spherisorb ODS-2 10µm 300x3.9 mm Mobile phase: 0.033 M potassium phosphate buffer, pH 2.2/MeOH (98:2)	Post-column addition of 0.3 M KH_2PO_4 pH 7.0 Fluorescence; Ex λ=328 nm Em λ=390 nm
Pig liver, vegetable, wheat flour/ PM, PL, PN *(54)*	Autoclave sample with 0.1N HCl at 120°C for 30 min. Adjust to pH 4.8, add acid phosphatase and b-glucosidase and incubate at 37°C for 18. Cool, adjust to pH 3.0 and filter	Nucleosil 120 C18 5µm, 250x4 mm kept at 30°C Mobile phase: 0.04M H_2SO_4 and 0.005M TCA (pH 1.0)	Post-column addition of 0.13 M $K_2HPO_4.3H2O$ pH 7.0 Fluorescence: Ex λ=333 nm Em λ=375 nm
Baker's yeast extract, egg yolk, milk / PMP, PM,	Homogenize sample with 1M perchloric acid. Add isopyridoxal as internal standard. Adjust pH to 3.0-4.0 with KOH, refrigerate for a few hours,	Phenosphere ODS2 5µm, 250x4.6mm Mobile phase: 0.15M NaH_2PO_4 adjusted to pH 2.5 with perchloric acid	Post-column reaction with sodium bisulfite Fluorescence:

PLP, PNP, PL, iso-PL, PN, 4-PA *(55)*	centrifuge and filter (0.45 mm)		Ex λ 290 nm Em λ 389 nm
Pork products/ PM, PL, PN *(56)*	4-deoxypyridoxine as internal standard. Acid and enzyme hydrolysis: mixsample in 60ml 0.1M HCl, shake and heat in water bath at 100°C for 30 min. Cool, adjust to pH 4.0-4.5 then add takadiastase and incubate. Precipitate proteins by adding 2ml 50% TCA, heat at 95°C for 5 min. Dilute to 100 ml with and filter	Spherisorb ODS C18 5µm, 250x4 mm Mobile phase: isocratic with 0.01M H_2SO_4 at flow rate of 1ml/min	Fluorescence Ex λ = 290nm Em λ = 395nm
Cooked sausages/PN, PL, PM, PLP, PMP 108, 2001 *(57)*	Extract with 20ml metaphosphoric acid 5%, dilute to 100ml with water, centrifuge and filter the supernatant	Hypersil ODS C 18 5µm, 100x4.6 mm Mobile phase: isocratic with 50 mM phosphate buffer (pH 3.2):MeCN (99:1)	Fluorescence Ex λ = 290nm Em λ = 395nm
Various foods/ PL, PM, PN 109, 2003 *(58)*	Autoclave sample in 50ml 0.1 M HCl at 121°C for 30 min. Cool down, adjust to pH 4.5. Add acid phosphatase, incubate at 45°C for 18h. Add 5ml 1M HCl, dilute to 30 mL with 0.01 M HCl. Filter	Phenomenex Hypersil C18, 3µm, 150x4.6mm Mobile phase: (A) 2.2 mM 1-octan sulfonic acid in 81mM KH_2PO_4 and 19 mM 85% phosphoric acid and 4.0 mM triethylamine, adjusted to pH 2.75 with 3.5 M KOH or 85% phosphoric acid Isocratic with 93%A and 7% acetonitrile	Fluorescence Ex λ = 333nm Em λ = 375nm
Various foods/ PMP, PM, PLP, PL, PN, 4-PA *(59)*	Acid hydrolysis with 4ml 1M perchloric acid. homogenize, centrifuge and adjust supernatant pH to pH 7. Cool down in ice bath for 30 min. Centrifuge, dilute supernatant to 5ml with mobile phase. Enzymatic hydrolysis: adjust diluted supernatant to pH 4-4.5, add takadiastase and phosphatase mixture.	Discovery RP-Amide C16 5µm, 150x4.6 mm Mobile phase: isocratic with 50 mM KH_2PO_4, pH 7	Fluorescence Ex λ = 335nm Em λ = 389nm

	Incubate at 50°C for 1h. Adjust supernatant to pH 7, centrifuge, dilute and filter		

