SEPARATION AND QUANTIFICATION OF FOURTEEN CHEMICAL FORMS OF FAT SOLUBLE VITAMINS IN FOOD MATRICES USING SOLID PHASE EXTRACTION COUPLED WITH LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE GRADUATE SCHOOL OF THE

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DEPARTMENT OF NUTRITION AND FOOD SCIENCES

COLLEGE OF HEALTH SCIENCES

BY

PRIYANKA MATHUR B.Sc., M.Sc., M.S.

DENTON, TEXAS

DECEMBER 2014

TEXAS WOMAN'S UNIVERSITY

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To the Dean of the Graduate School:

I am submitting herewith a dissertation written by Priyanka Mathur entitled "Separation and Quantification of Fourteen Chemical Forms of Fat Soluble Vitamins in Food Matrices Using Solid Phase Extraction Coupled With Liquid Chromatography – Mass Spectrometry". I have examined this dissertation for form and content and recommend it to be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Nutrition and Food Sciences.

Nancy DiMarco, Ph.D., Major Professor

We have read this dissertation and recommend its acceptance:

Richard Sheardy, Ph.D.

Cynthia Warren, Ph.D.

Manal Omary, Ph.D.

Rene Scott, Ph.D.

Department Chair

Accepted:

Dean of the Graduate School

DEDICATION

For my family, friends, and roommates,

thank you all for your never-ending patience and support.

ACKNOWLEDGEMENTS

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Finally, I would like to thank my family, friends, and roommates for all their patience and cooperation throughout my research.

ABSTRACT

PRIYANKA MATHUR

SEPARATION AND QUANTIFICATION OF FOURTEEN CHEMICAL FORMS OF FAT SOLUBLE VITAMINS IN FOOD MATRICES USING SOLID PHASE EXTRACTION COUPLED WITH LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY

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A solid-phase extraction (SPE) method was developed for separation and quantification of vitamin A (retinol, beta-carotene, retinyl-palmitate, retinyl-acetate), vitamin D (D_2, D_3), vitamin E (α,β,δ,γ tocopherols, tocopheryl-acetate), and vitamin K (K_1, K_2, K_3) from different food matrices. The polarities of all fourteen fat-soluble vitamins (FSV's) were determined and chemical properties of many different organic solvents were studied. Quantification was conducted using liquid chromatography-mass spectrometry (LC-MS). All fourteen fat-soluble vitamins were found to be non-polar in nature; beta-carotene was found to be the most non-polar vitamin in the group, followed by vitamin esters. Tocopherols, ketones (vitamins K_1, K_2, K_3), and alcohols (vitamins D_2, D_3, and retinol) were found to be relatively more polar. All fourteen forms were soluble in dimethyl sulfoxide (DMSO) and were thus extracted with DMSO and methanol from food matrices.

To determine the recoveries of known concentrations of vitamin standards methanol and water were found to be suitable polar solvents for conditioning of the SPE column. Hexane was used for elution. This methodology was further applied to separate fourteen forms of FSV's from food matrices. 80-100% recoveries of FSV's were observed after SPE of provitamin samples (containing 2-3 ingredients). Poor recoveries of FSV's were observed after SPE of complex food samples such as multivitamin capsules, vitamin fortified drink mixes, and chewable nutrition tablets, which consisted of many different ingredients with varying polarities. The presence of other ingredients interfered with the SPE process leading to the elution of other compounds in the hexane solution along with FSV's.

Repeated measures ANOVA compared means of total FSV's after SPE and total FSV's from food labels. No significant differences were observed in the means ($p \leq 0.05$). A strong reliability ($\alpha > 0.9$) was observed between the three SPE extractions from each food sample. This SPE methodology can therefore be used for consistent and efficient separation of FSV's from products which do not have compounds that interfere with the extraction process.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Vitamins include a broad range of organic compounds that are vital for the normal functioning, growth, and maintenance of the human body. They can be classified into two main groups: water soluble and fat soluble vitamins. The water soluble vitamins include the B-group vitamins and vitamin C. The fat soluble vitamins (FSVs) are A, D, E and K. These compounds play a significant role in the metabolic processes of humans, and their deficiencies in the body can lead to development of various health disorders (Chatzimichalakis et al., 2004; Moreno and Salvado, 2000). The diet is the main source of vitamins for the body. However, some foods that contain these vitamins may have reduced vitamin content due to losses during processing, storage, or traditional cooking methods. Moreover, certain staple diets lack the presence of all the essential micronutrients, and therefore put these individuals at risk of development of deficiency disorders.

The rising consumer interest in consumption of a balanced and healthy diet has led to an increase in the demand for multivitamin supplements and vitamin-fortified foods. As a result, many people consume these products to supplement their diets to meet the daily recommended requirements of vitamins. It is therefore important to develop vitamin assessment methods in fortified foods and nutritional supplements, not only to provide information to consumers, but to also control the intake of vitamins in the diet especially if supplements are consumed along with the

regular diet. Further, the federal food labeling regulations also require quantitative information of the nutritional content, per specified weight, be mentioned on the nutrition label of the product (Breithaupt and Kraut, 2006; Code of Federal Regulations, 2013).

Commonly used fat soluble vitamins for fortification include vitamin A, which is usually added in its palmitate or acetate form; vitamin E, which is mainly used in its acetate form; and Vitamin D, which is usually added as vitamin D_3 (cholecalciferol). It is important to study the physical and chemical properties of each of these fat soluble vitamins and their various bound forms, to separate them most efficiently from different food systems (Breithaupt and Kraut, 2006).

Various analytical methods have been developed for quantification of fat soluble vitamins, including capillary electrophoresis, colorimetry, fluorimetry, chromatography, and spectrophotometry (Delgado-Zamarreño et al., 2002; Tütem et al., 1997; Pérez-Ruiz et al., 1999; Amin, 2001; Heudi et al., 2004). Currently the most widely used technique for separation of vitamins and vitamin esters is high performance liquid chromatography (HPLC). HPLC is a rapid, sensitive and accurate method for quantification of vitamins. It is economical to use, and can be combined with other techniques of separation, and further requires small amount of samples. Further, most oil-based samples can be directly injected in the HPLC system without sample preparation, and only dilution of the oil sample.

