8-2013

ANALYSES OF MULTIVITAMINS IN NUTRACEUTICALS BY REVERSE PHASE HPLC WITH DAD, ELSD AND MS

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ANALYSES OF MULTIVITAMINS IN NUTRACEUTICALS BY REVERSE PHASE HPLC WITH DAD, ELSD AND MS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Food, Nutrition, and Culinary Science

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by
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August 2013

Accepted by:
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ABSTRACT

Vitamins are essential to human beings and support long-term healthy lifestyle. They exist widely in food, but in small amount. Hence, dietary supplements and fortified food products are more and more popular in the nutritional markets. According to the Infant Formula Act of 1980, the range of each of the nutrients should fall within a specific range or above the required minimum, so there is an increasing interest of accurate measurement of vitamins, particularly the B-group vitamins. However, traditional methods for vitamin B analyses are time-consuming and often in poor accuracy. As a result, rapid and reliable analytical methods for simultaneous determination of water-soluble vitamins in multivitamin and supplemented foods are important, indispensible and needed by food and nutraceutical industries for quality control during production, and for accurate evaluation of data and label clams.

Due to low concentrations of B8 and B9 in nature, there is a critical need for preconcentration to facilitate their isolation and purification from a complex matrix like food. In order to minimize the consumption of solvents, solid phase extraction (SPE) has been introduced for sample preparation. In this study, two types of SPE, i.e., reversed-phase C18 and strong anion-exchange phase, were used for sample clean-up and pre-concentration. The average recoveries for vitamin B_3, B_5, B_7 and B_9 were 101.3%, 102.9%, 92.8% and 102.6%, respectively, by using C18 SPE. In contrast, the strong anion-exchange SPE provided the average recoveries of B_3, B_5, B_7 and B_9 in 91.5%, 93.0%, 109.1% and 106.7%, respectively.

Furthermore, simultaneous determination of water-soluble vitamins was developed by a HPLC system installed with a ZORBAX Eclipse XDB-C18 (250mm x 4.6mm, 5 μm particle size, Aglient Technologies, Inc., Loveland, CO, USA) with a

guard column (12.5mm x 4.6mm, 5 μm particle size), and compared by three detectors, including DAD, ELSD and MS. The B-complex supplement, multivitamin/multimineral tablets and powder and vitamin drink were tested to verify HPLC method. According to the results, LC-MS is the best to do the simultaneous determination of the B-group vitamins in lights of its analytical accuracy, precision, sensitivity and versatility.

DEDICATION

I dedicate this work to my parents, Chen-Yuh Huang and Hsiu-Mei Chuang, my sisters Yu-Wen and Yi-Ling Huang, my grandparents, Kuan-Nan and Mei-Niu Hung Huang, and my undergraduate advisor, Chia-Feng Kuo, with love and pride.

ACKNOWLEDGMENTS

First, I would like to give my deepest appreciation and gratitude to my major advisor, Dr. Feng Chen, for providing me this opportunity with consistent support and valuable guidance and assistance throughout the completion of this thesis and my master degree program.

I would also like to thank you my committee members Dr. Ashby Bodine and Dr. Kurt Young for their instruction, advice, and encouragement during my study and writing this thesis. I also thank my lab mates, Dr. Yu Qi and Gregory Jones, for their help on technical training on HPLC systems and assistance on my study.

Finally, I want to appreciate my family and friends in Taiwan for their endless support and encouragement for my entire education and oversea study. My success is attributed to their love and support.

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

The term “Vitamin” has become a word commonly used in daily discussion (1). Vitamins are essential to humans in order to maintain a long-term healthy lifestyle and support normal growth (2-5). Vitamins are a minor dietary constituent that are widely consumed although in small amounts in the daily diet (6,7). They can be classified based upon their solubility into two groups: fat-soluble vitamins (e.g. vitamin A, D, E and K), and water-soluble vitamins (i.e. vitamin C and vitamin B, e.g. thiamin (B_1), niacin, folic acid (B_9), biotin (B_7), pantothenic acid (B_5), etc.) (8,9). B-group vitamins as coenzymes or co-factors have a critical role in the metabolism of carbohydrates, proteins, and lipids. However, they are unable to be synthesized by tissues in sufficient amounts; therefore, their deficiency or excess in the human body depends on the dietary consumption and supplementation to either which insufficient or excess vitamin consumption could lead to serious dieses (3,8,10-12). Although, the main resource for obtaining B group vitamins is from the diet, these vitamins are unstable and easily destroyed and/or degraded by heat, light, oxygen, and other food components. They are also subject to loss during food processing, cooking and long periods of storage. It is well known that insufficient levels of vitamins can cause many problems in maintenance of body functions and normal growth (4,8,11,13). For this reason, many foods are supplemented with vitamins, especially for infant and baby products in order to meet the nutritional requirements. In addition, the use of a multivitamin supplement is another way to circumvent the lack of sufficient dietary

intake of vitamins and decrease the risk of hypovitaminosis, as a consequence of malabsorption, bad eating habits, pregnancy, or acute and chronic diseases. Multivitamin supplements are also used for curing individual deficiencies (10,12,14,15).

Currently, the general population recognizes the importance of pursuing a healthy lifestyle, so there is a demand for food and pharmaceutical companies to produce functional food products, including vitamin fortified foods (e.g., vitamin fortified cereal, milk, and even water). Besides, functional beverages enriched with dietary multivitamin/multimineral supplements are also popular in modern society (16,17). The labeling of nutritional facts for foods and beverages is required by the U.S. Food and Drug Administration (FDA), and is regarded as a basis for people to assess their daily intake (10,16,18). The Nutrition Labeling and Education Act (NLEA) of 1990 requires food producers to provide specific nutrition information on individual products that are sold in the USA. In addition, according to the Infant Formula Act of 1980, the range of each of the nutrients should fall within a specific range or above the required minimum (19,20,18). Currently, there is an increasing interest in the accurate measurement of vitamins, particularly the B-group vitamins, in dietary intake from all sources; therefore, it is necessary to develop rapid and reliable analytical methods for simultaneous determination of water-soluble vitamins in multivitamin and supplemented foods. This is important for confirmation of the recommended dietary allowance (RDA) in final food products, for quality control during production, and for accurate evaluation of data and label clams, which has been of particular interest to the nutritional field and food industries (2,9,12,21,22). The complexities of vitamins in terms of their chemical compositions in dietary

supplements and fortified foods as well as their different chemical structures and properties, development of a robust analytical method for vitamin analysis is a big challenge, because vitamins structures 1) may be inter-convertible; 2) have a variety of chemical properties in terms of stability, polarity and acidity that can vary within the sample preparation and determination; 3) can be readily degraded in solution; 4) have varying sensitivities to light; 5) their stabilities in solution are pH dependent, and 6) the amount of individual vitamins is different in multivitamin tablets. For the above reasons, single analysis, particularly simultaneous analysis of vitamins is difficult and challenging (2,7,9,14,23,24).

The traditional analytical methods and the current official methods for determination of water-soluble vitamins are based on spectroscopic, chemical, enzymatic, and microbiological assays, which are usually tedious and time-consuming, because various indispensable steps for sample preparation are required to remove the interfering chemicals. Regardless of the shortcomings of the above mentioned methods, both non-bioavailability and bioavailability measurements often give overestimated amounts of vitamins and lead to inaccurate assessed results (6,10,12,17,21,25).

In the last decade, investigators in food laboratories have shown great interest in development of simultaneous determination methods for water-soluble vitamins. As a result, many techniques, including capillary electrophoresis (CE), UV-Vis spectrophotometry, fluorimetry, chemiluminiscence, atomic absorption, micellar electrokinetic chromatography, micellar liquid chromatography, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), and HPLC/mass spectrometry (LC/MS), have been

investigated and reported (7,9,11,12,21,24). Among them, the most common method for vitamin determination is HPLC (26,27), because improved quality and properties of stationary phases and chromatographic equipment has enabled significant improvement of chemical separation (resolution) and signal detection (sensitivity) (21). Although many HPLC methods are still not officially approved as the standard methods by the AOAC because of different conditions of columns (9), HPLC technique can obviously provide many benefits for determining vitamins in various products. For example, it is fast, sensitive, accurate, precise, and can minimize required solvent and sample quantities. As a result, it is better than the traditional methods (10,11).

Liquid chromatography (LC) is a kind of separation technique that can be classified into normal-phase, reverse-phase (RP), ion-exchange or ion-pairing chromatographies with isocratic elution and/or gradient programs. Currently, new techniques are emerging with quit varied and improved bonded stationary phases and column packing materials, which can be used to determine individual vitamins and analyze more complicated matrices. At present, methods for separation of B group vitamins are commonly done using RP-LC with or without ion-pair reagent, such as a buffered solution. Many mobile phases use phosphate buffer solutions at a variety of pH values. However, the phosphate buffer is incompatible with some detectors such as evaporative light-scattering detector (ELSD) and mass spectrometry (MS). Moreover, these buffered ion pair eluents require a longer time for column equilibration and total run time compared with LC-RP without an ion-pair reagent. Although, LC-RP without ion-pairing reagents has low reproducibility for some vitamins, an aqueous tolerant column can be used to alleviate low reproducibility

(10,12,13,21-23). LC methods can be used with different detectors, such as UV, fluorescence, diode array detection (DAD), ELSD and MS. UV detection is the most commonly used even though MS has higher selectivity and specificity (2,7,27).

The most important step for an HPLC analysis is sample preparation. As previously mentioned, some B group vitamins (e.g. biotin, folic acid, B_{12}) exist in very small amounts and are mixed with other components in a complex system such as dietary supplements, so a good sample preparation, including clean-up and concentration steps, are usually required prior to the chemical analysis. These steps are very important, especially, for quantitative tests, in which yields and recovery at each step of sample preparation must be monitored to validate the HPLC method. The extraction step needs to prevent B group vitamin degradation under heat, oxygen, light, pH, and oxidizing conditions. If it is necessary, the analysis should be conducted under reduced-light, and should avoid compound conversion under unsuitable conditions, in order to obtain accurate and precise results. Based on different types of matrices, a suitable type of extraction such as liquid-liquid extraction (LLE), solid-phase extraction (SPE), or supercritical fluid extraction (SFE), should be selected. The parameters for extraction, such as solvent, sample/solvent ratio, time of exaction, temperature, pH, and percentage or concentration of acid or base in a solvent, should be considered for optimization of an extraction method (17,22). Many interfering compounds, such as fat-soluble vitamins, sugars, minerals, complexing agents, etc. whose existence in dietary supplements and functional beverages makes the sample extraction more complex, should be removed. Recently, solid-phase extraction (SPE) has been more frequently applied to purify samples by eluting the absorbed compounds on the stationary phase by different polarities of solvents (10).

Another benefit from applying SPE is to concentrate the target analytes in the sample. Due to the small quantities of biotin and folic acid in microgram levels in dietary supplements and soft drinks, it is hard to perform accurate quantitative analysis. Biotin lacks chromophores for UV detection and only has a short wavelength absorption near 204 nm, a wavelength at which many compounds, including common mobile phase components absorb (10,12,28). For the above mentioned reasons, analyses of biotin and folic acid are particularly difficult.

1.2 The Vitamins

The vitamins are organic compounds with low-molecular-weights (1,11). Thirteen compounds have been generally recognized as vitamins including nine water soluble vitamins (ascorbic acid, thiamine, riboflavin, niacin, pyridoxine, biotin, folic acid, pantothenic acid, and cobalamin) and four fat-soluble vitamins (vitamins A, D, E, and K) (29,30). The term "vitamer" is defined for structurally distinct chemical compounds that have similar biological activity as the native vitamin (31). Vitamers are either convertible to final active form in the reaction of metabolism or their structures have similarity to react in the same activity (Table 1.1) (32).

1.3 Niacin

Niacin (B_3, preventing pellagra (PP) factor or antipellagra vitamin) was discovered in 1936, and can be found from a significant number of sources such as brewer's yeast, meats, milk, high-protein products, nuts, breads and cereal (1,31). Generally, the term niacin describes both nicotinic acid (NA) and nicotinamide (NAm) which are contained in foods (33,34). NAm is also commonly used in cosmetic skincare products (35). NA occurs predominately in plants, and NAm presents as part

of nicotinamide-adenine dinucleotide (NAD (H)) and nicotinamide-adenine dinucleotide phosphate (NADP (H)). Niacin is required by law to be added to wheat and other grain products in United States (1). In the human body, niacin can be synthesized from tryptophan. In living animals, niacin is required for biosynthesis of NADH and NADPH, and is used as coenzymes for proton and electron carrier in many redox reactions. NADH carries hydrogen reducing equivalents from tricarboxylic acid (TCA) cycle to electron transport chain for releasing energy from metabolism of carbohydrates, fatty acid, and amino acid (1,30,31) while NADPH is used for its very high reducing potential in certain enzyme reactions and biosynthesis.

1.3.1 Pharmacological Use of Niacin

Nowadays, NA in large dose has been used as treatment to reduce serum cholesterol for hypercholesterolemia and to prevent atherosclerosis and coronary diseases, and also been used as vasodilator (1). In clinical use, NAm can reduce urinary excretion of tryptophan and increase available tryptophan to synthesize serotonin for enhanced anti-depressive effects. In animal models and clinic trials, NAm shows to delay or prevent the development of non-obese or insulin-dependent diabetic symptom, because Nam protect pancreatic β-cell function through NAD^+ and the DNA-protective enzyme poly (ADP-ribose) polymerase activity ([1,36]). NAD^+ can also repair skin DNA damage from UV light (1,36).

1.3.2 Chemical, Physical and Spectral Properties

Two vitamers of niacin are nicotinic acid (NA) and nicotinamide (NAm). NA and NAm are chemically named as pyridine 3-carboxylic acid ($C_6H_5O_2N$, molecular weight (MW) = 123.1) and pyridine 3-carboxamine ($C_6H_6ON_2$, MW= 122.1),

respectively (Figure 1.1). They have equal biological activity and are easily inter-convertible. The free acid can be converted to the amide form and further to formation of NAD and NADP (Figure 1.2) in humans (30,37).

1.3.2.1 Chemical and Physical Properties

Both NA and NAm forms are colorless and odorless with white needle-shaped crystals. Their melting points are 235℃ and 129℃, respectively. NA has a tart taste, but NAm tastes bitter. NA is poorly soluble in water (1.67 g/100 mL) at 25℃ and ethanol (0.73 g/100 mL), but is freely soluble in boiling water, alcohol, alkali hydroxides and carbonates. It is insoluble in acetone and diethyl ether. NA is amphoteric, its pKas are 4.9 and 2.07, and the pH of its saturated aqueous solution is 2.7. NA solution can be autoclaved for 10 minutes at 120℃ with no decomposition. Moreover, NA can tolerate autoclaving with 1 to 2 N mineral acid or alkali. NAm is easily dissolved in water (100 g/100 mL) and in ethanol (67 g/100 mL). It can also be dissolved in acetone, chloroform, and butanol, but is slightly soluble in diethyl ether and benzene. The pKa of NAm is 3.3. The pH value of a NAm aqueous solution is 6. Both NA and NAm are bases and form quaternary ammonium salts in acidic solution. NA forms a carboxylic acid salt in basic solutions, but NAm does not provide acidic properties. Both forms of niacin are suitable for fortification and supplementation. NAm is added in dry and liquid products, while NA is used in products that do not rely on high solubilities (30,37,38).

Manufacturers are cautious of the NA dose added in products because NA is a powerful vasodilator, which can cause a serious flushing through inhalation of the dust. The oral dose for reducing serum cholesterol is enough to cause flushing in

patients (30,36,37).

1.3.2.2 Spectral Properties

NA and NAm have similar absorption properties near 260 nm. The ultraviolent (UV) absorption spectra of NA and NAm standards are shown on Figure 1.3 and Figure 1.4, and their intensity is dependent on pH. Free forms of niacin do not emit fluorescence. However, the nicotinamide ring and adenine ring enable the UV absorption in maxima at 260 nm and 340 nm, respectively. Their corresponding fluorescent coenzymes emit fluorescence at 470 nm when excited at 260 nm or 340 nm. Therefore, there is a problem in detecting NA and NAm in biological materials, due to their lack of fluorescence. Moreover, when NA and Nam are determined by LC-UV, a time-consuming extraction and clean-up steps are required in order to remove interference which can disrupt the UV absorption (37).

1.3.3 Stability

Niacin is the most stable water-soluble vitamin, as its biological activity is unaffected by air, oxygen, light, acid, alkali, or heat in the dry state and in neutral aqueous solution (1,30,37). Hence, acid and alkali hydrolysis are utilized in many biological samples for niacin extraction. Such processes release niacin from coenzymes, and also convert NA to NAm for quantitative analysis after autoclaving, because nicotinic acid is stable in acid conditions. NADH and NADPH (reduced form) are readily destroyed in acid, but are very stable in alkali. After a long-time storage in alkali solution, NADH and NADPH are subject to be oxidized. However, NAD^- and $NADP^-$ (oxidized form) have opposite properties to the NADH and NADPH. They are stable in acidic conditions (30,33,37).

In food processing, niacin would not be lost during storage, cooking and heating, but is subject to loss in leaching during food preparation. In particular, the available niacin is almost fifty percent lost when combined soaking and cooking together (37). Naturally, niacin in uncooked food mainly exists as NAD^- and $NADP^-$, so it perhaps undergoes hydrolysis during cooking to liberate NAm from coenzymes (30).

1.3.4 Bioavailability

Niacin exists in a variety of foodstuffs; however, its bioavailability in natural foods is often low. Natural cereal grains, which include maize, wheat, rice, barely and sorghum, have been found to contain a significant amount of niacin, but 85-90% of niacin is present in chemically bound forms, which cannot be absorbed or utilized if absorbed (30,37). The NA often occurs in food, bound to polysaccharides on the carboxyl group of NA, referred as niacytin, or combined with peptides via amide linkage between amino group and peptide, as niacinogen. In cereal grains, niacinogen is unavailable for utilization in body (30,33,37). In other foods, the methylated derivative (1-methylnicotinic acid or trigonelline) of nicotinic acid is a plant hormone not biologically active in animal tissues. It is heat labile, so heating can liberate nicotinic acid, and suddenly converts nicotinic acid to nicotinamide (1,37).

In order to accurately quantify niacin in food, an acid or enzymic hydrolysis is often used to liberate nicotinic acid from its bound forms prior to extraction. For cereal samples, acid hydrolysis does not hydrolyze most bound nicotinic acid, so acid hydrolysis is usually followed by an alkaline hydrolysis to release nicotinic acid from macromolecules. This allows the total content of niacin to be determined, but can

overestimate the available niacin from food (33,37).

1.4 Pantothenic Acid

Pantothenic acid is also one of B group vitamins (vitamin B_5), which is discovered and identified by R. J. Williams in 1931 and 1933. “Panthos” is from Greek, meaning everywhere, and is reflective of the widespread occurrence of pantothenic acid in nature. Pantothenic acid is essential for releasing energy from carbohydrates, amino acids and fat, and is also involved in the synthesis of lipids, neurotransmitters, steroid hormones via cholesterol, and hemoglobin. Pantothenic acid is widely distributed in animal and plant tissues as its structure is the part of co-enzyme A (CoA) and acyl-carrier protein (ACP), which exist in all tissues. CoA and ACP are important to metabolic pathways, including tricarboxylic acid (TCA) cycle and fatty acid metabolism, as an acyl transfer agent (30,33,36,37). Owing to its availability from food, human deficiency is rare, and has only been diagnosed in severely malnourished patients. The daily intake range of pantothenic acid in United States is from 4 to 7 mg/day, so the Reference Daily intake (RDI) has been established at 10 mg/day according to the USDA (37).

Pantothenic acid is widespread and measurable in most food from 10 to 50 μg/g (w/w), due to its diverse metabolic functions as a part of constituent of CoA. It exists in the free forms, (i.e. pantethine), or bound forms, (i.e. CoA, CoA esters, ACP) in foods. The most significant resources are yeast, organ meats, egg yolk, avocado, some vegetables and whole grain (1,33,37,39). Although, pantothenic acid occurs in all living matter, processing and refining foods, such as sugar, oil, cornstarch or grains, can cause as much as 50% loss of pantothenic acid (33,39). Generally, there are three

commercial forms: sodium and calcium D-pantothenate and D-pantothenol. Sodium and calcium D-pantothenate are often added in foods, feeds and pharmaceuticals in order to increase stability and promote easy handling. D-pantothenol, which is a stable liquid, is used to be added in liquid products or in cosmetics (33,36).

1.4.1 Pharmacological Used of Pantothenic Acid

Some benefits have been reported through the supplementing of pantothenic acid and/ or its metabolites. For example, consuming high doses (500-1200 mg/day) of pantothenic acid can reduce serum cholesterol, decrease low-density lipoprotein (LDL)-associated cholesterol and triglyceride, and increase high-density lipoprotein (HDL)-associated cholesterol. Moreover, pantothenic acid has been observed to reduce the time of morning stiffness, the degree of disability, and the severe pain for rheumatoid arthritis patients (1).