Table A.13 Selected HPLC methods for vitamin B12 analysis

Sample matrix/Analyte	Extraction/Clean up	Column/Mobile phase	Detection
Pharmaceutical preparations/ cyanocobalamin *(60)*	Mix sample with 0.05 M NaH_2PO_4. Centrifuge and filter. Clean up with SAX and C18 SPE	µBondapak C18, 300x3.9 mm Mobile phase: gradient with 0.02M KH_2PO_4 and methanol at flow rate of 1.5ml/min	UV 550nm
Multivitamin, multimineral tablets/ cyanocobalamin *(61)*	Mix ground tablets with 30ml water containing 0.25g ammonium pyrrolidinedithiocarbamate, 1g citric acid and 10ml dimethyl sulphoxide. Shake, stand in water bath at 40 °C for 15min. Centrifuge, and dilute 15ml supernatant with100 ml water.	µBondapak C18 , 10µm, 150x3.9 mm Mobile phase: gradient with water and methanol at flow rate of 1ml/min	UV 550nm
Elemental diet/ Cyanocobalamin *(62)*	Mix 20g sample with 60ml deioinized water, put in the water bath set at 50°C then add 10g NaCl. Let the mixture stand for 30 min. Dilute to 100 ml with deionized water before removing fat with hexane. Sample clean up with Sep-Pak C18	Capcellpak C18, 5µm, 250x4.6mm Mobile phase: isocratic with water:acetonitrile (87:13) at flow rate of 0.6 ml/min	Visible 550nm
Foods/ Cyanocobalamin *(63)*	Mix 50g sample with Na_2SO_4 15% solution containing 1 mM sodium EDTA, filter. Sample clean up with Bond-Elut C18	Spherisorb ODS-2, 5µm, 150x4.6 mm Mobile phase: isocratic with 50 mM KH_2PO_4 (pH 2.1):MeCN (90:10) at flow rate of 1ml/min	Visible 550nm
Pharmaceutical preparations/ cyanocobalamin *(64, 65)*		Brownlee Aquapore C18, 7µm, 100x1 mm & Vydac C8, 5µm, 150x1 mm Mobile phase: gradient with 25 mM acetate (pH 4) and methanol at flow rate of 0.04ml/min.	MS-ESI positive ion mode w/ SIM or MS/ MS-ESI

			positive ion mode w/MRM
Multivitamin tablets/ cyanocobalamin *(66)*	Extract sample with 0.1M phosphate buffer, pH 7.0, centrifuge and filter	µBondapak C18, 10µm, 300x3.9 mm Mobile phase: isocratic with water and methanol (70:30) at flow rate of 0.8ml/min	Fluorescence Ex λ = 275 nm Em λ = 305 nm
Multivitamin tablets/ cyanocobalamin *(67)*	Extract ground tablets in 350 m mixture of methanol-water (50:50) for 30 min and filter. Dilute 25ml filtrate with 50 ml of extractant.	Column with phenylpropanolamine support, 150x4.6 mm Mobile phase: isocratic with 0.03M phosphate buffer (pH 3):MeCN (94:6) at flow rate of 1 ml/min	UV 361nm
Standards/ Cyanocobalamin *(68)*		Kromasil C18, 5µm, 250x4.6 mm Mobile phase: gradient with 0.4mM TFA and acetonitrile at flow rate of 0.7 ml/min; or Zorbax Extend C18, 3.5 µm, 150x2.1 mm Mobile phase: gradient with water containing 10mM 1-methylpperidine and acetonitrile at flow rate of 0.2 ml/min	MS/MS-ESI+ with MRM or SIM
Infant formula, nutritional supplement/ cyanocobalamin *(69)*	Sonicate sample in 5mM KH_2PO_4 for 10min, dilute with KH_2PO_4 and centrifuge. Collect middle layer, centrifuge. Add chloroform to remove lipids, centrifuge. Collect aqueous layer, centrifuge and filter (0.45 mm)	Pre-separation column: Capcellpak MF C8 (octyl) 5µm, 150x4.6 mm. Focusing column: Capcellpak MG C18, 5 µm 35x2.0mm Analytical column: Capcellpak UG C18, 5µm 250x1.5 mm. Column temperature 40^{o}C Mobile phase: gradient with (A) 5mM KH_2PO_4/ methanol (80:20) and (B) 5mM KH_2PO_4	Visible 550 nm
Foodstuffs/ Cyanocobalamin *(70)*	Extract with water for free vitamin B12. Digest with pepsin for total vitamin B12. Clean up with immunoaffinity column	LiChrospher 100 RP18 5µm, 250x4 mm Mobile phase: gradient with water and methanol at flow rate of 1ml/min	Fluorescence Ex λ = 250 nm Em λ = 312 nm