One major disadvantage of using HPLC for vitamin quantification is it can only measure a few vitamins at a time (Po et al., 1997). Simultaneous determination

of vitamins is a challenge due to their different chemical properties including polarities. Vitamins can be extracted simultaneously from complex matrices using gradient elution programs. In such a case, the mobile phase may be a mixture of three to four different solvents (van Vliet et al., 1991). Vitamin assay kits are also used extensively for quantification of vitamins. These kits include all the reagents required for preparation of samples. For example, precipitation reagents help in the precipitation and removal of substances with higher molecular weights, such as proteins. After treatment with precipitation reagents, the sample is centrifuged, and the supernatant is injected in the HPLC system. These assay kits are quick and easy to use, but are very expensive and can only analyze one vitamin at a time.

Another challenge in the quantification of FSVs is the interference of other food components such as fatty acids, and other nutrients bound to fatty acids during the extraction process. Traditionally, alkaline saponification is used to remove free fatty acids from mono, di, and triglyceride molecules in the sample matrix. These compounds interfere in the extraction of FSVs from the food matrix. In the saponification process, the sample is treated with a strong alkali such as sodium or potassium hydroxide, at ambient or elevated temperatures. The breakdown of fats at high temperatures, and saponification of free fatty acids helps in the removal of interfering fat compounds. Saponification however, leads to oxidation of vitamins, thereby leading to poor recoveries of FSVs. Also, it is a laborious time-consuming extraction process (Xue et al., 2008). The conventional liquid extraction method involves the use of organic solvents such as hexane, ether, or chloroform, to extract the fat soluble vitamins. This process leads to excessive solvent consumption and

incomplete extraction (Yakushina and Taranova, 1995; Wieli ski and Olszanowski, 2001).

One methodology that can be used to overcome most of these shortcomings is solid phase extraction. Solid phase extraction is a rapid, cost-efficient method that can be used to remove interferences by using minimal solvent, and can be used to simultaneously separate and quantify different vitamins from food matrices. It can also be combined with liquid chromatography to get more accurate results (Chatzimichalakis et al., 2004). Solid phase extraction has been used extensively for separation of fat soluble vitamins. Xue et al., 2008 have reported the simultaneous extraction of five fat soluble vitamins (menadione, retinyl acetate, cholecalciferol, alpha tocopherol and alpha tocopherol acetate) from feed, and quantification of those vitamins using HPLC. Further, Chatzimichalakis et al., 2004 have reported the extraction of eight fat soluble vitamins (retinol, menadione, menaquinone, alpha-tocopherol, cholecalciferol, delta-tocopherol, alpha-tocopherol acetate and phylloquinone) from biological fluids, using solid phase extraction coupled with HPLC with a photodiode array detector. Furthermore, Iwase, 2002 reported the extraction of vitamin A and beta carotene from nutritional supplements using solid phase extraction and HPLC.

The objective of this study was to develop a solid phase extraction method that could be used for the separation and quantification of 14 chemical forms of FSVs that are commonly added in nutritional supplements and vitamin fortified foods. There was no study in the literature that used solid phase extraction technique that could be used for the extraction of fourteen different forms of fat soluble vitamins. This study

could be beneficial to the food industry, as it could be a rapid and cost-effective method for vitamin analysis. Further, this method would allow simultaneous quantification of all 14 different forms of fat soluble vitamins, with a minimal number of solvents.

CHAPTER II

REVIEW OF LITERATURE

Fat Soluble Vitamins

Fat soluble vitamins include vitamins A, D, E and K. These vitamins are present in various bound forms which have different chemical properties.

Vitamin A

Structure and general properties. Vitamin A (Figure 1), refers to a group of compounds including retinoids (retinol, retinal, and retinoic acid), and carotenoids (beta carotene). In animal tissues, vitamin A activity is primarily in the form of retinol or its esters, retinyl palmitate and retinyl acetate (Figures 2 and 3), retinal or retinoic acid. Retinyl acetate and retinyl palmitate are synthetic forms of vitamin A which are used widely for food fortification. Beta carotene and other carotenoids are mainly derived from plants. Some carotenoids can be converted to retinol in the body. These are known as provitamin-A carotenoids (Groff, 1995).

A compound needs to have at least one non-oxygenated β ionone ring, and an isoprenoid side chain terminating in an alcohol, aldehyde or carboxyl group to exhibit provitamin-A activity. Beta-carotene exhibits maximum provitamin activity, as compared to other carotenoids. However, in case the beta carotene molecule undergoes oxidation, both the rings on the molecule become oxygenated, and this limits its provitamin activity. Dietary beta carotene is cleaved in the body to release

two molecules of retinol; however, due to the inefficiency of the process, it produces only half the activity of vitamin A, as compared to retinol. The activity of all vitamin A compounds is measured by determining the retinol activity equivalents for each compound (Damodaran, 2008).

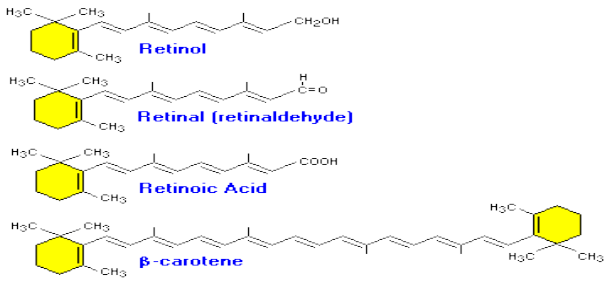


Figure 1: Structure of vitamin A and its different forms

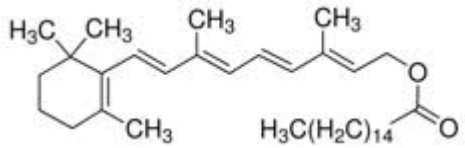


Figure 2: Structure of retinyl palmitate

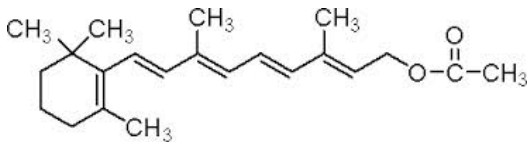


Figure 3: Structure of retinyl acetate

Retinoids and provitamin compounds are non-polar compounds, and are therefore associated with lipid components of cells and cell organelles. In food products, these compounds are dispersed in lipids and fat globules present. Vitamin A compounds are sensitive to oxidation reactions, just like unsaturated lipids. Autooxidation usually begins at the isoprenoid side chain of these compounds.

Vitamin A also has the tendency of undergoing photodegradation on exposure to light.