Pantothenol has been used in cosmetics, and also for treatment of skin disorders and wound healing. However, oral supplementation was reported to have no effect on wound healing. For seizure patients, valproate administration to young children is used to control seizures, but it would deplete CoA and damage liver. On the contrary, pantothenate administration can prevent seizures and reduce the risk of liver damage (33).

1.4.2 Chemical, Physical and Spectral Properties

The D-isomer of pantothenic acid (Figure 1.5) is the only naturally occurring vitamer that has biological activity. Pantothenol also has biological activity, which is a synthetic compound, because it can be oxidized to pantothenic acid (32). Pantothenic acid ($C_9H_{17}O_5N$, molecular weight= 219.2) is composed by pantoic acid (butyric acid)

linked to an amino acid (β-alanine) through a peptide bound (30). The free form of pantothenic acid and its sodium salts are too unstable and hygroscopic for commercial application. Therefore, the form for human supplements and therapeutical use is usually the calcium D-pantothenate [$(C_9H_{16}O_5N)_2Ca$, MW = 474.5] (30,33,36). The United States Pharmacopeial Convention (USP) standard is D-(+)-pantothenic acid. Other commercial forms include sodium and calcium salts and the alcohol, pantothenol (37).

1.4.2.1 Chemical and Physical Properties

Pantothenic acid is yellow, viscous, oily, and readily soluble in water, alcohol and dioxane, but is rarely soluble in diethyl ether and acetone. It is insoluble in benzene and chloroform (36). Calcium pantothenic acid is a color, odor free microcrystalline powder, and has a bitter taste. Pantothenic acid is highly hygroscopic, while the calcium pantothenate is moderately hygroscopic and melts at 195-196℃. The solubility of calcium salts is 40g/100 mL in water, and is slightly soluble in ethyl acetate, and insoluble in diethyl ether. The pKa value of the calcium salt is 4.4 (dissociation of the carboxyl group). A 5% solution of the calcium salt has a pH of 7.2-8.0 (30,37).

1.4.2.2 Spectral Properties

Pantothenic acid and other isomer compounds do not have a chromophore. Only the carbonyl group has weak absorbance below 210 nm (Figure 1.6) (37).

1.4.3 Stability

The stability of pantothenic acid and calcium pantothenate are highly pH

dependent, and moderately stable under light and atmospheric oxygen, if protected from moisture. Pantothenic acid is most stable at pH 4.0-5.0, while the calcium pantothenate is stable in pH at 5.0-7.0. Therefore, because of greater stability at near neutral conditions calcium pantothenate, as opposed to the free pantothenic acid, is more often utilized in fortified foods and for pharmaceutical use. Although calcium pantothenate is more stable, it is easy degraded during autoclaving or similar conditions. For example, when vegetables are cooked in water, the pantothenate salt is lost in a large extent (30,33,36,37).

In aqueous solutions with pH values under 5.0 or above 7.0, calcium pantothenate becomes thermally labile, and will undergo hydrolytic cleavage to produce pantoic acid, its salts and β-alanine. Compared with other B vitamins, pantothenic acid is more stable at higher pH values (30).

In plant or animal foods, pantothenic acid is present as both free and bound forms, but most of them are present in the bound form in food, because of CoA and ACP. Pantothenic acid is the most stable form in food storage; however, it is potentially leached 15 to 50% from cooked meats and 37 to 78% from blanch vegetables (1,30). In the processing industry, pantothenic acid may be destroyed through freezing, canning and refining processes (31).

1.4.4 Bioavailability

The bioavailability of pantothenic acid in foods and feedstuffs has not been fully investigated. It was reported that the bioavailability of pantothenic acid ranged from 40 to 61%, with a mean of 50%, based on urinary excretion from male subjects and tested by microbiological assay. In previous research for the study of

bioavailability of pantothenic acid in five different types of foods: i.e., wheat, coarse wholemeal bread, steamed potatoes, boiled pork, and boiled beef, which were fortified with minerals, fat-vitamins, enriched soybean oil and amino acids, the results showed that 65 to 81% of pantothenic acid was digestible in studied animal (e.g., pig), and indicated that the feed did not yield significantly different levels in the pigs. The bioavailability of pantothenic acid decreased in the following order: wheat diet>pork diet> potato diet> beef diet, and the coarse wholemeal bread diet only reach 28% (1,30,37).

1.5 Biotin

Biotin is a B-group vitamin also referred to as vitamin B_7 or vitamin H. This vitamin has been found in yeast and meat extracts as a growth factor "bios" in 1901. Then, in 1927, it was shown that biotin was associated with raw egg white in rat and human. The reason is that raw eggs contain a compound, avidin, which will tightly bind with biotin, and cause a functional biotin deficiency. The symptoms of deficiency lead to severe dermatitis, alopecia, and neuromuscular dysfunction. In 1942, scientists isolated, crystallized, and named this growth factor, biotin. Other names of biotin include bios II, factor X, coenzyme R, and anti-egg white injury factor (36,37,39). In mammals, biotin functions as a coenzyme in four carboxylase enzymes and plays an important role in gluconeogenesis, fatty acid biosynthesis, amino acid metabolism, and odd-chain fatty acid catabolism (33,40,41). The function of carboxylases is to incorporate CO_2 into numerous substrates using bicarbonate as the carrier of CO_2. For example acetyl-CoA carboxylase is the rate limiting enzyme in fatty acid synthesis, and pyruvate carboxylase is for oxaloacetate formation during

gluconeogenesis, etc (37).

Although biotin is present in all natural foodstuffs, its content in food is very low, even in the richest biotin content food. The richest resources of biotin are from liver, eggs, and soybeans, while good sources include yeast, wheat bran, and some vegetables (1). Because dietary intake is sufficient to provide levels to maintain our health, deficiency of biotin has rarely been diagnosed. Biotin can be found as a protein bound form in animal products, nuts, cereals, and yeast, but as a free form in vegetables, green plants, fruits, and milk. Due to the fact biotin exists in all foods and it can be synthesized by bacteria in intestinal microflora, its deficiency is rare when having a well-balanced diet (30). The main cause of deficiency is diagnosed by having genetic disorders in biotin metabolism, but it can be cured by administering large doses of biotin (1).

Biotin has no established Recommended Dietary Allowances (RDA) by Dietary Reference Intake (DRI) committee, but has Adequate Intakes (AIs) ranging from 5 μg/day for 0 to 6 months, which is based on biotin content in breast milk, 35 μg/day for lactating women, and 30 μg/day for adults. The Reference Daily Intake is 300 μg for calculating nutrition label information (37,39).

1.5.1 Chemical, Physical and Spectral Properties

The systematic chemical name of biotin ($C_{10}H_{16}O_3N_2S$, MW = 244.3) is *cis*-hexane-2-oxo-1-H-thieno [3,4-d] imidazole-4-pentanoic acid (Figure1.7). Biotin contains three asymmetric carbon atoms, so it has eight stereoisomer structures (2^3) in nature, but only one isomer, (+) *d*-biotin, is biologically in humans (33,37,42,43). In animals and plants tissues, small amounts of biotin occur in the free form. Mainly,

biotin bound with protein by covalent bound on biotin-dependent enzymes, through linkage of amide bound between carboxyl group of biotin and the ε-amino acid group of a lysine residue. Proteolysis of the enzyme complex can liberate a biologically active compound, biocytin (ε-N-biotinyl-L-lysine), which is a natural water-soluble fragment (30). In nature, plant tissues have greater amounts of the free form of biotin (23 to 80%) than in animal tissues (11 to 29%). However, according to a one report, there was more than 95% free biotin in breast milk (37).

1.5.1.1 Chemical and Physical Properties

Synthetic *d*-biotin is produced as fine colorless crystalline needles. It can decompose and melt at 232℃. In its acidic form, it is very slightly soluble in water at 25℃ (20 mg/100mL) and in 95% ethanol (80 mg/mL), but is more soluble in hot water. It is more soluble in weakly alkali solutions than in weakly acidic ones. It is insoluble in organic solvents, such as chloroform, diethyl ether, and petroleum. A 0.01% aqueous solution of biotin has a pH of 4.5 with the pKa value of 4.51. The salt form of biotin is greater water-solubility than its acid form. The United States Pharmacopeia Convention (USP) standard form for biotin is *d*-biotin (30,33,37).

1.5.1.2 Spectral Properties

Biotin has low absorbance at 200-220 nm, with its maximum absorption at 204 nm from its carbonyl group (37). Therefore, it is a challenge to analyze biotin using a LC/UV method. The spectrum of biotin is shown in Figure 1.8.

1.5.2 Stability

Dry, crystal biotin has a good stability in air and to heating, but it is usually

destroyed by UV light. Biotin is stable in a solution with pH value between 4.0-9.0 under temperature up to 100℃. It is also stable in frozen food and/or to autoclaving without any loss of its activity. However, biotin will lose its stability when heated in more acidic and alkali solutions. The sulfur atom in biotin is readily oxidized to isomeric *d*- and *l*-biotin sulfoxides and its stronger oxidant form biotin sulfone, resulting in loss of its biological activity. Although humans have the ability to reduce *d*-biotin sulfoxides back to *d*-biotin, such capability is limited and not enough. The sequential sulfur oxidation can degrade biotin, but the exact reasons for loss of biotin during the food processing and storage have not been well elucidated. Moreover, biotin has a good stability during soaking and cooking without loss due to leaching. Furthermore, the protein bound form of biotin in vegetables is less sensitive in the cooking medium, which is different from other water-soluble vitamins that are easily lost during cooking, processing, and long-term storage (30,36,37). Biotin is commonly extracted with an aid of strong acid hydrolysis with autoclaving to liberate it from its bound form or with a proteolytic enzyme from biological samples. Some food processing, such as canning and heating, can cause the loss of biotin in food, but losses can be reduced with addition of antioxidants, such as vitamin C, vitamin E, and butylated hydroxytoluene (BHT), etc (1,30).

1.5.3 Bioavailability

Bioavailability of biotin mainly depends on some enzymes in the gastrointestinal tract, for example, proteases and peptidases that convert protein-bound biotin to biocytin; then, biotinidase releases biotin for further metabolism (30,42). On the contrary, biotin-binding proteins, for example, avidin,

which exists in raw egg whites, decrease biotin's bioavailability. Traditionally biotin bioavailability was measured by estimating biotin from urine, but this assay underestimated the value because the measurement did not include the biotin metabolites. Later, LC analysis combined with avidin-binding method was developed, which is a more precise and specific measurement of bioavailability for biotin and its metabolites (30,37).

1.6 Folate

In 1931, folate deficiency was discovered in India, where there is a high prevalence of anemia in women. In 1938, this anti-anemia factor was called vitamin M, and later called folic acid that was derived from a Latin word "folium", which means leaf (37) because the chemical was separated from spinach. Folic acid is a water-soluble B vitamin and occurs naturally in food, but it is not suitable for fortified foods or supplements. The derived form of folate is folic acid that can be added in supplements and foods due to its increased stability compared to its natural form (36,44)

Pteroyl-L-glutamic acid (folic acid) is the parent compound of folate, which can be reduced to tetrahydrofolate (THF) that usually attaches up to 12 molecules of glutamic acids as polyglutamate. Dietary folate could be converted to the THF form during absorption in small intestinal to portal blood, then to tissues for utilization. Folate is a component of many enzymes and involves in many metabolic pathways for transferring "one-carbon" units. "One-carbon" moieties, such as formyl (-CHO), active carboxyl (-COOH) or hydroxymethyl ($-CH_2OH$), are attached on the positions of N_5 and N_{10} of the folic acid, or linked across both positions to yield a lot of

biologically active metabolites of folic acid. These transferring reactions occur in amino acid and nucleotide metabolisms, including homocysteine interconversion to methionine, transfer RNA methylation, and *de novo* purine and pyrimidine synthesis (33,36).

The folate deficiency can be caused by poor absorption of folate from daily diet or increasing consumption of folic acid by the body (45). This deficiency will cause serious problems such as failure of DNA replication which leads to impaired cell division. An example is the hemopoietic tissues in bone marrow that under deficient conditions result in enlarged and fragile erythrocytes (pemicious anemia) that occurs especially in pregnant women. Another example is the epithelial cells of the gastrointestinal tract cause gastrointestinal disturbances and result in an altered nutrition status. With unrepaired and mis-repaired DNA caused by folate deficiency, the wrong cell division can cause mutations and may result in cancer. Additionally, folate deficiency leads to elevated homocysteine levels due to lack of methylation of homocysteine to methionine which increases the risk of heart disease. However, taking 200 μg of daily folate could reduce deaths associated with coronary artery diseases (1,30,36,45). The most important discovery about folate relevant to the public health is that it can prevent neural tube defects for infants (30,36). Hence, in 1998, the United States government required food manufactures to supplement grain products with folic acid at 140 μg/100g in bread products, flour, cornmeal, rice, and noodles. This is due to the fact in North America, more than half of pregnancies have folate deficiency which can cause adverse problem to embryonic brain development for infants (30,31). Folate has–also been suggested as a treatment/preventative Alzheimer's disease (27).

Folate also plays an important role in B_{12} deficiency, because B_{12} induced anemia can be alleviated by treating with folic acid; however, if folic acid supplements are taken without B_{12}, an irreversible neurological damage may occur. Therefore, folic acid is always co-supplemented with B_{12} in prophylactic vitamin supplementation (30).

Liver is the richest source of folate in the diet while other rich sources include wheat bran, egg yolk, some cheeses and green leafy vegetables (33).

1.6.1 Pharmacological Uses of Folic acid

High doses of folate (400 μg/day) can efficiently treat patients who have macrocytic anemia caused by the folate deficiency. Folate (<1 mg) also can be used to reduce elevated homocysteine and normalize plasma homocysteine levels. In addition, folate treatment with vitamin B_{12} can fortify its effectiveness to lower elevated homocysteine. Also, high doses of 5-methylfolate (15 mg/day) have been shown to benefit acute psychiatric disorders. The U.S. Public Health Service (PHS) recommends that childbearing women to take 400 μg/day of folate from daily foods or supplements. The FDA has recommended that pregnant women should take 4 mg/day of folate to prevent potential neural tube defects in infants (1,45).

1.6.2 Chemical, Physical and Spectral Properties

Folate, with its structure drawn in Figure 1.9, is the term referring to all of derivatives of pteroic acid that exhibit vitamin activity in the body. The vitamers differ in many ways, including oxidized forms of the molecule, the length of the glutamate side chain (which usually prefers conjugation with one or more glutamic acid molecules), and the one carbon units attached on specific positions (30,37,39).

The naturally occurring folate in foods presents as tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MeTHF), and 10-formyltetrahydrofolate (33,46). The primary form in fresh foods of polyglutamates is 5-MeTHF. Under storage conditions, it will slowly degrade to monoglutamates and be oxidized to less available folate. Folic acid ($C_{19}H_{19}N_7O_6$, MW = 441.4) is a synthetic compound, its glutamate group is an essential part of the biological activity (33) (shown in Figure 1.10). Folate is sensitive to heat, acid, oxygen, and light, but folic acid is stable. Therefore, folic acid is added in fortified foods and used for supplementation of commercial products and in pharmaceutical formulations (36,37). Nevertheless, folic acid must to be reduced to dihydrofolate or THF for biological use. The USP Reference Standard is folic acid (37).

1.6.2.1 Chemical and Physical Properties

Folates with vitamin activity usually contain linkage of one or more glutamates, which is a requirement for its biological activity (33). Folic acid is synthesized for commercial use for food fortification, and it may be chemically modified to contain one glutamate group, or made available as its disodium salt (30,33). Synthetic folic acid is an orange-yellow microcrystalline compound. It is nearly odorless and tasteless. Its melting point (Mp) is at 250℃ in dark. Commercially used folic acid has 8.0 to 8.5% water by weight, but can be removed at 140℃ under vacuum with hygroscopic products. The free form of folic acid is insoluble in cold water, but slightly soluble in boiling water (20 mg/100 mL), whereas, the salt form is quite soluble (1.5 g/100 mL at 0℃). It is also slightly soluble in methanol and less soluble in ethanol. It is insoluble in acetone, diethyl ether,

chloroform, and benzene. Folic acid can be dissolved in warm hydrochloric acid, but its degradation generally increases along with the elevated temperature or strengthened acidity; By contrast, folic acid is soluble and stable in alkaline solution (30).

Folate has ionogenic and amphoteric characteristics. Its ionization depends on the pH value of solution and the pKa of ionogenic groups. The pKa of N-5 positions of THF is 4.8, while the γ-pKa and α-pKa of glutamate carboxyl groups are 4.8 and 3.5, respectively. Polyglutamyl folate presents greater ionic character than monoglutamyl folate, due to the carboxyl group of glutamates (30).

1.6.2.2 Spectral Properties

According to its UV absorption spectra, folate has three absorbance maxima in 256, 283, and 368 nm. The UV absorption spectrum of folic acid is given in Figure 1.11. One of its absorbance maxima is attributed to its *p*-aminobenzoyl-glutamic acid (PABG) moiety that has an absorption around 270-280 nm. PABG moiety also contributes the fluorescent property of folate, of which the intensity increases when pH value of solution goes down. For LC methods, fluorescence is considered to be the most specific and most sensitive detection for determination of folate (37). At pH value of 2.3, THF, 5-MeTHF, and 5-formyl-THF can increase limit of detection level to *p*mol level. However, dihydrofolate, 10-formyl-THF and folic acid are not sufficiently for quantitation, because folic acid and the reduced pterins without the *p*-aminobenzoyl moiety do not exhibit fluorescence (37).

1.6.3 Stability

Each of the folate vitamers has different degree of stability under different

conditions in terms of temperature, pH value of solution, oxygen and light, but it is uncertain of the effect of the length of the glutamate chain on the stability of folate (30,33,37).

Commercially, folic acid is preferred to be used due to its better stability compared to dietary folate. It is moderately stable to atmospheric oxygen. Loss of vitamin activity is attributed to the C-9-N-10 bond by oxidative cleavage (37). Folic acid is stable in solutions around pH 7; however, it becomes unstable when the pH value increases over pH 7 or decreases lower than pH 5. Its main oxidation product is *p*-aminobenzoylglutamate (PABG). On the other hand, folic acid is readily to be cleaved into PABG and 6-formyl pterin under ultraviolet radiation (UV). The latter can then be degraded into pterin-6-carboxylic acid. Although folic acid is stable at 100℃ even after 10 hours, its stability can be seriously affected by sunlight, so that it should be protected from sunlight. Moreover, the oxidative cleavage could be acceralated in the presence of riboflavin. In order to prevent oxidation of folate and folic acid during food processing and storage, reducing agents, such as ascorbic acid, 2-mercaptoethanol and dithiothrietol, are used for purpose of stabilization and delay of oxidation. However, such oxidation will not occur in dry products (30,33,37).

1.6.4 Bioavailability

The bioavailability of folate in foods is incomplete and varies; making it difficult to be quantified (1,30). Many factors can affect the vitamin bioavailability, including: (1) extrinsic factors: chemical form and physical form of the vitamin, concentration of the vitamin, food matrix, and antagonists; as well as (2) intrinsic factors: age of individual, animal species, and health of small intestinal (1,37,39).

Folic acid has 50% more bioavailability than folate (1,45). The reason is that folic acid has a higher degree of resistance to thermal processing and degradation. By contrast, the folate found in foods (mainly in the THF form) has 75% degradation under cooking conditions, which were determined by the microbiological assays and HPLC analysis (30,37,45).

1.7 The B-Groups Vitamins Analyses

The conventional analytical methods usually adopt the microbiological assays (MBA) for the measurement of B-groups vitamins (47,48,49), like the riboflavin analysis in 1939. However, the MBA has many disadvantages, such as the requirement of laboratory facilities, microbiologically-trained, and in particularly, such assays are quite time-consuming (6,33). Despite those drawbacks, the MBA method is suitable for determining a particular vitamin in a wide range of concentrations. Because of the many shortcomings of the MBA, many methods have been developed, such as the HPLC, for determination of water-soluble. HPLC provides many advantages as opposed to the MBA methods. HPLC offers high selectivity of individual vitamers, while the MBA shows response to all vitamers at the same time, and usually overestimates the amount of vitamins in foods (33). Moreover, the obtained analytical values of HPLC are truly closer to the real bioavailable concentrations of vitamins in foods compared to the microbiological value (34). Despite these advantages, HPLC has some shortcomings in terms of the sensitivity of detectors, chromatographic resolution, etc. For example, the chromatographic separation is easily disturbed by interfering compounds so that a sample pre-treatment or clean-up is usually required to remove the interferences (33).