Food products/ Cyanocobalamin *(71)*	α-amylase digestion for free vitamin B12; α-amylase and pepsin digestion for total vitamin B12. Clean up with immunoaffinity column	Ace 3 AQ C18, 150x3 mm Mobile phase: gradient with 0.025% TFA in water (pH 2.6) and acetonitrile at flow rate of 0.25 ml/min	UV 361nm
Fortified products, multivitamin tablets/ cyanocobalamin *(72)*	α-amylase digestion for free vitamin B12; α-amylase and pepsin digestion for total vitamin B12.	XTerra MS C18 5µm, 150x3.9 mm Mobile phase: gradient with water and acetonitrile at flow rate of 1ml/min	MS/MS-ESI+ with SIM

Table A.14 Selected HPLC methods for vitamin C analysis

Sample matrix/Analyte	Extraction/Clean up	Column/Mobile phase	Detection
Fruits, vegetables/ ascorbic *(73)*	Extractant containing 6% HPO_3, 1mM EDTA and 0.1mM diethylthiocarbamate	µBondapak C18, 10µm, 100x8mm	1.5% NH 4 H 2 PO 4 buffer, pH 3
Citrus juices / vegetables/ ascorbic *(74)*	Mix sample with 0.3M TCA, dilute and filter. Add 4.5M acetate buffer (pH 6.2) and ascorbate oxidase. Incubate at 37°C for 5 min. Add 0.1% O-Phenylenediamine; react at 37°C for 30 min.	Hypersil-ODS 3µm, 125x4.6mm Mobile phase: 0.08M KH2PO4 pH 7.8/MeOH (80:20)	Derivatization with quinoxaline Fluorescence Ex λ 365 nm Em λ 418 nm
Orange juice/ ascorbic *(75)*	Mix sample with 6% HPO_3 (1:1), centrifuge and filter	Brownlee RP-18, 5µm, 220x4.6 mm (or 100x4.6 mm) Mobile phasae: 2% $NH_4H_2PO_4$, pH 2.8	Amperometric: glassy carbon electrode, + 0.6V vs. Ag/AgCl
Fresh fruits/ ascorbic *(76)*	Extract samples with 8% acetic acid and 3% HPO_3 in water, dilute with the same extractant and filter	Spherisorb ODS-2, 5µm, 250x4.6mm Mobile phase: Water adjusted to pH 2.2 with H_2SO_4	UV 254nm
Wine and beer/ ascorbic *(77)*	Filter sample through 0.2µm membrane	Nucleosil 120 C18, 7µm, 250x4 mm Mobile phase: 0.5% aqueous methanol containing 0.05M acetate buffer (pH 5.4) and 5mM n-octylamine	UV 266 nm
Fruit juices/ ascorbic *(78)*	Dilute sample with water and filter. For AA: add a-methyl-L-DOPA into sample (as an internal standard), then add 2% HPO_3. For total vitamin C: add internal	Inertsil ODS-2 5 µm, 150x4.6mm Mobile phase: 0.1M KH2PO4 buffer (pH 3) containing 1mM EDTA.2Na	Amperometric: + 0.3V vs. Ag/ AgCl