Functions. Retinyl esters are stored in the retina, and are eventually broken down and isomerized to form 11-cis retinol. This compound binds with a protein called opsin, to form the visual pigment rhodopsin. Rhodopsin and rod cells function together to detect light, and are therefore responsible for maintaining vision (Ross, 1999). Isomers of retinoic acid function as hormones and regulate gene expression (Semba, 1998). Vitamin A also plays a pivotal role in maintenance and functioning of the immune system. Retinol helps in differentiation of white blood cells, and maintains the integrity of other cells that are involved in the body's defense system. Further, retinol and retinoic acid are involved in embryonic development, and regulate the gene that is responsible for functioning of growth hormone (Semba, 2001). Retinoids are also responsible for normal differentiation of stem cells into red blood cells (Ross, 1999).

Recommended dietary allowance. The recommended dietary allowance for vitamin A is 900 mcg retinol activity equivalent (RAE) (3000 IU) per day in adult females, and 700 mcg RAE (2333 IU) per day in adult males. The tolerable upper limit has been given as 3000 mcg RAE (10, 000 IU) per day. One mcg RAE corresponds to 1 mcg retinol, 12 mcg of beta-carotene, and 24 mcg of alpha carotene (Food and Nutrition Board, 2001).

Food sources. Vitamin A is usually present as retinyl palmitate (a precursor and storage form of retinol) in animal foods. In plants, especially yellow and orange

vegetables and fruits, it is present as carotenoids. Carotenoids are precursors for vitamin A. Green vegetables also contain carotenoids; however, the color of this pigment is masked by the green color of chlorophyll (Groff, 1995).

Vitamin D

Structure and general properties. Vitamin D activity in foods is observed in two different forms (Figure 4): vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Ergocalciferol is derived from plants, which produce ergosterol. Ergosterol is converted to ergocalciferol in the presence of ultraviolet light. Cholecalciferol, on the other hand, is usually synthesized in the human skin upon exposure to ultraviolet radiation or sunlight, from 7-dehydrocholesterol (Damodaran, 2008; Hollick, 2003).

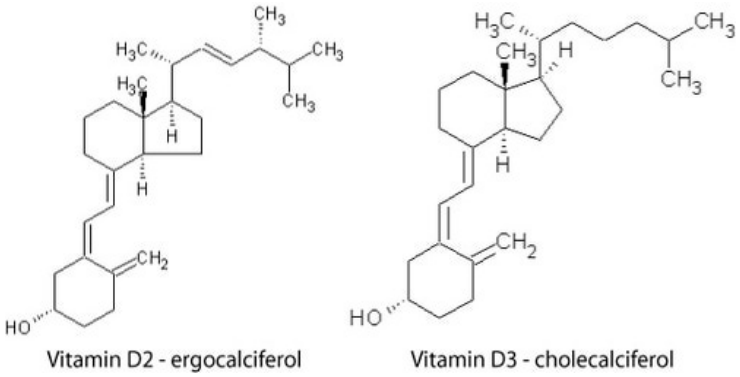


Figure 4: Structure of Vitamin D

Functions. Vitamin D_3 is hydroxylated in the liver to produce 25-hydroxy vitamin D (calcidiol), which is once again hydroxylated in the kidney to produce 1, 25-dihydroxy vitamin D (calcitriol). Calcitriol is the physiologically active form of vitamin D in the body (Hollick, 2003). Vitamin D is known for maintaining calcium levels in the body, which are required for the maintenance of the nervous system,

bone growth, and maintenance of bone density (Hollick, 2004). The parathyroid glands secrete parathyroid hormone if serum calcium levels drop too low. This leads to increased production of 1, 25 dihydroxy vitamin D which helps increase absorption of calcium from the kidneys (Hollick, 2003). Vitamin D_3 is also known to play a role in cell differentiation and proliferation (Hollick, 2004). Further, vitamin D_3 helps maintain the immune system, by enhancing innate immunity and lowering the risk of development of auto-immunity (Griffin et al., 2003). Some other functions of vitamin D_3 include maintenance of blood pressure and insulin secretion in the body (Sigmund, 2002; Zeitz et al., 2003).

Recommended dietary allowance. The recommended dietary allowance for vitamin D is 15 mcg (600 IU) per day for both adult males and females. The tolerable upper limit for vitamin D has been given as 100 mcg (4000 IU) per day for adults (Food and Nutrition Board, 2001). One mcg cholecalciferol is equivalent to 40 IU vitamin D.

Food sources. Foods containing vitamin D include some fatty fish such as mackerel, salmon, and sardines, fish liver oils, and eggs from hen which are fed a vitamin D rich diet. Milk, breads, cereals, and orange juice are usually fortified with vitamin D (Food and Nutrition Board, 1999).

Vitamin E

Structure and general properties. Vitamin E is the general term for a family of eight antioxidants including alpha, beta, gamma, and delta tocopherols, and alpha, beta, gamma, and delta tocotrienols. Alpha tocopherol is the biologically active form

of vitamin E which is found in the blood and tissues (Traber, 1999). The alpha, beta, gamma, and delta forms of tocopherols differ depending on the number and position of the methyl groups in their structures (Figure 5). The beta and gamma tocopherols have two methyl groups each, at different positions. They are therefore referred to as isomers. The other tocopherols including alpha and delta tocopherols, alpha and beta tocopherols, alpha and gamma tocopherols, and beta and delta tocopherols are referred to as homologues, as they differ only in the number of methyl groups in their structures.

The naturally occurring configuration of alpha tocopherol (RRR-α-tocopherol) has the maximum activity. The R form refers to the chiral configuration of the 2', 4', and 8' positions of the carbon atoms in the structure of the tocopherol molecule (figure 5). The other configurations which include the S form are referred to as the synthetic forms of tocopherols. The synthetic forms of vitamin E are used in food fortification. Tocopheryl acetate is a compound which is formed when an acetic acid molecule reacts with the phenolic group of the tocopherol molecule leading to the formation of an ester bond (Figure 6). This introduces stability in the molecule as it eliminates the radical quenching activity of the tocopherol molecule (Damodaran, 2008).