On the other hand, with an increase in health consciousness, more and more nutraceutical products containing vitamins are sold in supermarkets; therefore, rapid, robust, sensitive and high through-put methods need to be developed to fit the analytical requirement from the nutraceutical industry.

1.7.1 Extraction Methods for Water-soluble Vitamins from Supplements

Analytical techniques require sample pretreatment for extraction of vitamins from a complex food matrix and/or multivitamin supplement in order to improve their precision and accuracy. An appropriate extraction method should take into account of many factors, such as the nature of the food matrix, the forms of the vitamins in foods and supplements (particularly bond forms), amount and type of potential interfering compounds, stabilities of the vitamins (such as thermal sensitivity, pH value, light, and oxygen), and the selectivity and specificity of the analytical methods employed (37). In addition to their physical and chemical properties, the vitamin concentrations present in food matrices or in multivitamin supplements are also a challenge for accurate quantification because many vitamins exist at such low levels that samples must be concentrated prior to analysis. Additionally, and equally important, is that many vitamins exist in a wide range of concentrations in their matrix. As an example, the amount of NAm in a multivitamin drink is four hundred times more than folic acid which is present a level of only 12.5 μg. Due to the wide range in concentrations of vitamins from microgram to milligram levels, it is hard to devise a one-step procedure to allow the total extraction all vitamins of interest. Additionally, multiple extraction steps using differing pH solutions can cause vitamin loss or degradation because vitamins have different stabilities at different pH values. Other ingredients of the food

or supplement matrix, such as sugar or citric acid, may also affect the vitamin analysis. Hence, a clean-up step using solid phase extraction (SPE) is often adopted to remove interferences and to concentrate the analyte simultaneously (10,17,34,50).

1.7.1 Vitamin Interaction

Fortified and supplemented products are mixed with several single vitamins, resulting in some possibilities of mutual interactions of vitamins, and/or interactions with other compounds present in the matrix. Thus, it perhaps reduces stability and shelf-life of the products. For instance, many studies have been published about vitamin mutual interactions, which indicated that combination of ascorbic acid (vitamin C) with four vitamins, i.e., thiamin, riboflavin, folic acid and cyanocobalamin (B_{12}), caused a deleterious effect. Therefore, the interactions between vitamins and their matrices need to be considered as a factor which may affect the stability of vitamins during extraction procedures (33).

1.7.1.1 Folic acid-Ascorbic acid, Thiamin, or Riboflavin

If a solution contains both folic acid and ascorbic acid, the former would undergo cleavage because of the reducing effect of the latter. The most rapid breakdown of folic acid occurs when the solution pH range is between 3.0 to 3.3, and the slowest at pH 6.5 to 6.7 (33).

The degradation of thiamin significantly affects the stability of folic acid, especially at pH 5.9 to 7.0, because the degradation product causes a rapid rate of breakdown of folic acid (33).

The stability of folic acid also is influenced by riboflavin and light in particular at pH 6.5, because of light induced riboflavin free radical oxidation

reactions which lead to cleavage of folic acid. This reaction can be reduced, but not be eliminated by de-aeration (33).

1.7.1.2 Other interactions

In addition to the above interactions which decrease stability, there are interactions which can increase the solubility of some vitamins that have less solubility in aqueous solutions. For example, NAm acts as a solubilizer for folic acid and riboflavin (33).

1.7.2 Solid Phase Extraction (SPE)

Solid phase extraction (SPE) is a useful extraction technique based on separation of target analytes from complicated matrices through different affinities for two phases, one of which is the solid stationary phase (3,10,51). The second phase (mobile phase) usually is liquid, but it can be a gas, a supercritical fluid or an emulsion. When comparing liquid-liquid extraction (LLE) and SPE, SPE is generally more efficient, more accurate, and more precise than LLE. It also saves time and solvent (52). SPE is very selective and excellent for sample extraction, concentration, clean-up and solvent transfer. It is mostly used to prepare liquid samples, containing semivolatiles or non-volatile analytes (10,53,54). There are two different SPE strategies, including: (1) retention-cleanup-elution strategy, by which analytes are at first adsorbed in sorbent. After washing, the analytes are eluted with an appropriate solvent. (2) Pass-through cleanup strategy: by which analytes pass through sorbent, and interferences are retained in sorbent. Usually, samples are started to be collected from the sample loading step.

The protocol of SPE operation include: (1) sample pretreatment, (2)

conditioning and equilibration, (3) sample loading, (4) washing, and (5) elution. The sample pretreatment step should consider pH adjustment, buffer addition, centrifugation, dilution, or filtration, which is based on sample matrix and compounds of interest. The conditioning step is in order to wet and activate ligands to make sure there is a constant interaction between analytes and functional groups on sorbent. The next step is the equilibration that is for maximizing retention time of compounds of interest or interference. Significantly purified and cleaned-up sample matrix is achieved by selection of an appropriate solvent and careful control of the flow rate of 1~2 drops per second or 1 mL per minute. A proper washing solvent needs to be strong enough to wash out interfering compounds but weak enough to prevent desorption of compounds of interest. Furthermore, pH adjustment of elution solvent may improve recovery due to ionization of moieties in the compounds of interest which enhances their polarity thus weakening their interaction with a reverse phase sorbent (55-57).

SPE provides accuracy and precision which can be achieved by selecting an appropriate type of stationary phase. SPE products have three different types of solid phase, i.e: reverse phase bed (RP), normal phase bed (NP), and ion exchange bed that includes cation and anion exchange phases (30).

Reverse phase packings are hydrophobic, commonly constructed by silica-based materials with a non-polar stationary phase which involves in the separation of a polar or moderate polar sample matrix. Hydrophobic alkyl- and aryl-functional groups on the silica surface are classified in reverse-phase category. Retention of organic analytes from a polar solution are based on Van der Waals forces or dispersion forces between non-polar and non-polar by the attractive forces of

carbon-hydrogen bonds in the analytes and functional group on the silica surface. To elute compounds that are adsorbed on SPE, different polarity of elution solutions are used, in order to disturb the interaction and desorb target compounds from the stationary phase (58).

Ion exchange SPE can be used for the compounds which are charged when in an aqueous solution. The major retention mechanism is based on electrostatic attraction between charged functional groups on the analyte compounds and oppositely charged functional groups on the sorbent. In order to retain compounds on the sorbent, pH is the major factor to alter the compounds being carried positive or negative charged. When functional groups on the compound of interest or on the surface of the sorbent are neutralized, the electrostatic force between the two function groups is disrupted and the compound of interest is eluted. Alternatively, an eluent that has high ionic strength or contains ionic species can displace the adsorbed compound of interest on the sorbent and allow it to be eluted. Ion exchange can be categorized into two types of phases: anion exchanger and cation exchanger. According to ionization properties of acidic and basic functional groups, ion exchange resins can be classified as strong and weak exchanger. Functional groups are categorized depending on the degree of ionization of the functional group, as well as the pKa value of functional groups. The pH value in solution is directly influential to the ion exchange capacity. The maximum exchange capacity is achieved when all functional groups are ionized. Usually, the strong anion and cation exchanger sorbents have a wide range of pH; on the other hand, the weak exchanger has a limited usable pH range (30,59).

The two pH units rule means a solution that is used for elution should have 2

pH units above or below the pKa, which is based on the target analytes. Generally, buffer solutions provide flexible optimization (53). Table 1.2 summarizes the common buffers for the HPLC, and their pKa values, useful pH range and UV cutoffs. The definition of pKa of a molecular functional group is at a pH value, when 50% of groups in solution are charged and 50% unchanged. Change of a pH unit will affect the percentage of the compounds that carry positive or negative charges, being charged or uncharged by a factor 10. Hence, it is reasonable to choose a solution with pH of at least 2 units lower or higher than the pKa of analytes, in order to keep 99.5% of the functional group in a desired state of ionization. There are two ways to elute analytes of interest. For the weak anion acid, it can be successful by adjusting the pH value to be 2.5 or less units than the pKa, to neutralize the charge of the analytes. Another method is to prepare a high ionic strength buffer, in which the analyte is removed from the sorbent by competing with the other anion. The selectivity of common counter ions is as follows with order that the ion on the right can displace the ion on the left (59,60).

OH^- < acetate < formate < HCO_3^- < Cl^- < HSO_3^- < CN^- < citrate < benzene sulfonate

1.7.4 HPLC Analysis

High performance liquid chromatography (HPLC) is an efficient and accurate analytical technology with a variety of applications including analysis of pharmaceuticals, biomolecules, polymers, organic and ionic compounds. The advantage of HPLC relies on its high sensitivity provided by diverse detectors that have broad limits of detection ranging to nanogram (μg), picogram (pg), and even femtogram (fg) levels, and the high mass transfer , high theoretical plates, small

particle size stationary phases that allow excellent separation of compounds exhibiting very similar partition coefficients. HPLC is considered nearly universal in its applications as greater than 80% of known compounds can be determined with HPLC compared to only 15 % for GC. There are four major separation modes of HPLC column: normal-phase chromatography (NPC), reverse-phase chromatography (RPC), ion-exchange chromatography (IEC), and size-exclusion chromatography (SEC). Currently, the technology of designing stationary phases has been improved to devise other separation modes, such as hydrophilic interaction chromatography (HILIC), supercritical fluid chromatography (SFC), and affinity chromatography (38). In my research, reverse-phase chromatography was used as the separation mode, because reverse phase chromatography is the most commonly used mode, accounting for 70% of all HPLC analyses. The mechanism of separation is based on compounds' partitioning between a polar mobile and a non-polar stationary phase. Commonly, the stationary phase is comprised of solid particles coated with hydrophobic C18 (octadecyl) group bonded on silica support. The general polar mobile phase for RPC is methanol or acetonitrile with or without water, and with or without modifiers like trifluoroacetic acid, and the primary forces effecting partitioning and ultimately elution are attributed to solvophobic and hydrophobic interactions. RPC is applicable for analysis of polar, moderately polar, and some non-polar analytes. Also, ionic analytes can be separated by using ion-suppression or ion-pairing techniques with RP (4,38).

Regarding the fact that nicotinic acid, pantothenic acid, folic acid and biotin are ionizable analytes with carboxyl groups, the pH of the mobile phase can dramatically affect the retention of ionizable analytes, and impact selectivity and peak

shape (4). The ionized analytes are not partitioned on the non-polar stationary phase but prefer the aqueous mobile phase, so they are not retained, and elute from the column (38,53). Therefore, it is important to know the pKa of the analytes and keep them from becoming ionized. In RPLC, the acidic pH of 2.5-3.0 is utilized for many applications because choosing lower pH for acidic analytes can suppress the ionization and lead to higher retention time. But it is worthy of mention that most silica-based bonded phases are unstable at pH below 2.0 because the bonded group can be hydrolyzed by acid (5,38). Typically, phosphoric acid, heptafluorobutyric acid, trifluoroacetic acid (TFA), formic acid, and acetic acid are used to improve chemical separation (7,38).

The mobile phase interacts with both the solute and stationary phase. Then, it elutes components from the column, so it strongly affects the retention time and separation. Ideally, a mobile phase should have the following characteristics (38):

- High solubility for the sample
- High purity, UV transparency
- Non corrosive to the HPLC system
- Low to moderate toxicity, non-flammability, low viscosity

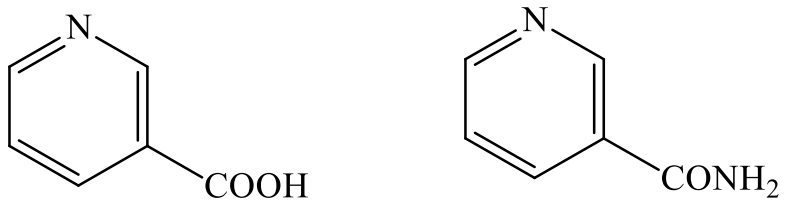
In addition to meeting the general requirements, there are some possible special limitations to different detectors; therefore, selecting a suitable mobile phase is very important. For example, the phosphate buffer commonly used as aqueous mobile phase in UV detectors is forbidden in MS detector due to phosphate precipitation and crystallization. After nebulizing and ionizing the mobile phase and analytes; the phosphate will precipitate in detector to cause significant interference and possible damage to the detector. Also, phosphate buffer may cause high concentrations of

inorganic salts to accumulate in the column thus disturbing the reproducibility of retention times for some vitamins and making the column equilibration longer and increasing total run time (13,21,24,38). Although UV detectors are commonly used for analysis of many compounds, it is not suitable for analysis of some vitamins, such as pantothenic acid and biotin that lack chromophores. In that case, MS or ELSD are more appropriate for simultaneously detection and quantification of all water-soluble vitamins (9,28,61).

1.8 Summary

The aim of my research is to develop optimized procedures and (an) efficient and accurate HPLC method(s) for multivitamin analysis, which will be able to: (1) obtain high recovery of analytes by conducting an optimized extraction process, particularly for biotin, pantothenic acid, folic acid and niacin in daily multivitamin supplements and soft drinks (Chapter 2); (2) develop a time-saving analysis for a simultaneous determination of water-soluble vitamins (Chapter 3). The method will be evaluated in terms of the analytical accuracy, precision, detective linearity, and reproducibility. Other parameters of the methods including limit of detection (LOD), and limit of quantification (LOQ) will also be estimated in regards of the following detectors, including DAD, ELDS and MS (38,62,63) (3).

1.9 Figures and Tables



(a) (b)

Figure 1.1 Structures of Niacin Vitamers: (a) Nicotinic acid and (b) Nicotinamide