	standard and L-cysteine diluted in 0.01M phosphate buffer, pH 6.8. Let the reaction occur for 15min		
Green beans/ ascorbic *(79)*	Homogenize sample with 4.5% HPO_3 solution for 15 min, filter, dilute with the same solution and filter again (0.22 mm)	Spherisorb ODS-2, 5µm, 250x4.6mm Mobile phase: Water adjusted to pH 2.2 with H_2SO_4	UV 254nm
Fresh fruits and vegetables/ ascorbic *(80)*	Mix macerated sample with 2% HPO 3 , shake, filter	Spherisorb ODS-2, 10µm, 250x4.6 mm Mobile phase: 0.01M KH_2PO_4/ 20% tetrabutyl ammonium hydroxide/ MeOH (970:1:30) adjusted to pH 2.75 using 85% phosphoric acid	UV 244 nm
Honey/ ascorbic *(81)*	Dissolve in metaphosphoric 0.25% then filter	Shimadzu C18- ODS 5µm, 250x4.6mm Mobile phase: methanol and water adjusted to pH 2.5 with HPO_3 (15:85)	UV 254nm
Fruit juices, fruit drinks/ ascorbic *(82)*	Add 1ml of 50mM dithiothreitol into1ml of sample, centrifuge and filter (0.22 mm)	J'sphere ODS-H80 4µm, 250x4.6mm Mobile phase: 2% acetic acid (pH 2.5)	UV 243nm
Multivitamin-mineral tablets/ ascorbic *(83)*	Extractant contains 1g pyrogallol and 19.21 g citric acid per liter. Extract sample with 100ml extractant solution and filter.	Hypersil BDS C18, 5µm, 250x4.0mm, Mobile phase: isocratic with acetonitrile: ion-pair solution (2:98) Ion-pair solution contains10 mM 1-hexane sulfonic acid, 10 mL acetic acid and 1.3 mL trimethylamine in 1L	UV 275nm
Sausage/ ascorbic *(84)*	Extract 5g sample with 20ml metaphosphoric acid 50% and 1ml EDTA, dilute to 50ml, centrifuge and	Spherisorb NH_2 , 5µm, 250x4.0mm Mobile phase: isocratic with 0.02M KH_2PO_4:acetonitrile (40:60), pH 3.6 at flow rate	UV 248nm

	fi lter	of 1 ml/min	
Beverages/ ascorbic *(85)*	Dilute with water if needed	Kromasil NH_2, 5µm, 250x4.6mm Mobile phase: isocratic with 0.1M acetic at flow rate of 1.5ml/min	UV 250 nm
Foods/ ascorbic *(86)*	Dilute sample in10ml mobile phase containing 20µM L-methionine.	Intersil ODS, 5 m, 150x3mm Mobile phase: isocratic with 0.2% H_3PO_4, pH 2.1at flow rate of 0.4ml/min	EC +400 mV v.s Ag/AgCl
Foods/ ascorbic *(87)*	Extract 2g sample with 5ml methanol and 25ml of 3% metaphosphoric acid and 8% acetic acid solution. Filter and ad 15ml 1% acetic acid to 10ml filtrate.	Waters Symmetry C18 , 3.5µm, 75x4.6 mm coupled to an Atlantis dC18, 5µm, 150x2mm Mobile phase: isocratic with methanol:0.05% acetic acid (70:30)	LC-MS-ESI-
Tropical fruits/ ascorbic *(88)*	Extract with 3% metaphosphoric acid and 8% acetic acid solution	Shodex RSpak KC-811, 5µm, 250x4.6mm Mobile phase: isocratic with 0.2% orthophosphoric acid at flow rate of 1.2 ml/min	UV 245nm
Fatty fish/ ascorbic *(89)*	Extract with 4.5% metaphosphoric acid	Waters Symmetry C18, 5µm, 250x4.6mm Mobile phase: isocratic with methanol:0.1% metaphosphoric acid (80:20) at flow rate of 1ml/min	UV 245nm
Fortified food products/ Ascorbic *(90)*	Weigh 10grams sample into 100ml flask, add 40ml 250ppm TCEP. HCl (Tris (2-carboxyethyl) phosphine hydrochloride), mix thoroughly and fill to the mark with 1% TCA. Shake and filter.	LiChrospher RP-18; 5 µm; 250x4.6mm Mobile phase: 1.6g decylamine, 80 ml acetonitrile, 100 ml of sodium acetate solution (0.25 M) pH 5.4 and 820 ml of distilled water. Final pH adjusted to 5.4 with phosphoric acid 85% and 50 mg TCEP. HCl. Isocratic at a flow rate of 1 ml/min	UV 265nm

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