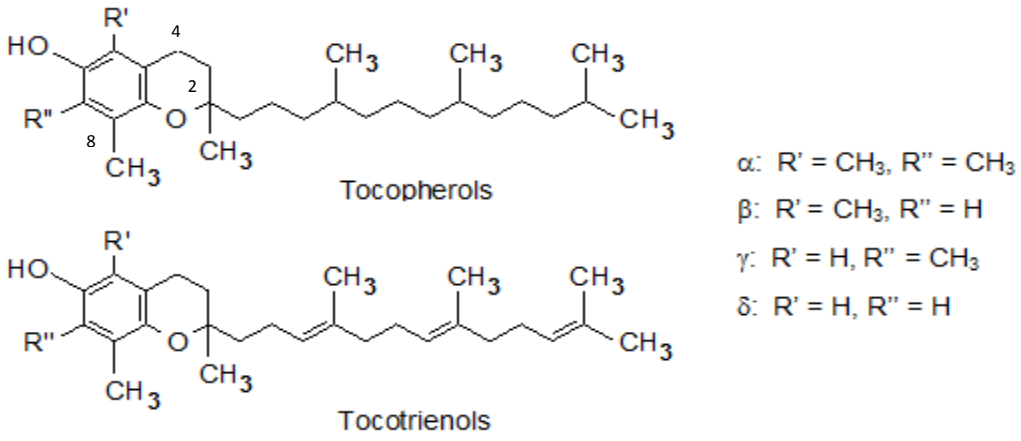


Figure 5: Structures of tocopherols and tocotrienols

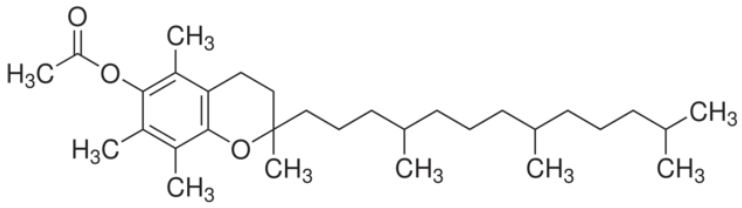


Figure 6: Structure of tocopheryl acetate

Functions. Tocopherols function as antioxidants, as they react with free radicals by donating an hydrogen atom and an electron from the phenolic group in their structures. They are constituents of biological membranes, and maintain membrane stability by functioning as antioxidants. Alpha-tocopherol is a lipid soluble vitamin which is present in the lipid components of the cells, and helps in the prevention of oxidative deterioration of these lipids. Alpha tocopherol also prevents oxidation of low density lipoproteins, which leads to the development of various cardiovascular risk factors (Traber, 2006; Bruno et al., 2006). Alpha-tocopheryl acetate exhibits antioxidant activity when the ester bond is cleaved due to enzymatic

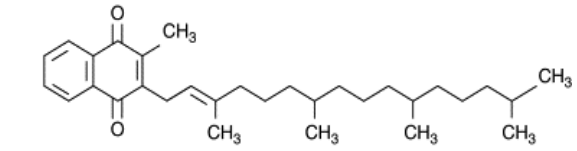
action within the cells. Alpha-tocopherols have also been used for the curing of bacon, as they prevent the formation of nitrosamines (Damodaran, 2008).

Recommended Dietary Allowance. The recommended dietary allowance for vitamin E is 15 mg (22.5 IU) per day for both adult males and females. The tolerable upper limit of intake for vitamin E is 1000 mg (1500 IU) per day for adults. 100 IU of natural vitamin E provides 67 mg of RRR-alpha tocopherol, and 100 IU of synthetic vitamin E provides 45 mg of RRR-alpha tocopherol (Food and Nutrition Board, 2001).

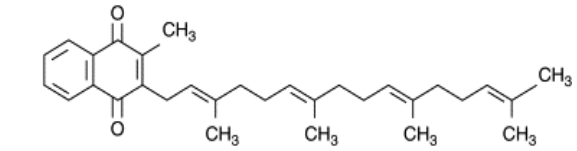
Food sources. Vegetable oils such as olive, sunflower, and safflower oils, are the major sources of alpha tocopherol in the diet. Alpha tocopherol is also found in nuts, whole grains, and green leafy vegetables (Food and Nutrition Board, 2001).

Vitamin K

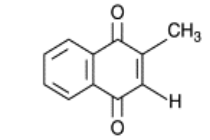
Structure and general properties. Vitamin K refers to group of compounds (Figure 7) including naturally occurring phylloquinone (vitamin K_1) and menaquinone (vitamin K_2), and synthetic forms including menadione (vitamin K_3), and menadione-hydroquinone (vitamin K_4). Phylloquinone is derived from plants, mainly green leafy vegetables, while menaquinone is produced from bacterial synthesis by intestinal micro-organisms. Menadione is used in vitamin supplements and for food fortification (Damodaran, 2008).



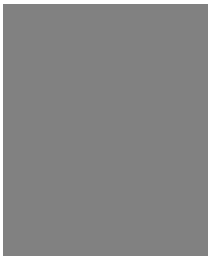
Phylloquinone (vitamin K_1)



Menaquinone-4 (vitamin K_2)



Menadione (vitamin K_3)



Vitamin K4

Figure 7: Structures of vitamins K_1, K_2, K_3, and K_4

Functions. Vitamin K acts as a cofactor for an enzyme that catalyzes a carboxylation reaction, where glutamic acid is converted to gamma carboxyglutamic acid (Furie et al., 1999). This compound binds to calcium and helps in blood coagulation, by activation of vitamin K-dependent clotting factors (Olsen, 1999). Vitamin K also helps in bone mineralization, as vitamin K dependent proteins such as osteocalcin, matrix Gla protein, and protein S are present in bone (Shearer, 1997). Gas6 is another vitamin K-dependent protein that helps in cell signaling and cellular growth (Ferland, 2006).

Adequate intake of vitamin K. The adequate daily intake of vitamin K is 120 mcg /day for adult males, and 90 mcg/day for adult females. No tolerable upper limit of intake has been defined for vitamin K (Food and Nutrition Board, 2001).

Food sources. The major dietary source of vitamin K_1 is phylloquinone. It is present in greean leafy vegetables and vegetable oils such as soybean, cottonseed, canola, and olive oils (Booth et al., 2001).

Physical Properties of Fat Soluble Vitamins

The physical properties of fat soluble vitamins and their various bound forms are indicated in Figure 8. It is important to determine the physical and chemical properties of fat soluble vitamins to separate and quantify them most efficiently in different food matrices (Table 1).