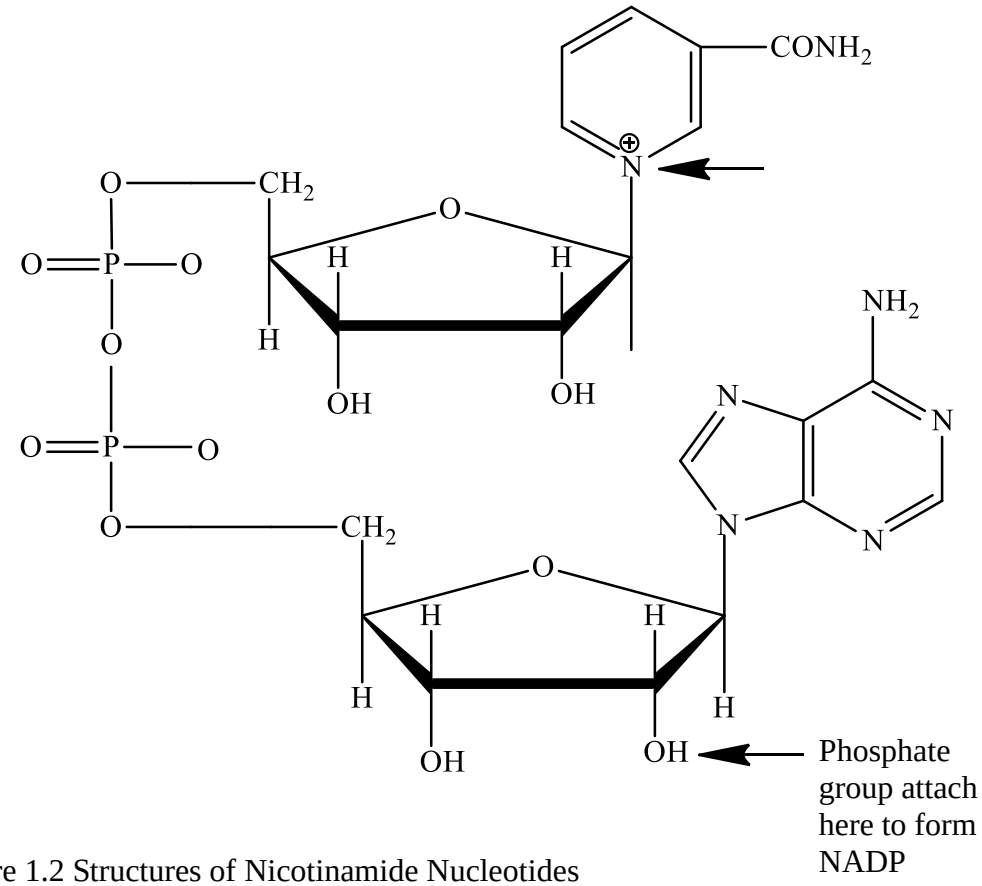


Figure 1.2 Structures of Nicotinamide Nucleotides

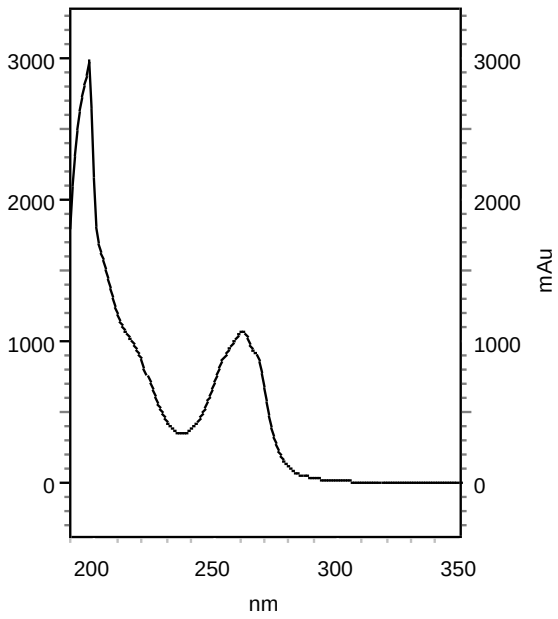


Figure 1.3 UV Absorption Spectra of Nicotinic Acid Standard

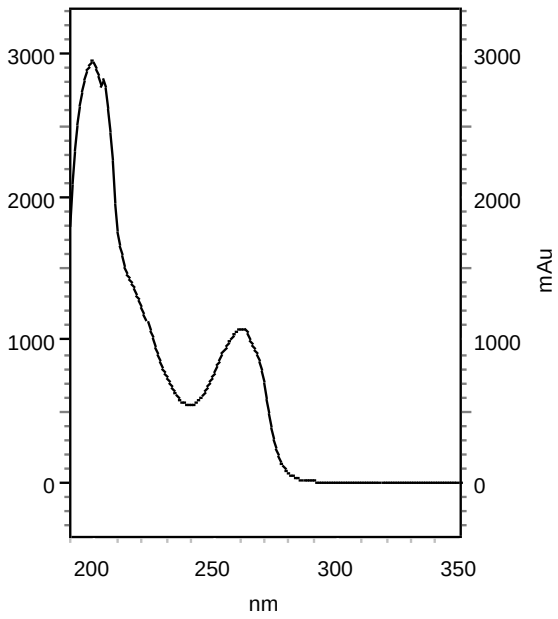


Figure 1.4 UV Absorption Spectra of Nicotinamide Standard

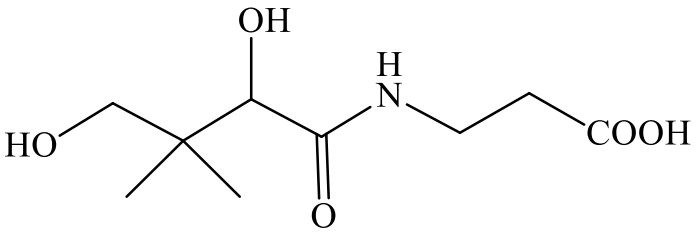


Figure 1.5 Structure of Pantothenic Acid

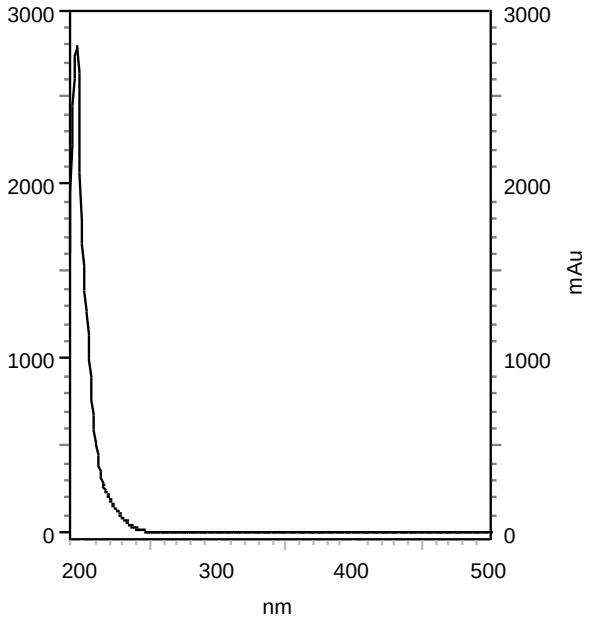


Figure 1.6 UV Absorption Spectra of Pantothenic Acid Standard

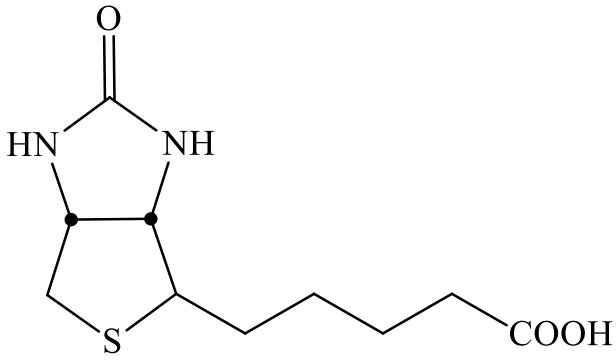


Figure 1.7 Structure of Biotin

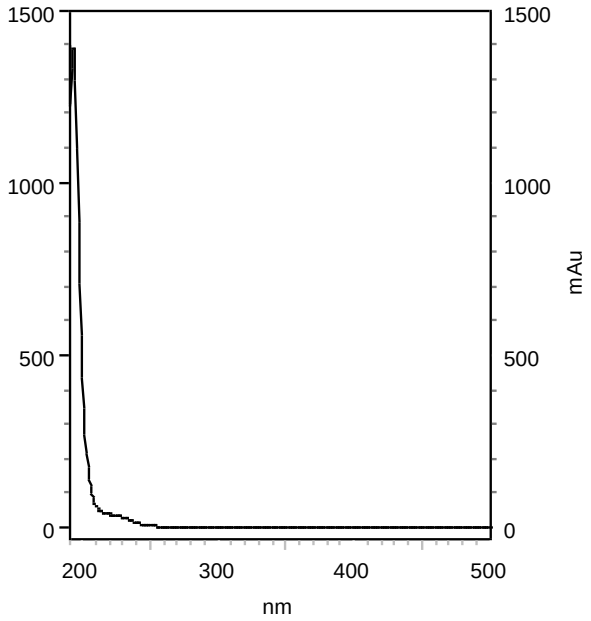
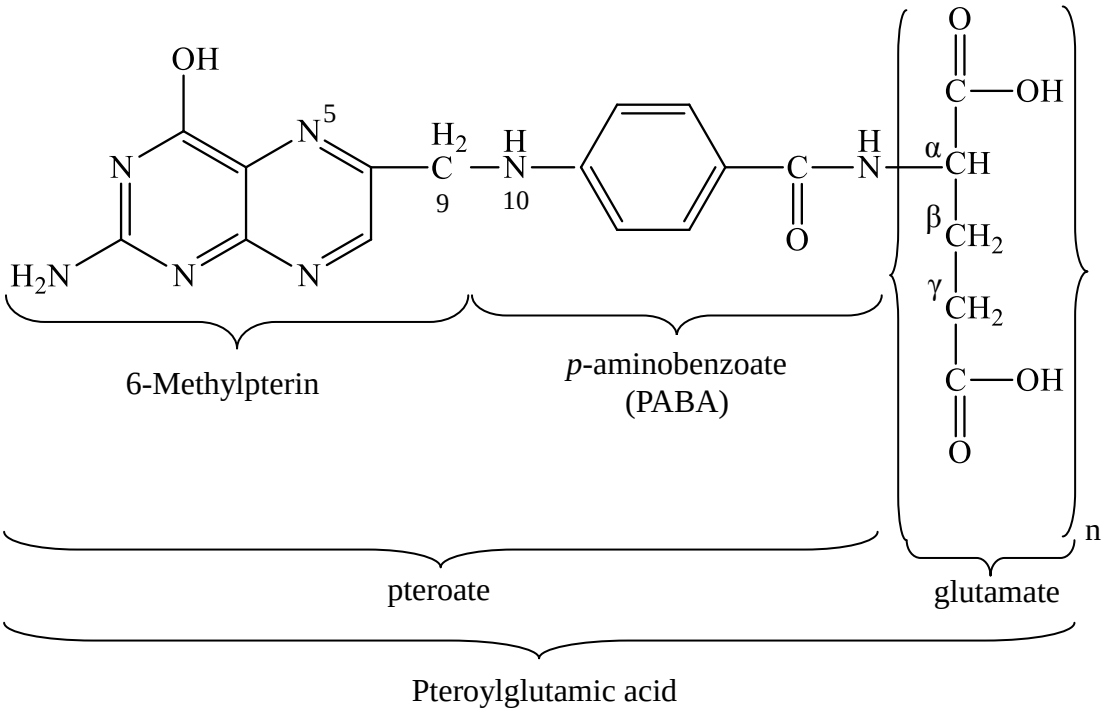


Figure 1.8 UV Absorption Spectra of Biotin Standard



*n: number of glutamate

Figure 1.9 Structure of Folate

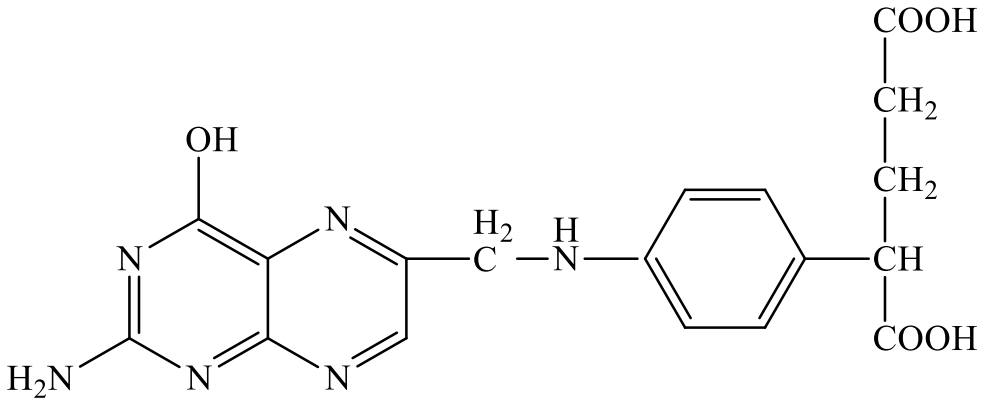


Figure 1.10 Structure of Folic Acid

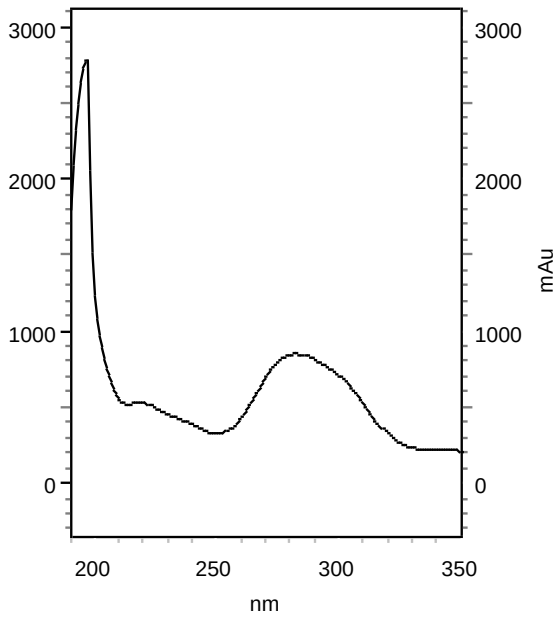


Figure 1.11 UV Absorption Spectra of Folic Acid Standard

Table 1.1 The Vitamins: Their Vitamers, Recommended Dietary Allowance (RDA), Function, and Deficiency Disease (1,24,31,32)

Vitamin	Vitamers	Function	RDA	Deficiency Disease
B_1	Thiamin	As coenzyme thiamin pyrophosphate (TTP) in energy metabolism	Female: 1.1 mg/day Male: 1.2 mg/day	Beriberi; Wernicke-Korsakoff syndrome
B_2	Riboflavin	Part of coenzymes FAD and FMN in energy metabolism	Female: 1.1 mg/day Male: 1.3 mg/day	Ariboflavinosis
Niacin	Nicotinic acid Nicotinamide	As coenzyme, NAD and NADP, release energy from carbohydrate, fatty acid and amino acid reactions.	Female: 14 mg NE*/day Male: 16 mg NE*/day	Pellagra
B_6	Pyridoxol Pyridoxal Pyridoxamine	As coenzyme in amino acid and fatty acid metabolism	Adults (19-50 yr): 1.3 mg/day	-
B_7	Biotin	As a coenzyme for numerous enzyme and involves in carboxylation	-	Rare
B_5	Pantothenic acid	As the part of coenzyme A and involve	10-100mg/day	Rare

		in numerous reactions.		
B_9	Folic acid Polyglutamyl folacins	Help DNA synthesis	Adults: 400 μg/day	Pregnant: neuron tube defects infant
B_{12}	Cobalamin	As part of enzyme in new cell synthesis; to maintain nerve cell; and break down some fatty acid and amino acid	Adults: 2.4 μg/day	Pernicious anemia
C	Ascorbic acid Dehydraoascorbic acid	As cofactor in collagen formation; as antioxidant	Female: 75 mg/day Male: 90 mg/day Smoker: +35 mg/day	Scurvy

*1NE equals 1mg of niacin and 60mg of dietary tryptophan.

Table 1.2 The Common Buffers for The HPLC and pKa Respectively, Useful pH Range and UV Cutoffs (38,53)

Buffer	pKa	Useful pH range	UV cutoff (nm)
TFA*	0.3	< 1.5	210
Phosphate	2.1, 7.2, 12.3	1.1-3.1, 6.2-8.2, 11.3-13.3	190
Citrate	3.1, 4.7, 5.4	2.1-4.1, 3.7-5.7, 4.4-6.4	225
Formate*	3.8	2.8-4.8	200
Acetate*	4.8	3.8-5.8	205
Carbonate*	6.4, 10.3	5.4-8.4, 9.3-11.3	200
Ammonia*	9.2	8.2-10.2	200
Borate	9.2	8.2-10.2	190
Diethylamine	10.5	9.5-11.5	235

* Volatile buffer systems, which are MS-compatible

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CHAPTER TWO

USE OF SOLID PHASE EXTRACTION FOR SAMPLE CLEAN-UP AND PRECONCENTRATION OF VITAMIN B_3, B_5, B_7 AND B_9 IN MULTIVITAMIN TABLETS

Abstract

Two types of solid phase extraction (SPE) based on reverse phase (C18) and strong anion-exchange materials are optimized for sample clean-up and preconcentration of four vitamins, i.e., nicotinamide (B_3), pantothenic acid (B_5), biotin (B_7) and folic acid (B_9), in multivitamin tablets. Sample concentrations of vitamins were determined by HPLC using gradient elution program with a ZORBAX Eclipse XDB-C18 (250mm x 4.6 mm, 5 μm) analytical column and a guard column (12.5mm x 4.6mm, 5 μm particle size). Mobile phases included 0.1% formic acid in water (A) and methanol (B). Detection was performed by evaporative light scattering (ELSD), diode array detection (DAD), and positive mode of ESI/MS. Investigated parameters of the analytical performance included accuracy, repeatability and recovery of the analyses. The repeatability of the vitamin extraction was measured by relative standard deviations (RSD), which were from 0.03% to 0.24% for the SPE C18 and from 0.07 % to 0.27% for the strong anion-exchange SPE. The recovery of vitamins extracted by SPE C18 and strong anion-exchange SPE were from 92.8% to 101.3% and from 91.5% to 109.1%, respectively. Linear regression analyses showed that the square of the correlation (R^2) were 0.9913, 0.9971, 0.9949 and 0.992 for B_3, B_5, B_7 and B_9, respectively.

2.1 Introduction

In the progress of quantitative analysis of food matrices, natural products and multivitamin/multimineral tablets, sample preparation is one of the most time-consuming and costly steps, which generally requires 61% of time to perform in a chemical analysis (1-3). The main purpose of extraction process is to provide a rapid and efficient way to isolate desired compounds from a complex matrix by using the minimum amount of solvents (4), and pre-concentration of the target components before their HPLC analyses, particularly in regards of some vitamins, such folic acid (B_9), biotin (B_7) and cyanocobalamin (B_{12}) (5-7), which are usually in low concentrations in samples (8). Solid phase extraction (SPE) is a very popular extraction technique that is typically applied to analyses of foods, environmental contaminants, and biological fluids. SPE is comprised of two phases: a solid phase and a liquid phase (9-12). The former is composed of small spherical particles with porous cavities that produce a large surface area able to interact with analytes and isolate analyte from liquid sample solutions (2,11,13,14). The latter is (a) solvent(s) depending on the used materials of the former. In recent years, SPE has been used successfully for rapid sample pre-concentration and purification (15). Also, it allows carrying out dissolution exchanging, trace enrichment, desalting, derivatization and class fractionation (1,2,11,16). SPE also provides the benefit of reducing solvent usage and exposure, disposal costs and extraction time for preliminary treatment of samples (7).

An appropriate selection of the SPE sorbent is based on interaction between the sorbent and compounds of interest, according to hydrophobic, polar and ionogenic properties of both. Generally, the mechanisms of retention are dependent on Van der

Waals forces (non-polar interaction), hydrogen bonding, dipole-dipole forces (polar interaction) and cation-anion interaction (ionic interactions) (11). Chemically, silica bonded C18 or C8 alkyl chains are commonly used materials for reverse phase extraction sorbents, and may also be used for extraction of non-polar and small polar analytes (9,11). Most polar solid phase is silica bonded with $–SO_3^-$ and $–N^+(CH_3)_3$ ionic function groups, which is suitable for extraction of acidic and basic compounds from aqueous solution (11).

Although many SPE methods have been published (11), few papers are published about using strong anion exchanges (SAX) SPE for vitamins sample enrichment. Also, most of C18 SPE methods for vitamins extraction are used for sample clean-up and always focused on single vitamin instead of multivitamins analysis. In this chapter, a C18 SPE and a strong anion exchange SPE method were developed for analyte extraction of multivitamin tablets. Appropriate conditions for SPE (Sep-Pak C18 and Oasis MAX) and HPLC, percentage of organic modifier, pH and strength of mobile phase, loading volume, and volume of elution were determined in order to enhance and increase recovery (17).

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

The nicotinamide (B_5), D (+)-biotin (B_7) and formic acid were purchased from Acros organics (New Jersey, NY, USA). Folic acid crystalline pteroylglutamic acid (B_9) and D-pantothenic acid calcium salt (B_5) were from ICN nutritional biochemicals (Cleveland, OH, USA). Ammonium acetate was obtained from Sigma-Aldrich chemical Co (St. Louis, MO, USA). Ammonium hydroxide was purchased from

Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). HPLC grade methanol, ammonium formate, acetic acid and sodium hydroxide, and 0.2 μm nylon syringe filters in size of 13 mm were purchased from Fisher Scientific (Suwanee, GA, USA). Water was pre-purified using a Millipore Synergy UV system (Billerica, MA, USA). The Sep-Pak C18 and Oasis MAX were obtained from Waters Corporation (Milford, MA, USA).

The multivitamin supplements Kirkland signature premium performance multivitamin (Costco Wholesale Corp., Seattle, WA, USA) was purchased from Costco in Greenville, SC.

2.2.2 HPLC /Mass Spectrometry (MS) Condition

The chromatographic measurements were carried out with HPLC system equipped with an Agilent 1200 series system (Agilent Technologies, Inc., Loveland, CO, USA), which includes a degasser (G1379B), a binary pump (G1312A), a standard autosampler (G1329A), a thermostatted column compartment (G1316A) and a variable wavelength detector (model G1314B) tandemed with a mass spectrometer (6110 series) that has an electrospray ionization (ESI) probe and single quadrupole analyzer. ChemStation software (Rev. B.03.02) controlled the LC system. Vitamin B_3, B_5, B_7, and B_9 were detected in positive mode ESI. Other detector parameters, such as capillary voltage , fragmentor, drying gas flow rate (N_2), temperature and nebulizer pressure, were set at 3.2k V, 80V, 10 L/min, 350℃ and 40 psi, respectively. The characterized ions of respective vitamins were as follows: B_3, 123.1; B_5, 220.1; B_7, 245.1; B_9, 442.1.

2.2.3 HPLC-ELSD/DAD Conditions

The HPLC system (Shimadzu Scientific Instruments Inc., MD, USA) was equipped with a column oven with a manual sampler (CTO-20A), a pump (LC-20AT), a degasser (DGU-20A_5) and a diode array detector (SPD-M20A). The system also was connected an ELSD detector (ELSD-LT II). Two wavelengths were chosen at 209 nm B_5 and B_7, and 270 nm for B_3 and B_9, while spectrum in the region 200-600 nm were also recorded. The ELSD conditions for the analytical method were set to:
Gain =11, Nebulizer Temperature = 40 ℃ and Nebulizer Pressure = 350 KPa.

2.2.4 LC Conditions

A reversed-phase column ZORBAX Eclipse XDB-C18 (250mm x 4.6mm, 5 μm particle size) and a guard column (12.5mm x 4.6mm, 5μm particle size, Agilent Technologies, Inc., Loveland, CO, USA) were used for sample separation under gradient elution. 0.1% formic acid in distilled water (pH 2.76) Solvent A, and methanol Solvent B were the mobile phases used for gradient elution. The gradient program is listed in Table 2.1. The flow rate was 0.8 mL min^{-1}, and the injection volume was 20 μL. The column temperature was kept at 37 ℃.

2.2.5 Preparation of Standard Solutions

Standard solutions of vitamins, i.e., B_3, B_5, B_7 and B_9, were prepared at different concentrations of 1.0, 0.2, 0.02 and 0.04 mg/L, respectively. The standard solutions were prepared daily to make analytical calibration curves. The investigated range of standard was diluted based on sample concentration.

2.2.6 Solubility and Storage Stability Studies

Four different pH values (pH 3, 5, 7, and 9) were used to test solubility of B group vitamins in solutions. The mixture of nicotinamide, folic acid, biotin, and pantothenic acid in each concentration of 10 ppm were dissolved in solutions at four different pH values mentioned above, and passed through 0.2 μm nylon filters prior to LC/MS analysis. The remaining mixture standards were stored under three different conditions: room temperature (25 ℃), 4 ℃, and -20 ℃ for testing storage stability, which were evaluated at 0, 24, 48, and 72 hours, respectively.

2.2.7 Study of Heating Sensitivity

Heating sensitivity of B_3, B_5, B_7 and B_9 were tested using a mixture of standards composed of 10 ppm of B_7 and B_9, and 40 ppm of B_3 and B_5, which were prepared in 10 mM ammonium formate at pH 9. The standards were heated for 20 minutes in a water bath at 25, 30, 35, 40 and 45 ℃, respectively.

2.2.8 Sample Preparation with SPE

2.2.8.1 Sample Preparation by SPC C18

Five tablets were finely ground with a mortar and pestle and mixed thoroughly. A portion of powder was weighed on equivalent as one average tablet weight or one average pack weight into a 100 ml volumetric flask. Fine powders of samples were dissolved by 0.1% NaOH (pH 12.1) solution, followed by degassing by ulta-sonication for 1 minute. The 0.1% NaOH (pH 12.1) was then added to the 100mL mark. The sample solution stood for 15 minute, followed by centrifugation at 5000 *rpm* for 10 min.

A SPE C18 cartridge was conditioned and equilibrated with 3 mL of methanol and 3 mL of distilled water. 1 mL sample was then loaded. Vitamins in samples were retained on the sorbent and then subsequently eluted by 3 mL of a mixture of 10 mM ammonium formate solution (pH 9) containing 80% methanol. The eluent was collected in 15 mL test tubes and evaporated to dryness at 25 ℃ under dry nitrogen stream for 20 minutes. The dry residue of the vitamin fraction was reconstituted in 0.1% NaOH solution to a final volume of 1 mL. Then, 30 μL of sample was injected into the LC/MS.

2.2.8.2 Sample Preparation by Strong Anion-exchange SPE

The finely powdered sample was dissolved in 0.1% NaOH solution, and was extracted for 15 minutes, followed by centrifugation at 5000 *rpm* for 10 minutes as given above. The SPE cartridge was pre-conditioned with 5 mL methanol and dried under vacuum (250mm Hg) for 10 minutes. Later, it was equilibrated with 5 mL of distilled water. One milliliter of sample solution was passed through the conditioned and equilibrated cartridge (strong anion exchange (SAX), Oasis MAX). The first fraction was eluted by 10 mL of 10 mM ammonium formate solution (pH 9) containing 15% methanol for B_3 and B_5, and collected in a 15 mL plastic tube. The second fraction was eluted by 5 mL of 2% formic acid in methanol (v/v) for collecting B_7, and 5 mL of 5% formic acid in methanol (v/v) for collection B_9. Both fractions were dried under N_2 at 25 ℃ water bath for 20 minutes. Then, the dried fractions were reconstituted by 10 mM ammonium formate (pH9) to 1.0 mL. Three vitamin fractions were injected into LC/MS in triplicates for chemical analyses.

2.2.9 Recovery of Vitamin B_3, B_5, B_7 and B_9

Multivitamin/multimineral supplement tablets and vitamin drinks contain known amounts of B_3, B_5, B_7 and B_9, and were spiked with three additional levels of their respective standards (50, 100, and 200% of the estimated values) to determine the recovery of SPE. The SPE procedure was performed on the spiked samples. The sample was then diluted and analyzed by LC/MS. The recovery was calculated by the following formula (18):

$$R (\%) = (Cs-Cp)/Ca \times 100$$

Where R (%) is presented as the percentage of added standard; Cs is vitamin amount of the determined spiked vitamins; Cp is vitamin amount of unspiked vitamins; and Ca is added amount of vitamins.

2.2.10 Statistic

Data collected in these studies were analyzed using SAS 9.3 version by one-way analysis of variance (ANOVA) and by independent samples F-test. A 3x4x3 factorial treatment design was analyzed by JMP Pro 10.0.0 version to fit regression model for solubility, storage conditions and storage duration.

2.3 Results and Discussion

The difficulties of determination of B group vitamins partially rely on their different stability and solubility under different pH conditions of their solutions (8,19,20). There exist significant differences in the amount of each vitamin from milligrams to micrograms in samples (21). Therefore, the sample extraction process is associated as a critical control for protocols used in vitamin analysis (22). In addition, before optimizing a chromatographic method, studying solubility and stability of

vitamins helps to keep the whole research under the same conditions and reflect actual values.

2.3.1 Effect of pH, Storage Conditions and Storage Duration on Vitamin Stability

The pH of solutions has a major impact on solubility of each vitamin, so solubility of B_3, B_5, B_7 and B_9 in a mixture solution was evaluated under 4 different pH values (23,24). The results are shown in Figure 2.1. In this study the significant level (α) was set at 0.05, and the *p* value < 0.05 indicated there was a significant difference. The solubility of B_9 significantly increased at higher pH values, for example, the signal response (i.e., solubility) of B_9 increased by 67% when pH of the solution shifted from 5 to 7 (25). B_7 is also more soluble at higher pH solution, but there is no significant increase in solubility from pH 5 to 7. Contrary to the solubility results of B_7 and B_9, B_3 has the highest solubility at pH 3. Under higher pH, B_3 exhibited less solubility. The solubility of B_5 varies depending on pH. In an acidic solution from pH 3 to 5, its solubility significantly increased along with the increase of solution pH. After pH 5, its solubility remained the same in the pH range of 5 to 9

Most of B group vitamins are well dissolved and stable in acidic solutions rather than basic solutions. Although folic acid, riboflavin and biotin are more soluble in basic solution, the acidic solution is preferred for vitamins extraction (26), because the use of basic solutions for simultaneous extraction has not been studied extensively. Moreover, some water-soluble vitamins are heat sensitive; for example, vitamin C is readily to be oxidized in the presence of minerals in multimineral supplements and easily disappeared, particular in the acidic solutions like pH 3 (27). In order to prevent vitamin degradation, it is important to test stability during samples storage under

different conditions. Assuming different storage conditions would influence vitamin stability, it is possible to occur in the autosampler while waiting for analysis. For instance, vitamin C was reported to decrease by 5% in an autosampler at 25 ℃ after 5 hours (24). Hence, the stability test of vitamins at 4 different pH solutions, 3 storage conditions (-20 ℃, 4 ℃ and 25 ℃, for 0, 24, 48 and 72 hours) were investigated with the aid of a factorial treatment design model to check if the three treatment factors had interactions. The results (Table 2.2) showed that pH, time, and pH interaction with time had significant influence on the peak areas (i.e., solubility of the vitamins), resulting in a reduced model to fit the discovery (see Table 2.3).

2.3.2 Study of Thermal Stability of Vitamins

A heating process was required in the SPE protocol, so that vitamin B_3, B_5, B_7 and B_9 were tested for their thermal stability under different temperatures to prevent the vitamin loss during the methanol evaporation precess. Figure 2.2 indicated four vitamins were stable at temperatures below 25℃, but B_9 was unstable at higher temperatures above 30℃.

2.3.3 Optimization of SPE C18 Extraction

Optimization of C18 SPE extraction involved the study of the effects of organic solvent volume, pH of solvent elution, and elution volume, which are all relevant to elution efficiency (15,28). In order to enhance recovery of four vitamins eluted from the SPE, different ratios of methanol in water were examined. As seen in Figure 2.3, recovery of vitamins B_3, B_7 and B_9 increased when the eluents contained higher percentage of methanol, except B_5 that was not significantly affected by change of the elution polarity. In this context, 80% methanol in water was adopted as

an optimal condition for B_3, B_5, B_7 and B_9 elution. However, vitamin B_7 and B_9 had recoveries of only 80%. Considering the pH effect on elution efficiency, 10 mM of 4 different buffer solutions (i.e. ammonium formate, ammonium acetate, ammonium acetate, ammonium formate buffer at pH 3, 5, 7 and 9, respectively) containing 80% methanol were tested. Figure 2.4 shows B_7 and B_9 have 100% recovery when pH of elution was 9. Besides, different volumes from 2 to 10 mL of 10 mM ammonium formate (pH 9) containing 80% methanol were tested to determine an ideal volume of elution solvent. Since 10 mL of elution solvent approached the best recovery, it was chosen to elute vitamin B_3, B_5, B_7 and B_9 from the C18 SPE.

2.3.4 Optimization of SAX SPE Extraction

Analytes are retained in the cartridge mainly due to the ionic interaction between the analytes and the sorbent, so carefully controlling the pH of the elution solvent is the key to isolating major compounds from the matrix (29,30). As mentioned before, samples were dissolved at high pH. Comparing the pKa values of 4 vitamins (i.e., B_3, B_5, B_7 and B_9), the last one has the highest pKa at 4.8. Therefore, 0.1% NaOH that has pH at 11.5 can fully dissolve all four vitamins because it has a much higher pH value than 6.8, which is required according to the two-unit rule for chemical dissolution mentioned in chapter one. In the basic solution, all 4 vitamins were negatively charged and adhered onto the sorbent because the sorbent were in positive charges all the time due to the pKa of SPE greater than 18.

According to the protocol for the strong anion-exchange SPE provided by the manufacturer, the chemical interferences that are neutral or basic compounds could be washed off by high pH solutions with methanol (0% to 20%) after the sample was

loaded. Then, 100% methanol was used to elute more hydrophobic compounds. The following steps used low pH solutions to wash down acidic compounds. Those three elution/washing steps were examined one by one to achieve 100% recovery.

2.3.4.1 Effect of Elution Modified by Methanol

The 10 mM ammonium formate (pH 9) containing different percentages of methanol (0, 5, 10, 15 and 20%) were used to test the efficiency of elution. At this step, only vitamin B_3 and B_5 were eluted from SPE. Figure 2.5 shows B_3 are less dependent on polarity of solvent, while B_5 was recovered in higher yields when the percentage of organic solvent was increased. If elution was conducted by 100% methanol, only a portion of vitamin B_3, B_5, B_7 and B_9 were washed down with a certain degree of loss of the analytes, so 100% methanol was not chosen to clean the hydrophobic compounds. This result also indicated that elution of vitamins from SPE are not only affected by pH of solution, also the solvent polarity.

The washing step was optimized by preparing a (2 to 5%) formic acid in methanol (v/v). The pH values of 2% and 5% formic acid in 100% methanol were 4.12 and 2.02, respectively. When the elution pH is lower than pKa of a vitamin, the vitamin will become neutral and/or positive; therefore, the vitamin will be desorbed from sorbent of SPE. In our study, the vitamin B_7 could be eluted by 2% formic acid in methanol, while B_9 was eluted by 5% formic acid in methanol.

2.3.5 Recovery Studies

The results of the recovery studies are shown in Table 2.4. For the C18 SPE, the range of recovery was 92.8 to 102.6% with RSD ranging from 0.03% to 0.24%. More specifically, the average recovery for vitamin B_3, B_5, B_7 and B_9 were 101.3%,

102.9%, 92.8% and 102.6%, respectively. For strong anion-exchange SPE, the range of recovery was 91.5% to 109.1% with RSD ranging from 0.07% to 0.27%. The average recoveries of B_3, B_5, B_7 and B_9 in multivitamin/multimineral tablets were 91.5%, 93.0%, 109.1% and 106.7%, respectively.

2.4 Conclusion

The SPE methods can facilitate isolation and purification of pharmaceuticals and/or nutraceauticals because the steps can remove interferences. Therefore, it can also simplify determination of analytes. In this chapter, two types of SPE were investigated to extract vitamins from multivitamin/multimineral tablets. Even though C18 SPE is more commonly used than the strong anion-exchange SPE, because the former can have an elution volume nearly as same as void volume with high recovery, the latter might be more desirable for some ionic vitamins in order to isolate them from complex sample matricies through the adjustment of the elution solvent pH . Therefore, C18 SPE is more suitable to purify vitamins B_3 and B_5, while the strong anion-exchange SPE are more suitable to concentrate vitamins B_7 and B_9. Nevertheless, further studies are needed to refine their applications to other foods.

2.5 Figures and Tables

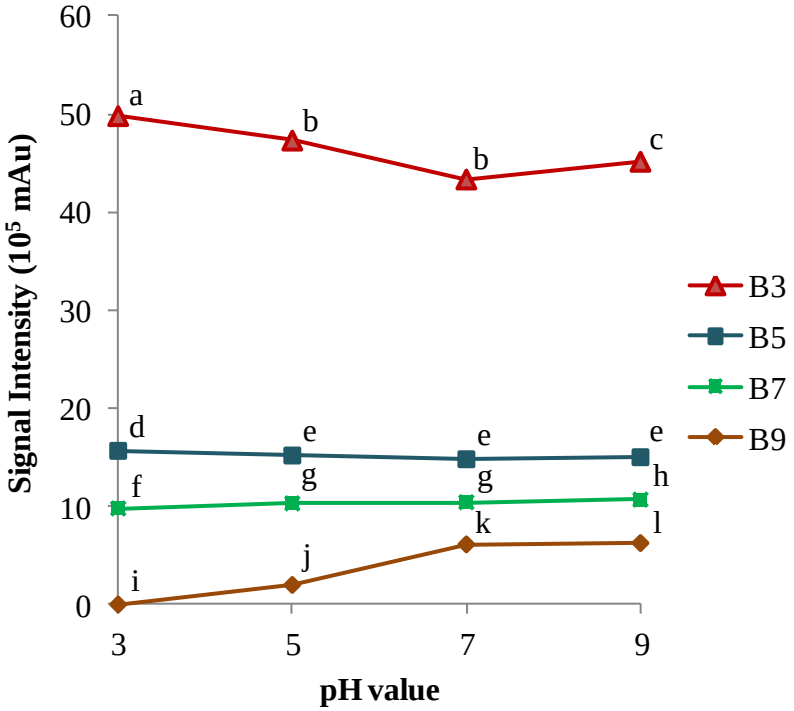

Figure 2.1 Four Vitamins-pH Profile. Solubility study (Level has same latter mean there is no significantly different)

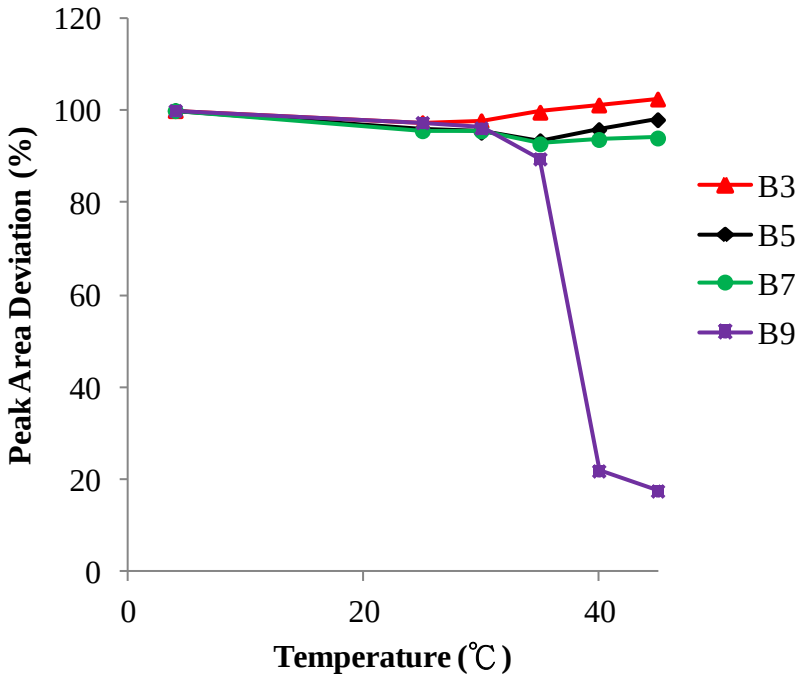

Figure 2.2 Deviation of the Peak Are for vitamin B_3, B_5, B_7 and B_9 at Six Different Heating Temperatures

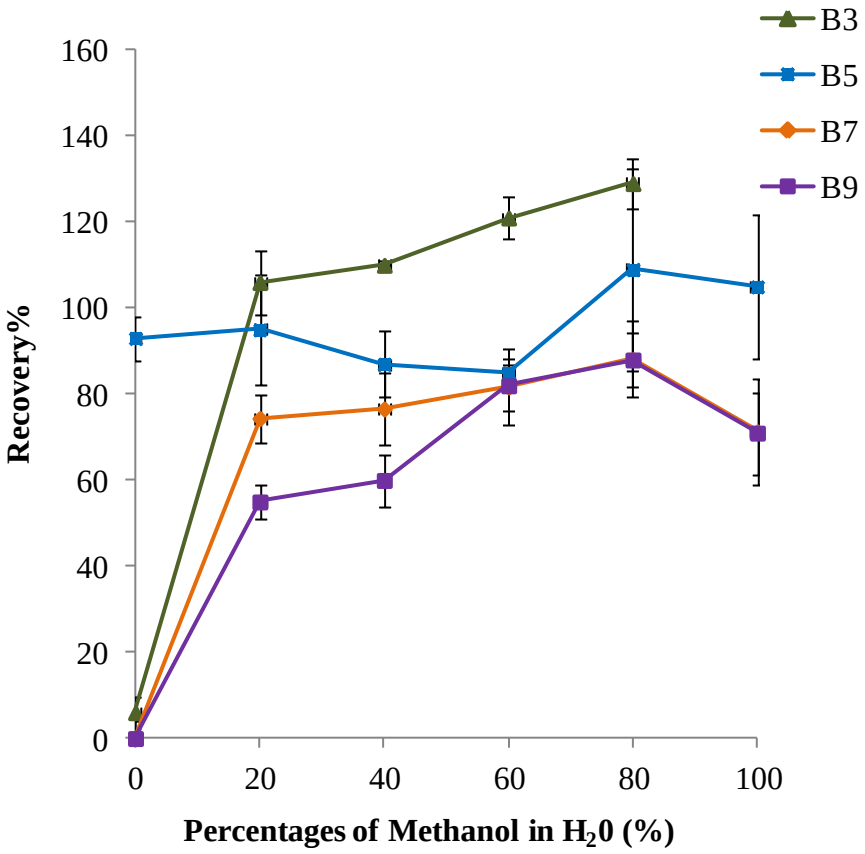

Figure 2.3 Selection of Volume of Elution Solvent for C18 SPE Using HPLC

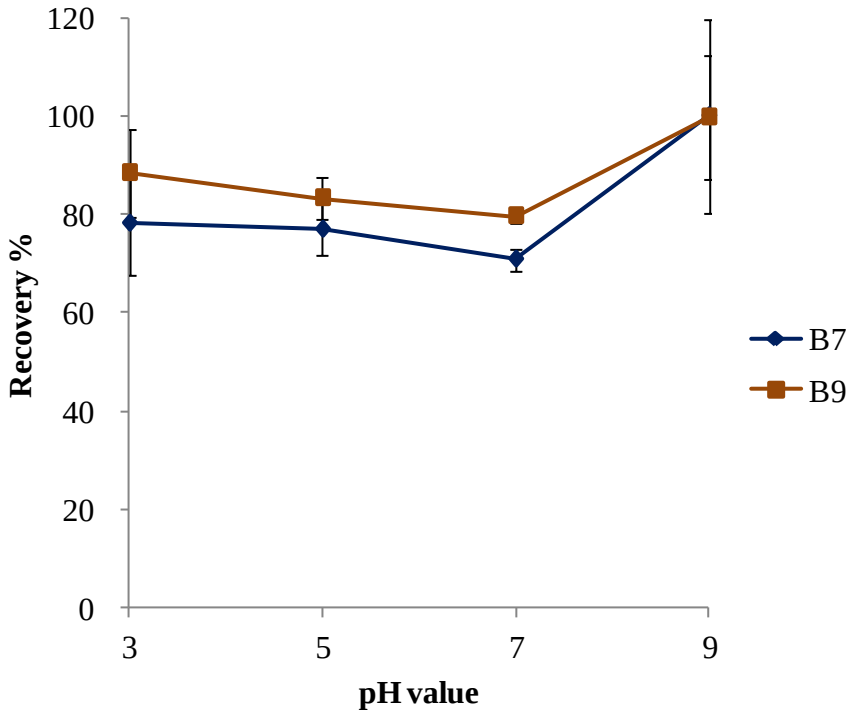

Figure 2.4 The Recovery of Vitamin B_7 and B_9 against pH Value in 80% Methanol of Elution for C18 SPE

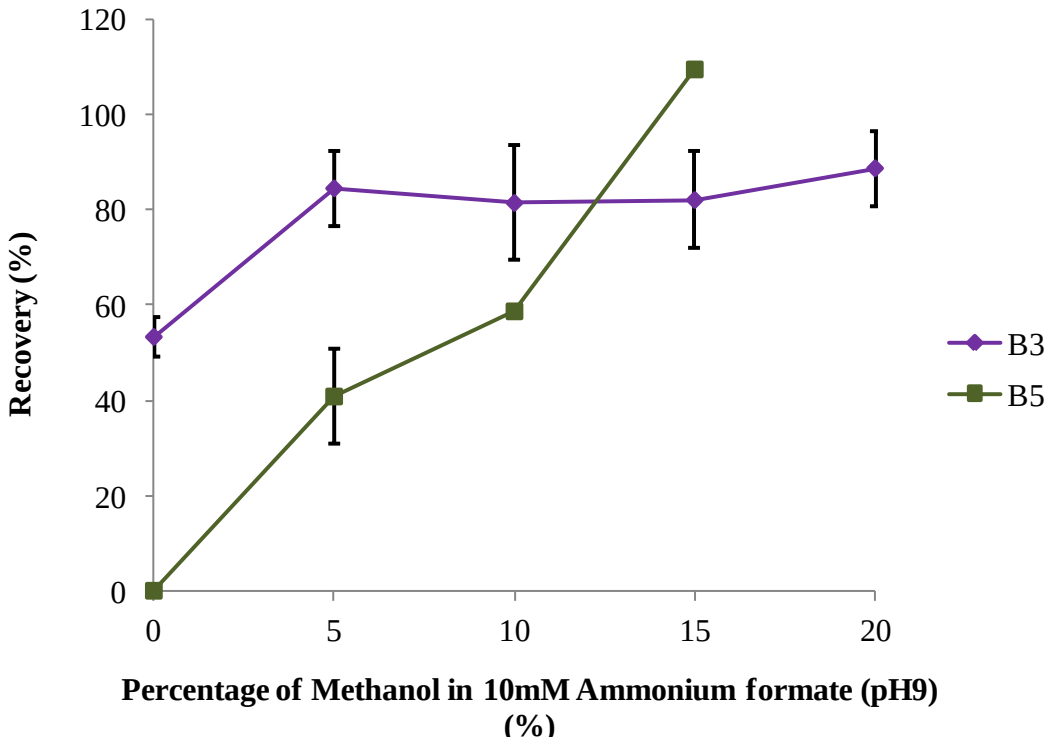

Figure 2.5 The Recovery of Vitamin B_3 and B_5 against Five Different Percentages of Organic Solvent in 10mM Ammonium formate (pH9)

Table 2.1 Gradient Elution Program of HPLC

Time	Solvent A (%, v/v)	Solvent B (%, v/v)	pH
0	100	0.0	2.77
5	97.3	2.7	2.77
8	95.0	5.0	2.80
9	65.0	35.0	2.94
13	52.0	48.0	3.16
15	51.9	48.1	3.17
19	51.9	48.1	3.17
21	51.8	48.2	3.18
22	51.8	48.2	3.18
25	0	100	6.30
35	0	100	6.30
40	100	0	2.77

Table 2.1 Full Factorial Treatment Effects Model

Vitamin	pH	Temp	Time	pH*Temp	pH*Time	Temp*Time	pH*Temp*Time
B_3	<.0001*	0.5866	0.8736	0.5209	0.5916	0.3166	0.2452
B_5	<.0001*	0.2566	<.0001*	0.474	<.0001*	0.2485	0.5824
B_7	<.0001*	0.6671	<.0001*	0.6698	<.0001*	0.6251	0.6742
B_9	<.0001*	0.8916	0.0080*	0.9022	0.0219*	0.8823	0.9539

* when p value < 0.05 , there is significantly effects

Table 2.2 Reduced Factorial Treatment Effects Model

Vitamin	pH	Time	pH*Time
B_3	<.0001*	0.8732	0.5903
B_5	<.0001*	<.0001*	<.0001*
B_7	<.0001*	<.0001*	<.0001*
B_9	<.0001*	0.0071*	0.0200*

* when p value < 0.05 , there is significantly effects

Table 2.3 The Average Recovery at Three Spiking Level for Vitamin B_3, B_5, B_7 and B_9 Analysis Using C18 SPE and Anion-exchange SPE

Vitamin	C18 SPE		Anion-exchange SPE	
	Recovery* (%)	RSD (%)	Recovery* (%)	RSD (%)
B_3	101.3±0.13	0.13	91.5±0.07	0.07
B_5	102.9±0.15	0.14	93.0±0.04	0.27
B_7	92.8±0.22	0.24	109.1±0.24	0.09
B_9	102.6±0.03	0.03	106.7±0.29	0.27

* Mean±standard deviation

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CHAPTER THREE

SIMULTANEOUS DETERMINATION OF WATER-SOLUBLE VITAMINS IN MULTIVITAMIN SUPLLEMENTS AND VITAMIN-FORTIFIED BEVEAGES BY HPLC WITH DIFFERENT DETECTORS

Abstract

Eight water-soluble vitamins: including thiamine (B_1), ascorbic acid (C), nicotinamide and nicotinic acid (B_3), pyridoxine (B_6), calcium pantothenate (B_5), folic acid (B_9) , riboflavin (B_2), and biotin (B_7), were separated on a ZORBAX Eclipse XDB-C18 (250mm x 4.6mm, 5 μm particle size, Agilent Technologies, Inc., Loveland, CO, USA) with a guard column (12.5mm x 4.6mm, 5 μm particle size). The separation was accomplished using a gradient elution with mobile phase consisting of 0.1% formic acid in distilled water pH 2.76 (solvent A) and methanol (solvent B) within 25 minute run-time and a flow rate of 0.8 mL min^{-1}. The calibration curves plotted with multi-concentrations of each vitamin were linear with a regression coefficient of R^2>0.990. With aid of three liquid chromatographic detectors, i.e., DAD, ELSD and MS, simultaneous quantification was applied for water-soluble vitamins, focusing on B_3, B_5, B_7 and B_9, in solid sample of dietary supplements, fortified powdered drinks and vitamin drinks. Chromatographic sensitivity, precision and accuracy were determined for assessing instrumental efficiency and method validation.