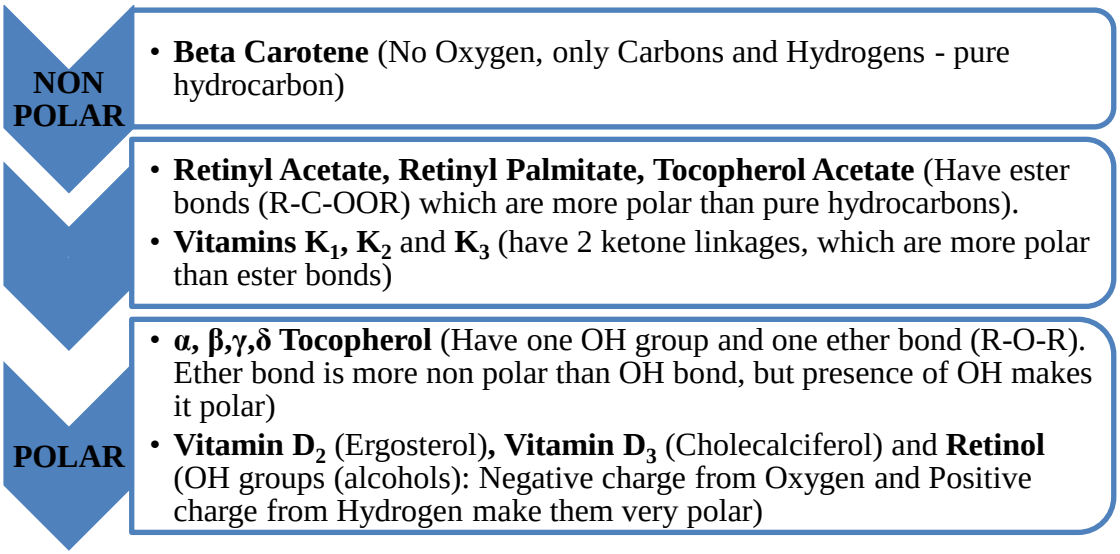


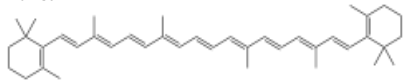
Figure 8: Polarity of fat soluble vitamins

Table 1

Physical Properties of Fat Soluble Vitamins

1. FUNCTIONAL GROUP – HYDROCARBON (NO OXYGEN)

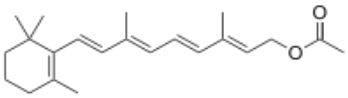
BETA CAROTENE
$C_{40}H_{56}$



Soluble in diethyl ether, acetone, benzene, choloroform, fats. Insoluble in cold/hot water.

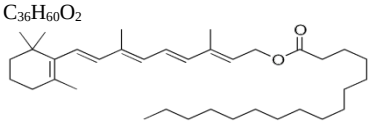
2. FUNCTIONAL GROUP – ESTER (R-C-OOR)

RETINYL ACETATE
$C_{22}H_{32}0_2$



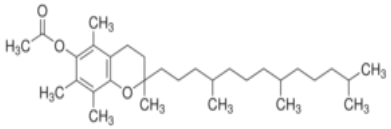
Insoluble in cold/hot water. Soluble in ethanol, chloroform. Slightly soluble in ethanol.

RETINYL PALMITATE
$C_{36}H_{60}O_2$



Insoluble in cold/hot water. Soluble in diethyl ether.

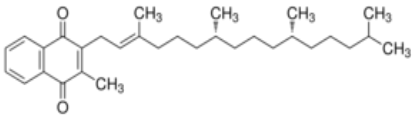
TOCOPHEROL ACETATE
$C_{31}H_{52}O_3$



Insoluble in water. Unstable in alkaline conditions. Soluble in choloroform, ethanol, ether, acetone, oils

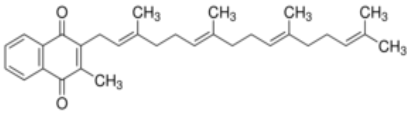
3. FUNTIONAL GROUP – KETONE (R-C(=O)-R)

VITAMIN K_1 (PHYLLOQUINONE)
$C_{31}H_{46}O_2$



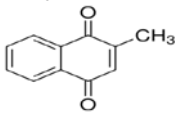
Soluble in ether, acetone, chloroform, slightly soluble in methanol. Insoluble in water

VITAMIN K_2 (MENAQUINONE)
$C_{31}H_{40}O_2$



Soluble in diethyl ether, chloroform and hexane. Insoluble in water

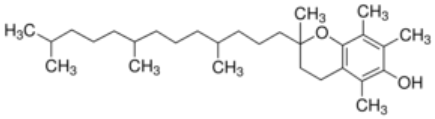
VITAMIN K_3 (MENADIONE)
$C_{11}H_8O_2$



Soluble in chloroform (might require heating), DMSO and ethanol.

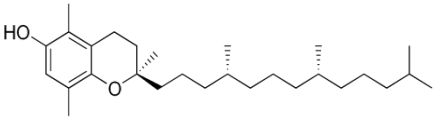
4. FUNCTIONAL GROUP - ALCOHOL (OH) AND ETHER (R-O-R)

ALPHA-TOCOPHEROL
$C_{29}H_{50}O_2$



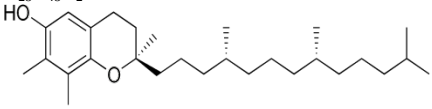
Insoluble in water. Soluble in ether, acetone, choloroform, oils

BETA-TOCOPHEROL
$C_{28}H_{48}O_2$



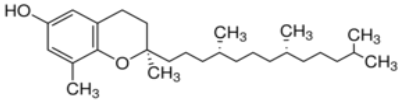
Insoluble in water. Soluble in ethanol, ether, acetone, chloroform.

GAMMA-TOCOPHEROL
$C_{28}H_{48}O_2$



Insoluble in water. Soluble in ethanol, ether, acetone, chloroform.

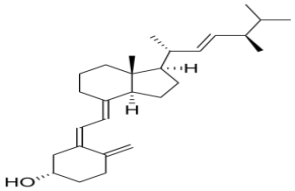
DELTA-TOCOPHEROL
$C_{27}H_{46}O_2$



Insoluble in water. Soluble in ethanol, ether, acetone, chloroform.

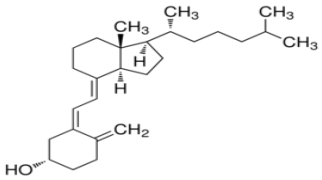
5. FUNCTIONAL GROUP – ALCOHOL (OH)

VITAMIN D_2 (ERGOCALCIFEROL)
$C_{28}H_{44}O$



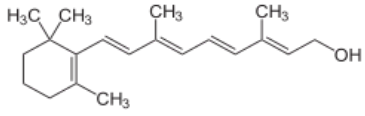
Soluble in acetone, ether, choloroform and alcohol. Insoluble in cold water

VITAMIN D_3 (CHOLECALCIFEROL)
$C_{27}H_{44}O$



Soluble in ethanol, acetone, ether, choloroform. Insoluble in water.

RETINOL
$C_{20}H_{30}O$



Insoluble in water/glycerol. Soluble in ethanol, methanol, chloroform, ether, fats, oils.

Fat soluble vitamins are non-polar in nature. However, depending upon the functional groups present in these vitamins, polarity could vary. Beta carotene is the most non-polar vitamin as it does not have an oxygen molecule in its structure. Similarly, vitamin esters are non-polar compounds due to the presence of ester bonds, as esters bonds tie up the alcohol and fatty acid groups. Tocopherols and retinol are alcohols, and are therefore slightly more polar as compared to vitamin esters and beta carotene. It is therefore essential to determine the appropriate organic solvent that could be effectively used to dissolve these vitamins so they could be separated and quantified.

Chemical organic solvents can be classified into two major types: polar and non-polar solvents. Polar solvents can further be classified into two categories, namely protic and aprotic solvents. Protic solvents contain a hydrogen atom attached to either an oxygen atom (hydroxyl group), or a nitrogen atom (amine group), and can therefore donate hydrogen ions. This property makes protic solvents highly polar in nature. Methanol and ethanol have high dielectric constants and can therefore be used for separation of polar compounds. Aprotic solvents, on the other hand, are unable to donate hydrogen ions. They have intermediate dielectric constants and polarity, and can therefore be used for separation of compounds that are slightly polar in nature (example: Acetonitrile). Non-polar solvents have the lowest dielectric constants and therefore have the least polarity. Hexane, benzene, chloroform and ether are non-polar solvents that are used for dissolving non polar compounds, such as beta carotene, vitamin esters, and other non-polar fat soluble vitamins (Table 2).

Table 2

Polarity of Solvents

Solvent	**Chemical Formula**	**Dielectric constant**
Hexane	CH_3-CH_2-CH_2-CH_2-CH_2-CH_3	2.0
Benzene	C_6H_6	2.3
Chloroform	$CHCl_3$	4.8
Diethyl ether	CH_3-CH_2-O-CH_2-CH_3	4.3
Ethyl acetate	CH_3-C(=O)-O-CH_2-CH_3	6.0
Acetone	CH_3-C(=O)-CH_3	21
Acetonitrile	CH_3-C≡N	37
Dimethyl sulfoxide (DMSO)	CH_3-S(=O)-CH_3	47
Ethanol	CH_3-CH_2-OH	30
Methanol	CH_3-OH	33
Water	H-O-H	80

Analytical Methods for Measurement of Fat Soluble Vitamins (FSVs)

Earlier, the methods that have been used for analysis of vitamins were biological in nature. These procedures were tedious and provided low precision (AOAC International, 1995). Various other analytical methods have been developed for quantification of FSVs over the past few years, including capillary electrophoresis, colorimetry, fluorimetry, chromatography, and spectrophotometry (Delgado-Zamarreño et al., 2002; Tütem et al., 1997; Pérez-Ruiz et al., 1999; Amin, 2001; Heudi et al., 2004). Currently the most widely used technique for separation of vitamins and vitamin esters is high performance liquid chromatography (HPLC). It is economical to use and can be combined with other techniques of separation. Further, it requires small amounts of samples. HPLC coupled with mass spectrometry (MS) provides a rapid, sensitive and accurate method for quantification of FSVs. Mass

spectrometry involves ionization of the analyte. This process deals with the conversion of atoms to ions, which are then measured by the detector. It is important to select the appropriate ionization method to obtain accurate measurements. (Lienau et al., 2002; Wang et al., 2000).

One major disadvantage of using LC-MS for quantification of FSVs is that it measures only few vitamins at a time. The use of atmospheric pressure chemical ionization (APCI) method of ionization improves the sensitivity of measurement of FSVs (Po et al., 1997; Lienau et al., 2002; Wang et al., 2000). Simultaneous determination of vitamins is a challenge due to their different chemical properties and polarities. Vitamins can be extracted simultaneously from complex matrices using gradient elution programs. In such a case, the mobile phase would be a mixture of three to four different solvents (van Vliet et al., 1991).

Vitamin assay kits are also used for quantification of vitamins. These kits contain reagents that are required for sample preparation before injection of the sample in the HPLC system. Following treatment with precipitation reagents, the sample is centrifuged, and the supernatant is injected in the HPLC system. These assay kits are quick and easy to use, but are very expensive and can analyze only one vitamin at a time.

Another method commonly used for removal of interfering materials from FSVs in food samples is saponification. Saponification with a strong alkali such as potassium or sodium hydroxide at ambient or elevated temperatures causes breakdown of fat molecules and removal of free fatty acids. This process however, it

can lead to oxidation of vitamins leading to poor recoveries. It is also a laborious time-consuming extraction process (Xue et al., 2008). The conventional purification method involves the use of organic solvents such as hexane, ether, or chloroform to extract the fat soluble vitamins; however this process can lead to excessive solvent consumption and incomplete extraction (Yakushina and Taranova, 1995; Wieli ski and Olszanowski, 2001).

Solid phase extraction (SPE) is a method that has been used to overcome the disadvantages of other extraction processes. It is a rapid, cost-efficient method that can be used to remove interferences using minimal solvent to simultaneously separate and quantify different vitamins from food matrices. It can also be combined with liquid chromatography to get more accurate and definitive results (Chatzimichalakis et al., 2004).

It has been reported that the use of solid phase extraction can provide efficient for the separation of fat soluble vitamins from food matrices. Simultaneous extraction and quantification of five fat soluble vitamins (menadione, retinyl acetate, cholecalciferol, alpha tocopherol and alpha tocopherol acetate) from feed using SPE coupled with HPLC was reported by Xue et al., 2008. Further, Chatzimichalakis et al., 2004 have reported the extraction of eight fat soluble vitamins (retinol, menadione, menaquinone, alpha-tocopherol, cholecalciferol, delta-tocopherol, alpha-tocopherol acetate and phylloquinone) from biological fluids, using solid phase extraction coupled with HPLC with a photodiode array detector. Furthermore, Iwase, 2002 has reported the extraction of vitamin A and beta carotene from nutritional supplements using solid phase extraction and HPLC.

Solid Phase Extraction

Solid phase extraction (SPE) is an elementary and rapid method of separation of analytes from complex matrices. This technique is used for extraction of nutrients and their various chemical forms and metabolites, from food, drugs, and biological fluids (Simpson and Horne, 1993; Simpson, 2000). SPE is a form of partition chromatography, where the solutes are separated based on their partitioning between a liquid mobile phase and a solid stationary phase. It can be of two types (normal-phase extraction and reverse-phase extraction), depending on the physical properties of the analyte and matrix, in particular, the solubility and polarity of the compounds (Thurman and Mills, 1998).