3.1 Introduction

Essential for our growth and health (1-3), most vitamins fortunately can be

supplied in our daily diet, but their amounts, particularly of some bioactive vitamins, may significantly decrease after storage, processing and/or cooking (1,4). Therefore, in many instances, people take multivitamin supplements and vitamin-fortified drinks to meet the daily requirements (5,6). Over ingestion of vitamins may cause adverse effects. Hence, it is important to accurately determine the vitamin content in such products. Many of current official analytical methods for vitamins are tedious, time-consuming and non-specific, resulting in inaccurate measurements (7,8). By contrast, HPLC has been widely used for numerous analyses due to its analytical speed, accuracy, precision, selectivity, detectors' sensitivity, wide detective linearity, etc., and is commonly used to analyze vitamins (5,9-11). On the other hand, during the last decade, simultaneous determination of vitamins has attracted more and more interests from, in particular, the food and supplement Industry. The industry continually seeks fast and efficient methods for quantitating nutrient content of food and supplement products as support for label claims. (3,6,12,13,14).

Combining the aforementioned considerations for vitamin analysis, the current priority for vitamin analysis is to develop simple but robust methods for achieving good separation and accurate quantification (5). In this context, several chromatographic methods, involving in the HPLC with DAD, ELSD, and/or MS coupled with a reverse phase (RP) C18 column under gradient elution, were investigated for simultaneous separation and determination of eight water-soluble vitamins in multivitamin tablets and vitamin-fortified drinks. The factors affecting the chromatographic peak shape and separation resolution were studied in terms of pHs of mobile phase, column temperatures, and gradient programs (15). The detectors' sensitivity (i.e., limit of detection (LOD) and limit of quantification (LOQ)), detective

linearity, analytical repeatability and reproducibility were assessed to evaluate both accuracy and precision of the method(s) (16).

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

All reagents were of analytical grade. The standards of nicotinamide (purity 98%), D (+)-biotin (purity 98%), and L (+)-ascorbic acid (purity 99%) and formic acid (purity 99%) were purchased from Acros Organics (New Jersey, NY, USA). Riboflavin 5'-monophosphate was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and riboflavin was purchased from Eastman Chemical Company. Vitamin B_{12} [Cyanocobalamin], thiamine-hydrochloride, and pyridoxol hydrochloride were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). D-pantothenic acid calcium salt and folic acid crystalline pteroylglutamic acid were obtained from ICN Nutritional Biochemicals (Cleveland, OH, USA). Ammonium acetate was from Sigma-Aldrich chemical Co (St. Louis, MO, USA). Ammonium hydroxide was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). HPLC grade methanol, ammonium formate, acetic acid, sodium hydroxide and 0.2 μm nylon syringe filters (13mm diameter) were purchased from Fisher Scientific (Suwanee, GA, USA). Deionized water was prepared using a Millipore Synergy UV system (Millipore Billerica, MA, USA).

The multivitamin supplements include the following samples. Sample A: Kirkland signature premium performance multivitamin (Costco Wholesale Corp., Seattle, WA, USA), sample B: Emergen-C Multivitamin+ dietary supplement (Alacer Corp., Foothill Ranch, CA, USA), sample C: GNC B-Complex 125 with C dietary

supplement (General Nutrition Centers, Inc., Pittsburgh, PA, USA) and sample D: Sparking ice (Sparkling ICE®, Preston, WA USA), were purchased from GNC shop store in Anderson, SC, from Costco in Greenville, SC, and at local supermarket in Central, SC. Table 3.1 lists the contents of water-soluble vitamins in each product as shown/claimed on their nutritional labels.

3.2.2 Chromatographic Conditions for Sample Analysis

A reversed-phase ZORBAX Eclipse XDB-C18 (250mm x 4.6mm, 5 μm particle size) chromatographic column with a guard column (12.5mm x 4.6mm, 5μm particle size, Aglient Technologies, Inc., Loveland, CO, USA) was tested for its separation of water-soluble vitamins in a single run with a gradient elution with mobile phase consisting of 0.1% formic acid in distilled water at pH 2.76 (solvent A) and methanol (solvent B). The gradient program was as follows: 0-5 min, linear gradient from 100:0 A:B (v/v) to 97.3:2.7 A:B (v/v); 5-8 min, linear gradient from 97.3: 2.7 A:B (v/v) to 95:5 A:B (v/v); 8-9 min, linear gradient from 95:5 A:B (v/v) to 65:35 A:B (v/v); 9-13 min, linear gradient from 65:35 A:B (v/v) to 52:48 A:B (v/v); 13-15 min, linear gradient from 52:48 A:B (v/v) to 51.9: 48.1 A:B (v/v); 15-19 min isocratic at 48.1% B; then, 19-21 min, another linear gradient from 51.9: 48.1 A:B (v/v) to 51.8: 48.2 A:B (v/v) and 3 min hold; 25-30 min, linear gradient from 51.8: 48.2 A:B (v/v) to 100% B for 10 min; 40 min, back to 100% A, and post-run time was 5 min for column equilibration prior to the next injection. The gradient program is listed in Table 3.2. The flow rate was 0.8 mL min^{-1}, and the injection volume was 20 μL for LC- ELSD / DAD and 30μL for LC-MS. The column temperature was maintained at 37 ℃.

3.2.3 High-performance Liquid Chromatography-Diode Array Detector (DAD)/ Evaporative Light-scattering Detector (ELSD) Conditions

A high performance liquid chromatography system (Shimadzu Scientific Instruments Inc., MD, USA) was equipped with a column oven with manual sampler (CTO-20A), a pump (LC-20AT), a degasser (DGU-20A_5) and a diode array detector (SPD-M20A) coupled with an ELSD detector (ELSD-LT II).

Two wavelengths were chosen: 209 nm for B_5 and B_7, and 270 nm for B_1, B_2, B_3, B_6, B_9, B_{12} and vitamin C, while spectra in the region 200-600 nm were recorded. The ELSD conditions for the analytical method were set to: gain=11, nebulizer temperature= 40 ℃, and pressure= 350 KPa.

3.2.4 HPLC /Mass Spectrometry (MS) Conditions

Another HPLC system used in these experiments was an Agilent 1200 series (Aglient Technologies, Inc., Loveland, CO, USA), which was equipped with a degasser (G1379B), a binary pump (G1312A), a standard and preparative autosamplers (G1329A), a thermostatted column compartment (G1316A) and a variable wavelength detector (model G1314B). The system was coupled on-line to a mass spectrometer (Aglient 6110 series single quadrupole analyzer) with an electrospray ionization (ESI) probe. The ChemStation software (Rev. B.03.02) controlled the LC system.

ESI was performed in positive mode for B_1, B_2, B_3, B_6, B_5, B_9, B_7, and B_{12}; vitamin C and B_9 could be also detected in a negative mode [6]. The voltages of capillary and fragmentor were set at 3.2 kV and 80 V, respectively. The drying gas flow rate (N_2) was 10 L/min and temperature was 350℃. The nebulizer pressure was

set at 40 psi. Full scan mass spectra were selected in the range from *m/z* 10 to 500, and selected ion mode was used for quantitation. Selected ions for each water-soluble vitamin are listed in Table 3.3.

3.2.5 Preparation of Sample Solutions

Five tablets were finely grounded and mixed. A portion of powder was weighed as equivalent to the weight of either one tablet or one average pack. Sample C was prepared on equivalent to half weight of a tablet because of its high concentrations of group B vitamins. Sample powder was transferred into a 100 mL volumetric flask and filled with 0.1% NaOH to the mark on the flask neck, except the Emergen-C Multivitamin+ (sample B). Sample B was added by 20 mL of 0.1% NaOH at first; after degas of the sample for 5 minutes in an ultrasonic bath, sample B was mixted with 0.1% NaOH until the flask mark. The sample solutions were shaken vigorously for 1 minute and under ultrasonication for 15 minutes at room temperature and covered by aluminum foil for protecting samples from light to prevent autooxidation. Then, the mixture was transferred into 50 mL centrifuge tubes and centrifuged at 5000 *rpm* for 15min under 4. The supernatants were filtered through 0.2 μm nylon syringe filters before being injected into LC. The other remaining extracted samples were stored at 4 ℃ until tested.

The vitamin beverage (sample D) was degassed for 5 minutes and then passed through 0.2 μm nylon syringe filters directly before the sample injection.

3.2.6 Standard Solution Preparation

Each of the standards – B_1, B_2, B_3, B_6, B_5, B_9, B_7, B_{12}, and C – was accurately weighed (25 mg) and poured into respective 25 mL volumetric flask to prepare a

single standard stock solution. B_1, B_2, B_5, and B_6 were prepared on a concentration of 0.2 mg/mL; B_7 and B_9 were prepared on a concentration of 0.025 mg/mL; B_3 and C were prepared on a concentration of 0.5 mg/mL. All standards were dissolved by 10 mM pH 9 ammonium formate solution. The stock solutions were stored at -80℃ until analysis.

The standard solutions were serially diluted by 10 mM ammonium formate at pH 9 to obtain several different concentrations for calibration curves.

The stock solution were diluted to 1.0 mg/L, 10 mg/L, and 100 mg/L by 10 mM pH 9 ammonium formate for testing separation of group B vitamins. Three different levels of mixed standard solutions were used by different LC detectors to test the detectors' chromatographic parameters such as sensitivity, recovery, accuracy, etc.

3.2.7 Column Temperatures

Column oven was set up at selected temperatures from 30 to 40 ℃. Temperature was studied to evaluate its effect on peak resolution of a mixed vitamin B group standard. The experiments were run in triplicate of each selected temperatures (28, 30, 32, 35, 37, 38 and 42 ℃).

3.2.8 Method Validation

3.2.8.1 Detector Linearity (calibration curve)

The detector's analytical linearity was constructed and tested by the peak area to the concentration of each vitamin in a range of the concentrations (5,17). The stock standards were mixed and tested with different dilution series. The test range for each individual vitamin was based on its content in samples, and the chosen concentrations

were above the limit of quantitation (LOQ).

3.2.8.2 System Suitability Testing

The system suitability testing (SST) is used to evaluate resolutions, column efficiency, and repeatability (9). SST was assessed by five replicate analyses of mixtures of group B vitamin standards. An acceptable SST value was set at ≦5% residual standard deviation (% RSD).

3.2.8.3 Limits of Detection and Quantitation

The limit of detection (LOD) is the smallest concentration of analytes that can be detected, while the limit of quantitation (LOQ) provides the lowest concentration that analytes can be quantified (9,17). Both were estimated by the signal-to-noise (S/N) ratio. LOD is defined as the peak height of lowest concentration that is three times the signal height of the baseline noise, and LOQ is the signal value that is 10X the baseline noise (12,17).

3.3 Results and Discussion

In this chapter, a simultaneous analysis of water-soluble vitamins was tested by HPLC coupled with DAD, ELSD, and UV/MS. The chromatographic method was selected based on optimized gradient elution, modifier of mobile phase, column temperature, and other LC parameters. The single standard solution of vitamins was used to determine their respective retention time and peak identification. A standard mixture of vitamins was analyzed to optimize the LC condition and maximize peak resolutions.

3.3.1 Optimization of Sample Separation

3.3.1.1 Mobile Phase (MP) Selection

There are two operation modes for HPLC separations, isocratic and gradient elution (9). The latter is most frequently used in chemical separation, by which the mixture of vitamins is eluted by changing of the concentration of the mobile phase (10). In order to efficiently separate the analytes, selection of an appropriate eluent and the pH of that eluent are two important factors to consider.

The pH of the mobile phase can impact the chromatographic selectivity, peak shape and retention, particularly for ionic compounds (17,18). The ionic form has more affinity for an aqueous mobile phase, and thus has a lower retention on non-polar stationary phases (18). Knowing the pKa of analytes helps to select the pH of mobile phase. Usually, the low pH is preferred for analysis of ionizable compounds by reverse phase column in order to avoid secondary interaction between ionized silanols on the silica surface and positive-charge compounds, which cause peak broadening and peak tailing at mid pH values (18,19).

Since the goal of this research is to develop a chromatographic method that is able to be applied to three detectors: i.e., DAD, ELSD and MS, it is important to select mobile phases compatible with these detectors. Even though some mobile phases containing potassium dihydrogen phosphate or sodium acetate can give very good baseline separation of water-soluble vitamins (10), phosphate salts that have limited solubility in organic solvents are unacceptable for use (20). For example, phosphate salts may precipitate in the ELSD when being used with high content of organic solvent (21,18,22). Therefore, the ion-paired reagents, such as trifluoroacetic

acid (TFA), pentafluoropropionic acid, heptafluorobutyric acid (HFBA), acetic acid, formic acid and ammonia, were adopted for improving the vitamin separation (6). Acetic acid and TFA have UV cutoff below wavelength at 240 nm, resulting in a shifted baseline when the detector's wavelength approaches their UV cutoff (23,18). This causes challenges to determine B_5 and B_7 that have no chromophores (24,25). Moreover, TFA and acetic acid were found exhibit intense ion suppression of analytes in the positive and negative mode of ESI, although acetic acid is good for negative mode. Formic acid and its ammonium salt are ideal to be used for the positive mode of ESI (6,18,26). Though volatile ammonia is frequently used to prepare a buffer solution, the high pH of the mobile phase will damage column because of dissolution of silica. As a result, acetate and formic acid are mostly used as ionic modifiers because they are not only volatile, but also soluble in organic solvents (18). Although there were some reports of using negative mode of ESI for determination of vitamin C and B_9 (6), these two vitamins were observed in positive mode of ESI in this study (27). Therefore, 0.1% formic acid in distilled water (pH 2.76) instead of phosphate buffer solution mixed with methanol was used in my research (28).

3.3.1.2 Mobile Phase Optimization

All water-soluble vitamins are simultaneously separated by an analytical C18 column within 20 minutes. Since vitamin B_1 is very polar, it nearly has no retention in the column. In order to improve the retention for the early eluted vitamins, such as B_1, Vitamin C, B_3, and B_6, different percentages of formic acid in water as MP (A) and initial B% were evaluated by retention factor (k), which should be maintained between 1 to 10 for resolution within reasonable run times (29). The pH of 0.05%,

0.1%, 0.125%, 0.15% and 0.2% of formic acid in water are 2.97, 2.85, 2.79, 2.74 and 2.51, respectively. Figure 3.2 shows the retention time of B_1, C, and B_6 did not change when the percentage of formic acid changed. The retention of B_3 decreased when increasing the formic acid ratio in the MP. The pH of mobile phase A not only affected the analytes' retention time (21), but also changed their asymmetry factor (As). The ideal chromatographic peak shape should have a Gaussian peak shape, for which As =1 (9). For most peaks, asymmetry factor is within 0.5 to 2.0. B_3 and B_5 lost their symmetry with higher pH, which can be seen in Figure 3.3. This suggested to have the pH of mobile phase close to their pKa. In contrast to B_3 and B_5, the asymmetry factor of B_9 increased when the pH of the mobile phase increased. Therefore, 0.1% formic acid was chosen to prepare the mobile phase A. To check whether the vitamins retention times were altered by percent of eluent B, B% started from 0%, and increased linearly to 0.5%, 1%, 2%, 2.5% and 3%, and then kept constant for 5 min. Figure 3.4 reveals that B_6 is more dependent on polarity. When the percentages of methanol increased, B_1, B_3 and B_6 could be eluted earlier, and B_6 had noticeable decreased k value than other vitamins. Once the initial B% rose to 3%, the poorest k value was obtained. As a result, the 0% of initial B% was chosen in order to increase the k value of vitamins. As many multivitamin tablets, powder and drinks may contain fat-soluble vitamins or more hydrophobic components, it is necessary to clean the LC column after each sample analysis to remove all the sample components from the column (30).

Isocratic elution was also tested to prevent co-elution of B_7 and B_2, once column temperature cannot be controlled, because temperature also plays an important role in separation. The separation of adjacent peak is evaluated by the

separation factor (α), which should have a value greater than 1.5 for baseline separation (9). When the flow rate reduced to 0.2 mL/min, a better α value equal to 1.05 was obtained. The effect of column temperature on improving separation of B_7 and B_2 will be discussed in the following section 3.3.1.3.

3.3.1.3 Column Temperature

The column temperature can affect the resolution of matrix components (18,10). Seven column temperatures were tested by analyzing a standard mixture of B group vitamins. Experiments were run in triplicate. The basic chromatographic parameter (retention factor) was evaluated in this experiment. The retention factor is calculated as $(k) = (t_R-t_0)/t_0$, where t_R is the retention time of analyzed compounds and t_0 is the retention time for an unretained peak (9). The changes in retention factors were influenced by viscosity of mobile phase. The results are shown in Figure 3.5 (A). As the column temperature increased, retention factors of B_1, B_3 and B_6 gradually decreased. Although the temperature change had less influence on the retention factors of other vitamins, i.e., B_2, B_5, B_7, B_9 and B_{12}, the chemical separation between B_5, B_9 and B_{12} was improved when the column temperature increased (figure 3.5 (B)). This was confirmed from calculation of separation factor or selectivity (α) between vitamin B_5 and B_{12} and between vitamin B_{12} and B_9 (9). Higher temperature often cause faster elution due to decreased viscosity of mobile phase (Figures 3.6) (10,31). The separation between B_7 and B_3 was also observed with a slight increase of 0.01 for α from temperature at 28℃ to 42℃ (Figure 3.7). Increasing column temperature by 1 ℃ will cause k values to decrease by 1 to 2% for non-ionic compounds (32). As a result, the separation of B_5 and B_{12} was improved when the column temperature

increased, but at the same time, B_{12} and B_9 were co-eluted. In this case, 37 ℃ was selected as an optimal temperature in light of its effective and better separation of water-soluble vitamins, and used in all experiments in this study.