Types of SPE Methods

Normal-phase SPE is used for the extraction of compounds with relatively high polarity using a polar stationary SPE column and a non-polar mobile phase, such as hexane. The stationary phase is usually made of adsorbent materials such as silica gel, alumina, magnesium silicate, or diatomaceous earth. Polar interactions between the column and the analyte form hydrogen bonds or dipole interactions result in the retention of the analyte on the column. This technique is usually used for extraction of compounds such as plant pigments and pesticides (Table 3).

Reverse phase SPE, on the other hand, is used for the extraction of compounds with low to moderate polarity, or hydrophobic compounds. It uses a relatively non-polar stationary phase, and a relatively polar mobile phase, such as methanol, or acetonitrile. The commonly used reverse phase stationary columns include n-octyl

(C8), n-octadecyl (C18), phenyl, and mixed polymeric phases. In reverse phase chromatography, hydrophobic interactions, such as van der waals forces, are responsible for the partitioning of the compounds. This separation technique is used for pharmaceutical analysis, protein and peptide mapping, soil and water sample analysis, and clinical analysis of blood and urine samples (Skoog et al., 1997).

Table 3

Types of SPE Methods

Column	**Type**	**Stationary phase**	**Mobile phase**	**Loading of sample**
C18	Reverse phase	Non-polar Alkyl hydrocarbon (octadecyl C18)	Polar Mixture of water and a polar solvent such as methanol. Polar compounds elute out first, non-polar compounds elute later. Retention increases, as the amount of water in mobile phase increases. Used for separations based on non-polar differences.	Polar solvent
Silica	Normal phase	Polar	Non-polar Hexane, mixed with a slightly polar solvent, such as isopropanol. Non-polar compounds elute first, polar compounds elute later. Retention decreases as the amount of water in the mobile phase increases. Used for analysis of solutes readily soluble in organic solvents, based on their polar differences	Non-polar solvent

Extraction Procedures

The SPE procedure is conducted in the following stages:

Sample dispersion. The first stage of sample extraction using SPE is to dissolve it in a suitable solvent in which the sample is soluble. The dissolution of the sample in the solvent also helps in reducing interferences in the extraction. The solvent system has two main functions; firstly, it helps in dissolution of the sample, and helps to carry it through the SPE column. Secondly, the solvent has to be weak enough to allow the analyte to have affinity for the adsorbent column (Nickerson and Colon, 2011).

Adsorbtion on the SPE cartridge. The adsorbent phase is selected depending on the analyte's physical properties, such as polarity, solubility, and concentration (Majors and Slack, 1997). It is essential to determine the breakthrough volume, which indicates the point at which the analyte can no longer be retained on the column, due to the sample volume at a given concentration exceeding the retention capacity of the adsorbent column. This is achieved by passing analytes of different concentrations through the column. Once the breakthrough volume is achieved, the column starts bleeding and the analyte passes out of the column instead of getting retained on it (Poole, 2002).

Elution. The elution step is the most critical step in solid phase extraction, as it determines the final concentration of the analyte that is separated through the column. It is important to use a strong solvent as it helps in better elution of the analyte and minimizes solvent use. For a reverse phase SPE column, the

recommended elution solvents are methanol, water, ethyl acetate, and acetonitrile. For normal phase SPE columns, hexane, chloroform, and ethyl acetate are generally used for analyte elution (Nickerson and Colon, 2011).

Extraction Methodology

The first step in the solid phase extraction procedure is conditioning of the adsorbent phase (column) with a solvent that has a polarity similar to the polarity of the sample solvent. Conditioning refers to the process of passing a polar solvent through the reverse phase column to clean and activate the stationary phase. Methanol is usually used to condition the column during reverse phase chromatography (Majors and Slack, 1997). The eluant is discarded as waste after the conditioning step. This is followed by loading of the sample onto the column, at a constant flow rate. The analyte is retained on the column, and the eluate from this step is discarded as waste. The next step involves washing of the column to remove any interferences from the sample matrix and the column. It is important to consider using an appropriate solvent for washing the column, as it should not dissolve the analyte of interest and elute it from the column. For reverse phase chromatography, a water solution is usually used to elute out any impurities or interferences from the matrix. The washing step is followed by elution of the analyte with an appropriate solvent that is capable of dissolving the analyte. The eluate from this step is collected and the recovery of the analyte is determined (Figure 9).

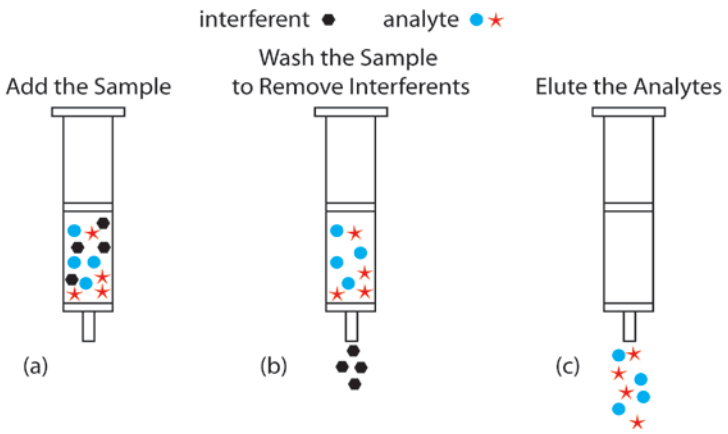


Figure 9: Steps involved in SPE analysis

Advantages of SPE over Other Extraction Methods

Solid phase extraction is one of the most efficient techniques of extraction of compounds. It has various benefits as compared to other extraction methods. First, it requires the use of smaller quantities of solvents as compared to other solvent extraction methods. Further, it is a quicker and more efficient method, and is also known to provide better recoveries of the samples, as compared to other liquid extraction techniques. In addition, there is less chance of sample contamination with this method, and easier automation of the extraction process. It is a simpler method to use, and is capable of concentrating the analytes on the adsorption matrix (Markell et al, 1991; Blevins and Schultheis, 1994; Majors, 1993).

Applications of SPE Method

Solid phase extraction technique is used widely for analysis of pharmaceutical compounds. It is used in the retention of analytes to increase their concentrations for analysis, retention of impurities in the sample matrix to separate them from the

analyte of interest, and removal of any compounds that may interfere with the analysis of the sample (Nickerson and Colon, 2011).