3.3.2 System Suitability Testing (SST)

SST includes repeatability of peak response (RSD), resolution (Rs), tailing factor (T_f), plate count (N) and capacity factor (k). The RSD is required less than 5%; Rs should be greater than 1.5; $T_f \leqq 2.0$. A larger N reflects better efficiency of the column, and k should be ≥ 2 and ≤ 8. Table 3.4 to Table 3.6 show results for the optimized HPLC method for simultaneous determination of water-soluble vitamins by ELSD, DAD and MS. Vitamins were analyzed individually by selected ions, but the values of Rs and k were calculated by total ion chromatography. According to Table 3.5 and Table 3.6, k value of B_1 is close to 0 which means B_1 was eluted so fast nearly without absorption on the stationary phase due to its high polarity. Therefore, its k is close to zero and Rs values are less than the recommended value. From Table 3.4, Table 3.5, Table 3.6 and Figure 3.1, it can be seen that base line separation of B_7 and B_2 was not achieved, due to Rs is less than 1.50. In this case, however, that would not interfere in the quantification of B_2, because the amount of B_7 was unable to be detected by DAD and ELSD.

3.3.3 Linearity, Limit of Detection, Limit of Quantification, Precision

Calibration curves ($y = ax + b$) were constructed by plotting the peak area (y) of analyte versus the concentrations (x) of the calibration standards. LOD and LOQ were assessed using standard solutions. The results are listed in Table 3.7. The LOQs for detectors were determined from quantification of individual vitamins.

The calibration curves generated from LC/ELSD for B_3, B_5, and B_7 were not linear within the concentrations in a range of 50-200 mg/L, 40-100 mg/L, and 10 mg/L, respectively. For example, the LC/MS calibration curve for B_5 has its R^2 =0.990. Interestingly, the calibration curves for B_3, B_5, and B_7 and B_9 generated from LC/DAD showed excellent linearity within the investigated ranges of 50-500 mg/L, 50-90 mg/L, 0.5-1.6 mg/L and 1-25 mg/L. Discrepancies in the linearity of the detectors may be caused by detector saturation/ion suppression during the ionization for yielding mass spectrum (33).

The precision of method was evaluated by the analytical repeatability and reproducibility. The repeatability was studied by 3 consecutive sample separations, and the reproducibility was tested by running the newly prepared samples within 3 consecutive days. The retention time and peak areas were determined and their RSDs were then calculated to estimate the precision. The data is presented in Table 3.8, which lists the chromatographic signal responses of DAD and ELSD for the vitamins of B_5 and B_7. ELSD provides better repeatability, with RSDs of 1.8% and 0.47% for the B_5 and B_7, respectively. The higher but acceptable RSDs for the DAD were 4.5% and 4.10% for the B_5 and B_7, respectively. The stability of the detector is based on RSDs of repeatability and reproducibility of the peak areas. RSDs for repeatability and reproducibility of peaks from the MS detector were in a range of 1.44%-3.42% and 0.152%-2.82% respectively. DAD RSDs were in a range of 2.12%- 4.51% and 2.14%-4.55%; while RSDs for the ELSD were in a range of 0.47%-4.56% and 0.62%-4.75%. Overall, regarding the stability of three detectors, MS and DAD are better than ELSD.

3.3.4 Sample Analysis

Four different types of samples, multivitamins/multimineral tablets, powder, B-complex tablet, and vitamin drink from different manufactures were purchased from local supermarkets. The results of the average values of three measurements of the vitamin analyses are shown in Table 3.9. Some of the measured values are different from those shown in their nutritional labels. The manufactures may add more amounts of vitamins in tablets and drinks than the required amounts in order to keep their contents in the samples more or equal before the expiration date of the products (7). This may be particularly true for vitamin drinks which are packaged in transparent bottle and susceptible to vitamin loss due to UV oxidation. Based on results shown in Table 3.9, the amounts of the vitamins determined by three detectors are similar except the sample C. The sample C contains extremely high levels of vitamins, which may either exceed the recovery power of the used SPE, or make the solution saturated. For example, vitamin B_3 has a significantly reduced solubility in a higher pH solution. This observation was discussed in chapter 2. Another situation was observed during analysis of the samples. After opened sample bottles, the sample may then absorb moisture, possibly resulting in degradation and concomitant underestimation of the actual vitamin content.

As the concentrations of some vitamins in the multivitamin/multimineral supplements or vitamin drinks are at microgram concentrations, e.g., B_7 and B_9, their quantitative analyses have often been a problematic. According to Table 3.9, B_7 and B9 were able to be detected by LC/MS because MS has a higher sensitivity than DAD and ELSD. By contrast, LC/DAD can only detect vitamin B_9, while LC/ELSD cannot detect either vitamins. In addition to the sensitivity of detector, the specification of

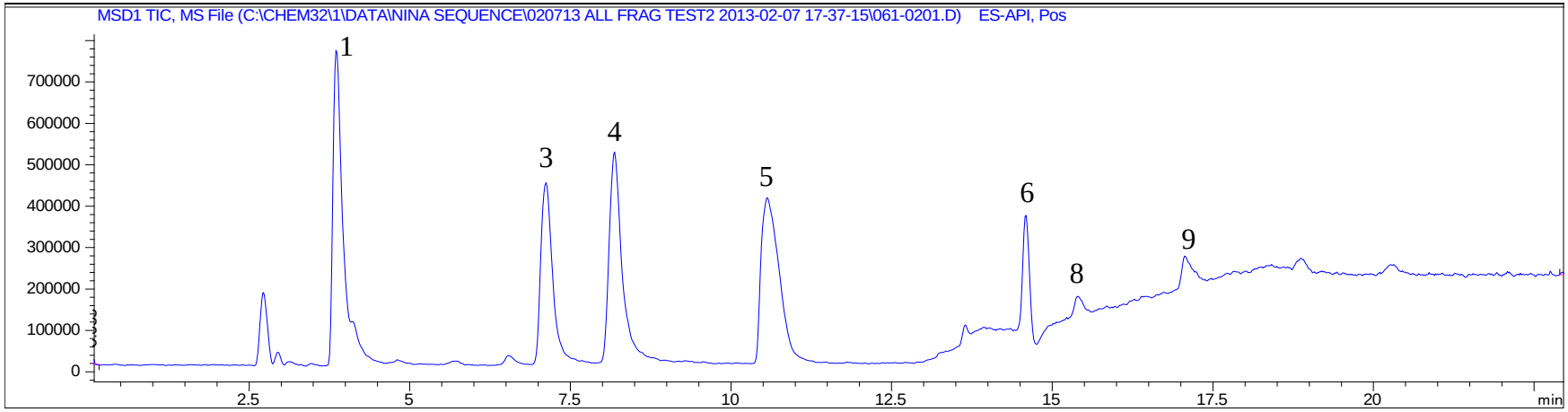
detector is another issue that should be discussed. Some LC detectors can provide additional information on the chemical structure of the analytes. For example, LC/DAD can not only monitor the UV absorbance of analytes, but also provide UV spectra of all the eluted peaks. Therefore, the UV detector is commonly used for peak quasi-identification and quantization. Among the nine of water-soluble vitamins, only two of them, i.e., B_5 and B_7, exhibited weak UV absorbance due to the lack of chromophores in their chemical structures (3,4,7). As a result, they cannot be quantified accurately and precisely due to interfere from mobile phase or other ingredients in the samples, even though they can be detected by DAD at the low wavelength (35). Nevertheless, the shortcoming of LC/DAD for determination of B_5 and B_7 can be overcome by LC/ELSD and LC/MS. Besides vitamin B_5 and B_7, other analytes with low UV absorbance, such as sugar, triglyceride, etc., are also amenable to detection by ELSD.

3.4 Conclusion

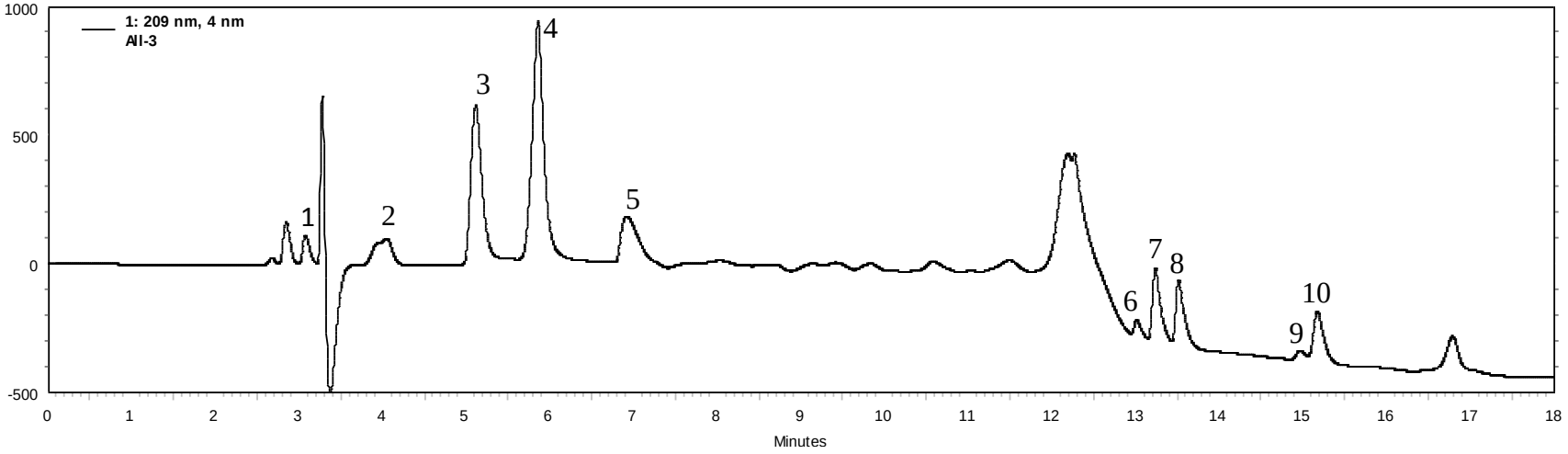
From the experimental results and above discussion, it is concluded that the LC/MS is the best system suitable for simultaneous multi-vitamin analysis because the MS detector demonstrated the highest sensitivity and precision, which make it to be able to analyze all water-soluble vitamins, including, B_7, B_9 and B_{12}, regardless of some limitations on its mobile phase required to be compatible with the detector. The LC/DAD is also good for peak identification and method development, though it is not suitable for analysis of vitamin B_5 and B_7, due to low repeatability and reproducibility. The LC/ELSD is an ideal detector for vitamin B_5 and B_7, but it is less stable and is limited in some applications due to its special requirements of the mobile

phase. In summary, simple, low-cost and time efficient methods based on HPLC connected with DAD, ELSD and LC/MS were developed and validated for the simultaneous analysis of eight vitamins (B_1, B_2, B_3, B_5, B_7, B_9 and C) in multivitamin/multimineral tablets and in multivitamin powder, and vitamin drinks. LC/MS is herein recommended if the simultaneous determination of multi-vitamins tablets, powders or drinks is required.

3.5 Figures and Tables



(A)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1000
500
0
-500
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2
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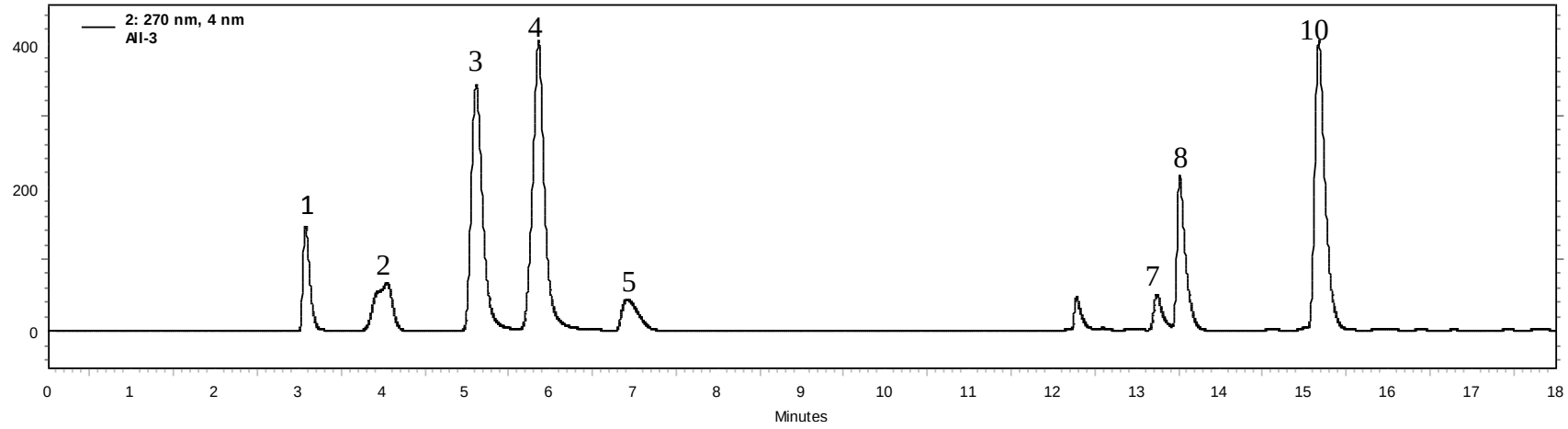
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Minutes

(B)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AII-3
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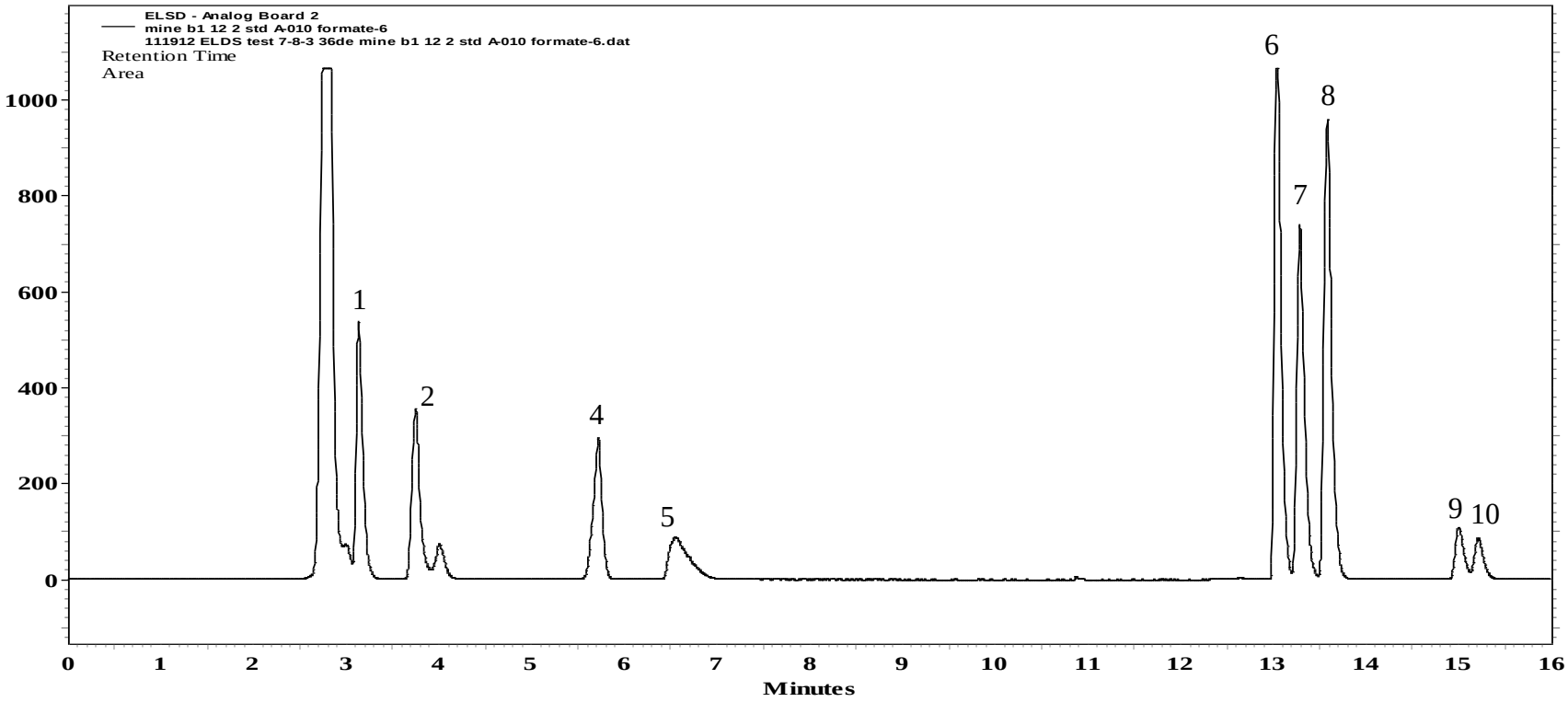
Minutes

(C)



(D)

Figure 3.1 Chromatography of Water-Soluble Vitamins Mix (Standard Prepared in 10mM pH 9 Ammoniun Formate) (A) LC/ESI+ Chromatograph of a 25 mg/L Standard Mixture (B) LC/DAD Chromatogram of 150 mg/L Standard Mixture at 209nm (C) LC/DAD Chromatograph at 270nm (D) LC/ELSD Chromatogram of a 150 mg/L Standard Mixture. Peak Number as Follow: (1) B_1; (2) C; (3) B_3-acid form; (4) B_3-amide form; (5) B_6; (6) B_5; (7) B_{12}; (8) B_9; (9) B_7; (10) B_2

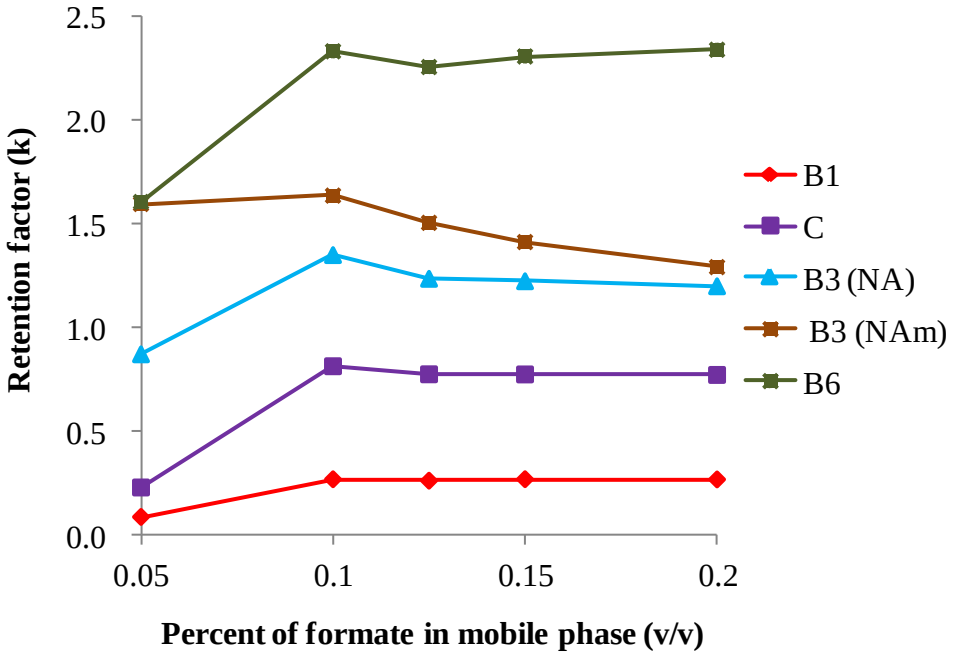


Figure 3.2 Effect of the Formic Acid Percentage in Mobile Phase A on the Retention Factor of Vitamin B_1, B_3 (Nicotinic Acid and Nicotinamide), C and B_6

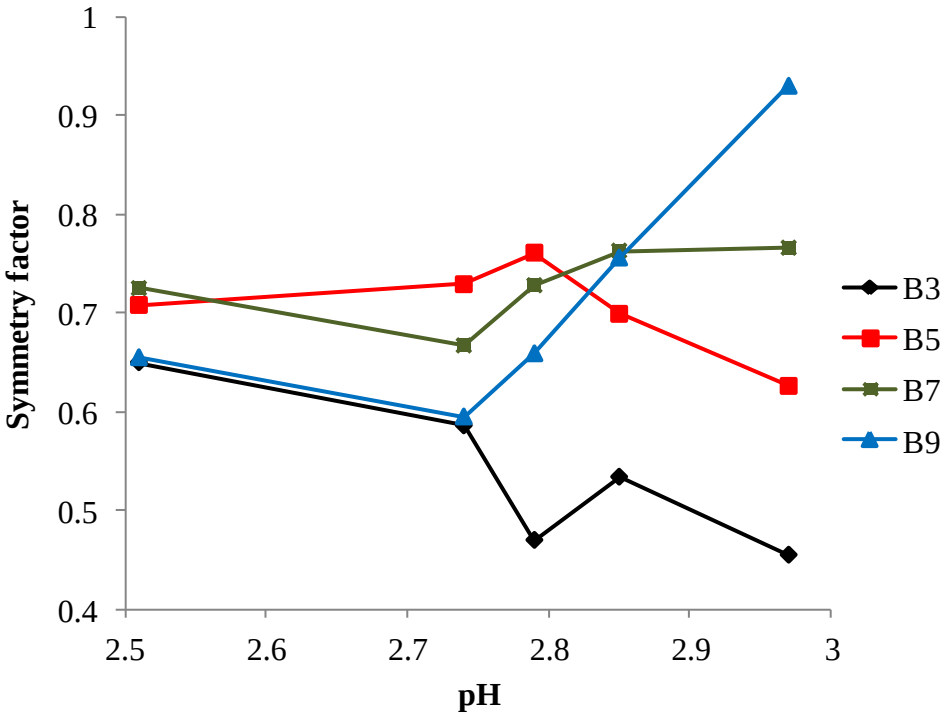


Figure 3.3 Effect of the pH on the Symmetry Factor of Vitamins

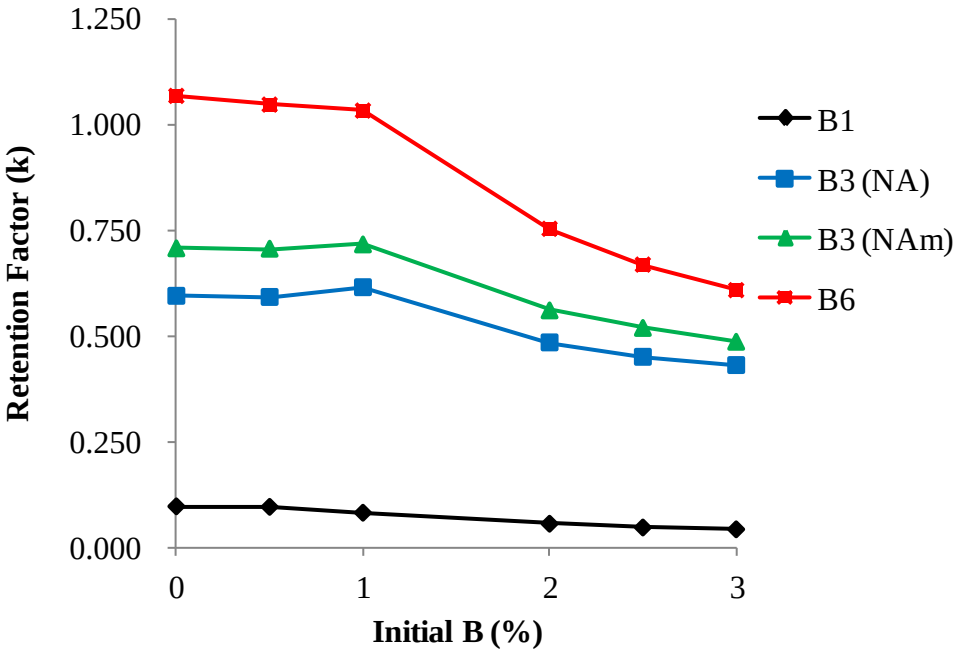
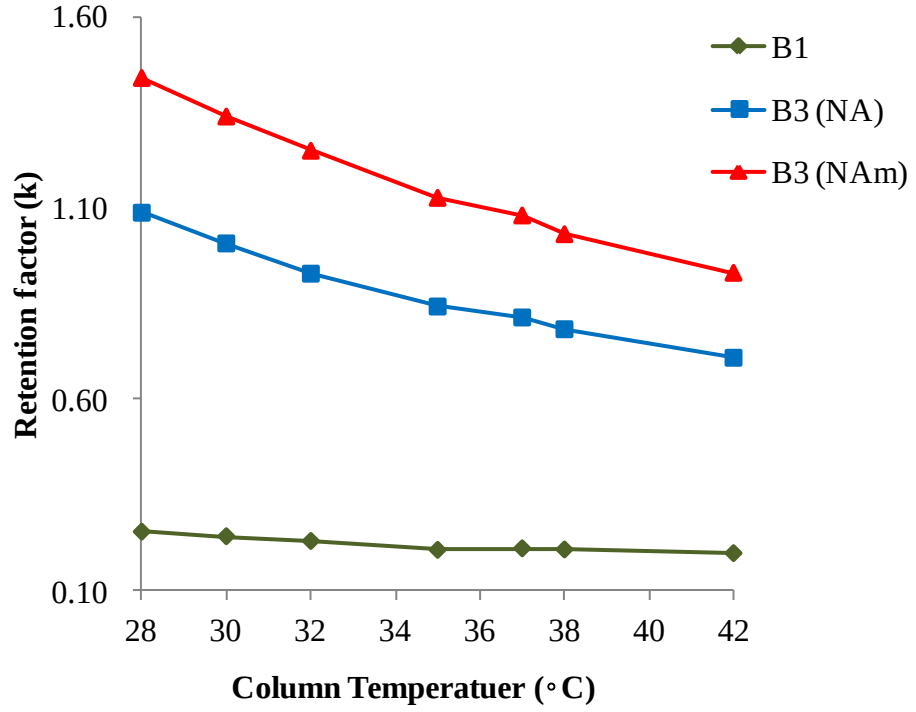
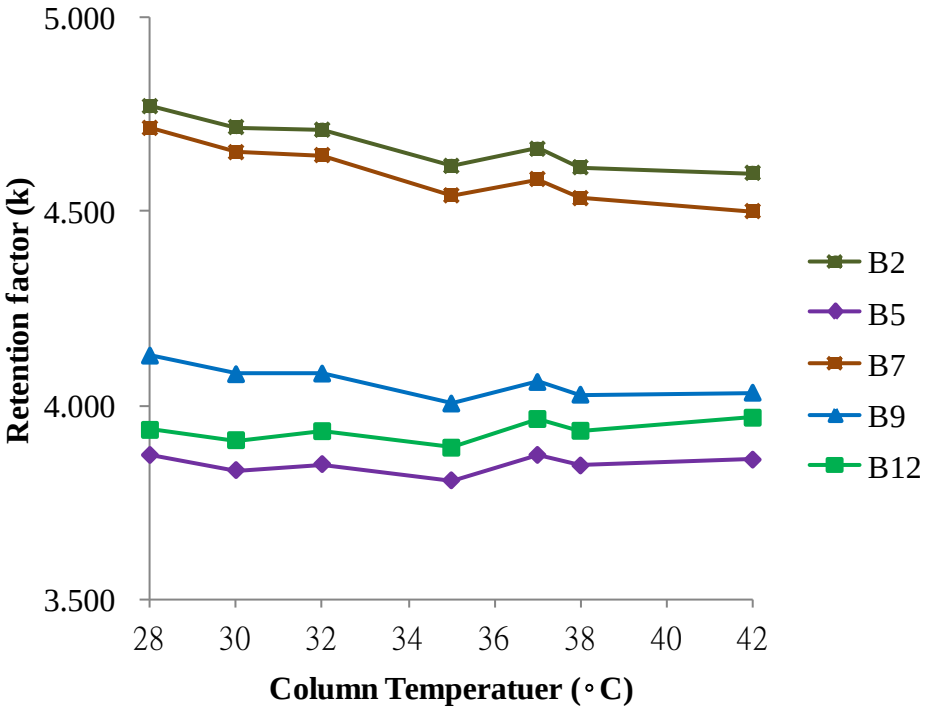


Figure 3.4 Effect of the Initial Organic Solvent in Mobile Phase on the Retention Factor of Vitamin B_1, B_3 (Nicotinic Acid and Nicotinamide) and B_6



(A)



(B)

Figure 3.5 Effect of the Temperature on the Retention Factor of (A) Vitamin B_1, B_3 (Nicotinic acid and Nicotinamide) (B) Vitamin on B_2, B_5, B_7, B_9 and B_{12}

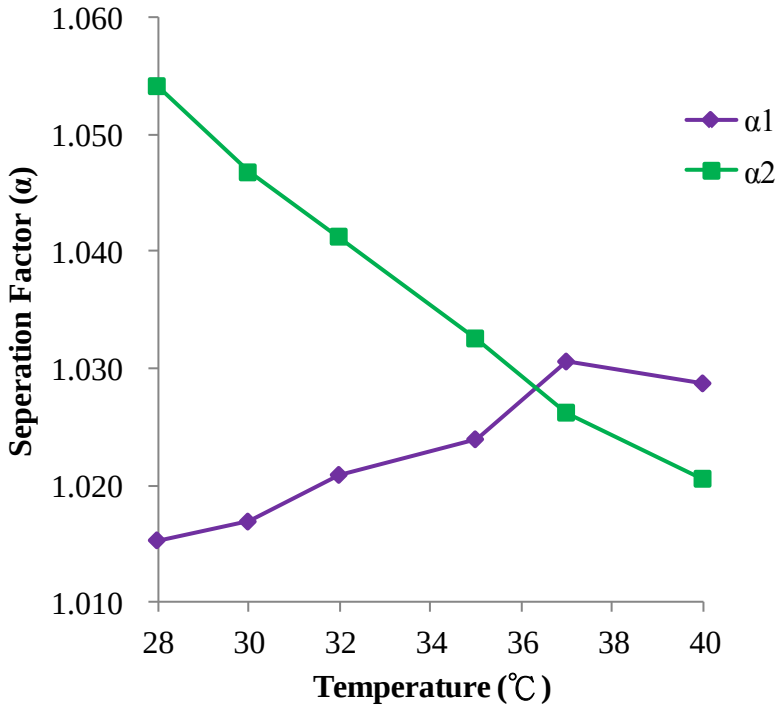


Figure 3.6 Effect of the Temperature on Vitamin B_5, B_9 and B_{12} Separation. (α1 is Separation Factor between Vitamin B_5 and B_{12}; α2 is Separation Factor between Vitamin B_{12} and B_9)

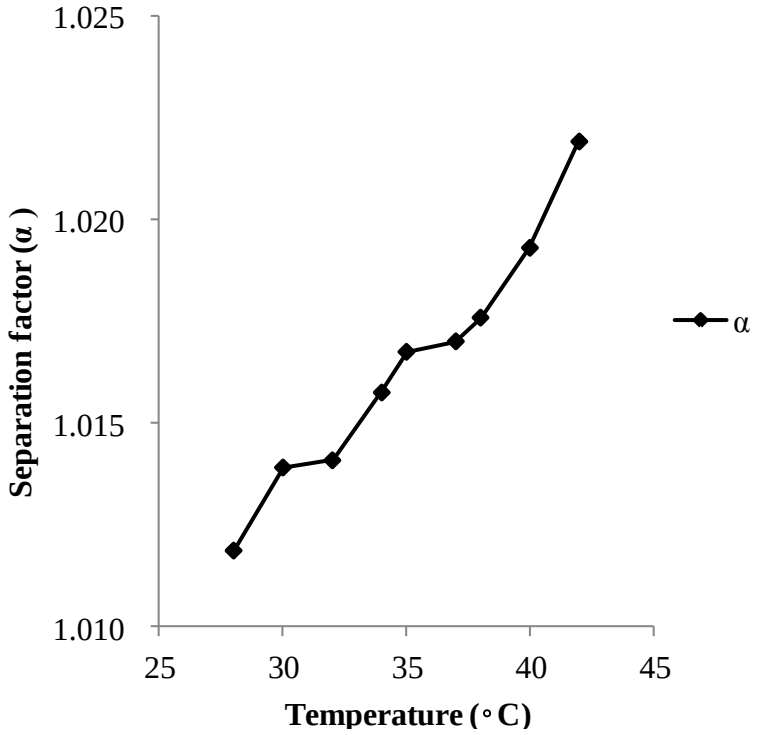


Figure 3.7 Effect of the Temperature on Vitamin B_7 and B_2 Separation. (α is the Separation Factor between Vitamin B_7 and B_2)

Table 3.1 Concentration of Vitamins in the Multivitamin Supplements, Fortified Powdered Drink, and Vitamin Drinks

Vitamins	Content in each tablet/pack			
	Kirkland signature premium performance multivitamin	Emergen-C Multivitamin+	GNC B-Complex 125 with C	Sparkling ICE Crisp Apple
	Sample A	Sample B	Sample C	Sample D
Vitamin C	120mg	-	125mg	-
Thiamin mononitrate (B_1)	5.25mg	-	125mg	-
Thiamin HCl (B_1)	-	1.5mg	-	-
Riboflavin (B_2)	6mg	-	125mg	-
Riboflavin 5'-phosphate sodium (B_2)	-	1.7mg	-	-
Niacin (as nicotinamide) (B_3)	50mg	20mg	125mg	2mg
Pyridoxine HCl (B_6)	10mg	2mg	125mg	200µg
Folic acid (B_9)	400µg	500µg	400µg	-
Cyanocobalamin (B_{12})	25µg	6µg	125µg	0.6µg
Biotin (B_7)	60µg	30µg	125µg	30µg
Calcium d-pantothenate (B_5)	10mg	10mg	125mg	1mg

Table 3.2 Gradient Elution Program of HPLC

Time	Solvent A (%, v/v)	Solvent B (%, v/v)	pH
0	100	0.0	2.77
5	97.3	2.7	2.77
8	95.0	5.0	2.80
9	65.0	35.0	2.94
13	52.0	48.0	3.16
15	51.9	48.1	3.17
19	51.9	48.1	3.17
21	51.8	48.2	3.18
22	51.8	48.2	3.18
25	0	100	6.30
35	0	100	6.30
40	100	0	2.77

Table 3.3 Molecular Information and Response Characters of Analytes

Analyte	Molecular weight	Ionization mode	Selected ion for quantification	Retention time[a]	Other ion
Ascorbic acid	176	ESI+	177	5.66 ± 0.040	141
Thiamine	337	ESI+	133	3.85 ± 0.005	265
Riboflavin	376	ESI+	377	17.15 ± 0.014	-
Nicotinamide	123	ESI+	123	8.17 ± 0.024	-
Nicotinic acid	124	ESI+	124	7.11 ± 0.013	-
Pyridoxine	169	ESI+	170	10.56 ± 0.035	-
Folic acid	441	ESI+	442	15.41 ± 0.027	-
Biotin	244	ESI+	242	17.07 ± 0.010	-
Pantothenic acid	219	ESI+	220	14.59 ± 0.005	-

[a] Represents the standard deviation of the retention time (n=6).

Table 3.4 System Stability Test Results for LC/MS

Vitamin	Retention time (min)[a]	Retention factor (k)[b]	Selectivity (α)	Resolution (Rs)	Plate count (N)	Symmetric factor (As)
B1	3.89±0.047	0.41	-	-	4163	0.59
C	5.56±0.05	1.08	2.63	4.07	1499	0.76
B_3 (acid)	7.02±0.059	1.34	1.24	2.65	2866	0.50
B_3 (amide)	8.08±0.061	1.99	1.84	2.14	2951	0.53
B_6	10.21±0.05	2.87	1.44	3.03	1877	0.32
B_5	14.59±0.005	4.34	1.47	7.48	65120	0.70
B_9	15.43±0.006	4.65	1.07	2.77	29959	0.75
B_7	17.05±0.006	5.25	1.13	4.01	25846	0.76
B_2	17.22±0.004	5.28	1.01	0.37	14280	0.73

[a] Represents the standard deviation of the retention time (n=5).

[b] The retention time of void peak is 3.06 min.

Table 3.5 System Stability Test Results for DAD

Vitamin	Retention time (min)[a]	Retention factor (k)[b]	Selectivity (α)	Resolution (Rs)	Plate count (N)	Symmetric factor (As)
B_1	3.08±0.001	0.15	-	1.32	1533.24	2.85
C	4.04±0.036	0.51	3.40	2.04	1078.47	0.85
B_3 (amide)	5.87±0.007	1.20	2.33	2.43	901.88	2.74
B_6	6.91±0.0.49	1.59	1.33	1.43	3634.63	2.80
B_5	13.01±0.004	3.87	2.44	16.48	93842.79	1.94
B_{12}	13.24±0.022	3.96	1.02	0.93	43208.91	1.50
B_9	13.52±0.047	4.06	1.03	0.81	28065.26	2.96
B_7	14.94±0.022	4.60	1.13	4.83	102633.68	1.12
B_2	15.17±0.021	4.68	1.02	0.55	18179.16	3.34

[a] Represents the standard deviation of the retention time (n=5).

[b] The retention time of void peak is 2.67 min.

Table 3.6 System Stability Test Results for ELSD

Vitamin	Retention time (min)[a]	Retention factor (k)[b]	Selectivity (α)	Resolution (Rs)	Plate count (N)	Symmetric factor (As)
B_1	3.13±0.009	0.12	-	2.26	9430	1.42
C	3.80±0.031	0.36	3.00	4.79	9981	1.33
B_3 (amide)	5.72±0.023	1.05	2.91	11.2	18807	0.87
B_6	6.52±0.047	1.33	1.27	2.85	4380	2.24
B_5	13.05±0.006	3.67	2.76	25.34	180581	1.57
B_{12}	13.25±0.096	3.79	1.03	1.75	136472	1.42
B_9	13.58±0.008	3.86	1.02	2.01	136621	1.45
B_7	15.01±0.011	4.35	1.13	9.37	130119	1.82
B_2	15.22±0.009	4.42	1.02	1.29	116127	1.01

[a] Represents the standard deviation of the retention time (n=5).

[b] The retention time of void peak is 2.80 min.

Table 3.7 Calibration Data, LOD, LOQ of Analytes

Analyte		Number of data point	Range (mg/L)	Linearity		Analytical measurement limits	
				y=ax+b	r^2	LOD (µg/L)	LOQ (µg/L)
MS	B_3	8	50-100	y = 65167x + 7E+06	0.992	0.25	5
	B_9	8	0.5-6.25	y = 45520x + 14725	0.996	1	5
	B_7	7	0.1-3	y = 165890x + 19157	0.995	25	100
	B_5	6	10-30	y = 12576x + 264189	0.990	10	50
						LOD (mg/L)	LOQ (mg/L)
DAD	B_3	15	10-150	y = 30042x + 29426	0.997	0.05	0.1
	B_9	15	0.5-15	y = 67852x + 3832.2	0.998	0.1	0.5
	B_7	4	22-18.5	y = 9088.4x + 5253.3	0.990	1	5
	B_5	7	50-100	y = 7035.3x - 103276	0.990	10	30
ELSD	B_3	10	20-500	y = 29698x - 3E+06	0.992	25	30
	B_9	8	10-22.5	y = 75228x - 457667	0.997	9	15
	B_7	7	10-25	y = 45426x - 269456	0.995	9	15
	B_5	7	20-70	y = 63127x - 923395	0.992	5	10

Table 3.8 The Precision of Method Was Evaluated by Vitamin Analysis for Repeatability and Reproducibility by LC/DAD/ELSD and MS.

Vitamins	MS				DAD				ELSD			
	Repeatability		Reproducibility		Repeatability		Reproducibility		Repeatability		Reproducibility	
	Retention time %RSD	Area %RSD	Retention time %RSD	Area %RSD	Retention time %RSD	Area %RSD	Retention time %RSD	Area %RSD	Retention time %RSD	Area %RSD	Retention time %RSD	Area %RSD
B_3	0.702	1.44	1.60	1.97	0.667	2.64	0.371	1.60	0.395	1.48	0.197	4.75
B_9	0.327	3.42	0.135	2.76	0.016	4.51	0.186	2.14	0.151	1.80	1.74	1.72
B_7	0.247	1.50	0.136	0.152	0.024	4.10*	0.433	4.55*	0.070	0.47*	0.173	0.62*
B_5	0.230	2.77	0.188	2.82	0.361	2.61	0.449	2.22	0.056	4.56*	0.23	4.03*

*The values were tested by vitamin standards.

Table 3.9 Declared and Found Vitamin Concentrations in Multivitamin/ B-Complex Tablets, in Vitamin Drink Powder and Vitamin Drink (n=5)

Detectors	Vitamins	Sample A (mg/g)		Sample B (mg/g)		Sample C (mg/g)		Sample D (mg/L)	
		Declared	Found	Declared	Found	Declared	Found	Declared	Found
MS	B_3	28.35	31.01	1.968	1.79	74.27	71.89	8.44	13.92
	B_9	5.67	9.58	0.984	1.40	74.27	90.08	4.22	8.02
	B_7	0.034	0.017	0.003	0.002	0.074	0.006	0.127	ND
	B_5	0.227	0.221	0.049	0.041	0.238	0.083	Nd	Nd
DAD	B_3	28.35	30.16	1.968	1.957	74.27	71.06	8.44	ND
	B_9	5.67	7.32	0.984	1.748	74.27	48.32	4.22	ND
	B_7	0.034	ND	0.003	ND	0.074	ND	0.127	ND
	B_5	0.227	0.249	0.049	0.062	0.238	0.101	nd	Nd
ELSD	B_3	28.35	34.52	0.984	ND	74.27	74.63	8.44	14.35
	B_9	5.67	9.69	0.003	2.75	74.27	57.40	4.22	ND
	B_7	0.034	ND	0.049	ND	0.074	ND	0.127	ND
	B_5	0.227	ND	1.968	ND	0.238	ND	nd	nd

nd: not declared, ND: not detect

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