Extraction of Vitamins from Food Samples

The most commonly used method for extraction of vitamins from food and pharmaceutical products is saponification of the sample, to separate the saponifiable compounds from the unsaponifiables, followed by extraction of the vitamins from the unsaponifiable compounds (Holler et al, 2003; Kienen et al, 2008). Solvent extraction is another frequently used method to separate vitamins from the sample matrix (Chavez-Servin et al, 2006; Ye et al, 2000). However, there are many drawbacks associated with the use of these methods. Firstly, saponification of the sample with alkali compounds causes degradation of vitamins leading to losses in the total vitamin content of the sample. Saponification also leads to variations in the recoveries of the vitamins from the sample. In addition, this method is time consuming and has low reproducibility (Luque-Garcia and Luque de Castro, 2001; Turner and King, 2001). The solvent extraction method also has various limitations, including inefficient extraction and low repeatability. Vitamin supplements and most pharmaceutical products contain gelatin, which makes the product stable and resistant to solvent degeneration (Thompson, 1986).

Liquid Chromatography- Mass Spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique that couples physical separation of compounds using high performance liquid chromatography (HPLC), with the determination of the masses of these

compounds, using mass spectrometry (MS). The high sensitivity and selectivity of this method enables it to be used in many applications (Arpino, 1992), such as separation of isomeric and homologue compounds with similar chemical structures and different masses, such as alpha, beta, gamma, and delta tocopherols.

Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is an analytical technique for separation of compounds. It is a widely used method for the analysis of biological, pharmaceutical and volatile environmental compounds. HPLC is a sensitive technique, which is applicable to non-volatile and thermally fragile compounds, and is adaptable for accurate quantitative measurements. Compounds that are commonly analyzed using HPLC include amino acids, proteins, carbohydrates, nucleic acids, hydrocarbons, drugs, pesticides, steroids, antibiotics, metal-organic compounds and inorganic compounds (Skoog et al., 1997).

Principle. There are various kinds of liquid chromatography that are used for determination of compounds. Some commonly used chromatography types include partition chromatography, adsorption chromatography, affinity chromatography, ion-exchange chromatography, and size exclusion chromatography.

Partition chromatography (Figure 10) is used to separate solutes from mixtures based on the partitioning between the liquid mobile phase and liquid bonded stationary phase column. This is a popular method of analysis used for low molecular weight compounds. Partition chromatography is of two types; normal phase chromatography, which involves the use of a non-polar mobile phase, and a polar

stationary phase; and reverse phase chromatography, which involves the use of a polar mobile phase and a non-polar stationary phase (Skoog et al., 1997).

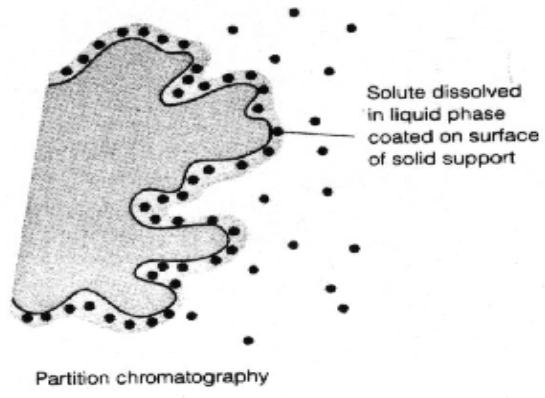


Figure 10: Partition chromatography

Adsorption chromatography is used for separation of solutes from mixtures, by their adsorption onto un-derivatized solid particles. Separation is based on the solutes' different degrees of adsorption onto solid surfaces. The stationary phase is made up of silica and alumina particles. This technique is used for separation of organic compounds and geometric isomers.

Ion exchange chromatography is based on the principle of separation of solutes from mixtures by adsorption onto a support containing fixed charges on its surface. Cation exchangers are stationary phases which have a negative charge, and are used for separation of positively charged ions. Anion exchangers have positively charged ions, and are used for separating negatively charged groups. Ion exchange chromatography is used for separation of organic and inorganic compounds and water deionization and purification.

Size exclusion chromatography, is another widely used type of liquid chromatography, which is used for separating compounds according to their different sizes or molecular weight. Compounds with larger sizes are eluted out first, while the smaller compounds are retained onto the column. Therefore, size exclusion chromatography is used for the separation of low molecular weight compounds, such as proteins and polymers.

Another type of liquid chromatography is known as affinity chromatography. The stationary phase in affinity chromatography is composed of immobilised biological molecules which act as substrates for specific compounds. Compounds which bind to only one or few closely related molecules are known as high-specificity ligands. For example, enzymes have specific substrates or inhibitors that they bind to. Other compounds which have the ability to bind to a family or class of related molecules are known as group-specific ligands. For example, lectins have the ability to bind to a group of compounds such as carbohydrates and glycoproteins (Skoog et al., 1997).

Instrumentation. High performance liquid chromatography is based on the principle of adsorption of solutes onto a stationary phase. A liquid sample is made to pass over a solid adsorbent material that is packed into a column, using a solvent which makes up the mobile phase. All the analytes in the sample interact differently with the stationary phase and are therefore adsorbed onto the column at different retention times. The efficiency of separation is dependent upon the size of the particles that pass through the column. Smaller sized particles improve the efficiency of the column, but require high pressure for separation (Skoog et al., 1997).

Components of an HPLC system. Mobile phase reservoirs – There are one or more glass or stainless steel reservoirs in an HPLC system. The solvent reservoirs are usually equipped with degassers, which are used to remove dissolved gases like oxygen and nitrogen. Degassing involves sparging of the solvent with bubbles of an inert gas of low solubility. A chromatographic separation where only one solvent is used as the mobile phase, is referred to as Isocratic elution. Gradient elution, on the other hand, refers to the use of two or more solvents as mobile phase (Figure 11).

Pumping system. The primary function of a pump is to deliver the mobile phase through the system at a constant flow rate and pressure. The pressure generated by the pump is usually very high (about 6000 psi) and the flow rate generated usually ranges from 0.1 to 10 mL/min. The pump maintains a pulse-free output and maintains flow reproducibility at 0.5% relative or better. There are three types of pumps used in an HPLC system, including reciprocating pumps, syringe or displacement-type pumps, and pneumatic or constant pressure pumps.

Sample injection system. The main function of the sample injection system is to introduce the sample into the column. Injection can be done either manually or automatically.

Column. Typical HPLC columns are made of stainless steel, are 10-30 cm long, with 4-10 mm internal diameter. These columns contain about 40,000 to 60,000 plates per meter. The stationary phase packing is made of micro-porous silica, which can withstand high pressure, and is abundant and inexpensive. Resin based packings can also be used as they can function over a wide range of pH.

Detectors. The common detectors that are used in an HPLC system include refractive index dectector, UV-Vis detector, fluorescence detector, conductivity detector, electrochemical detector, fourier transform infrared spectroscopy (FTIR), mass spectrometer, and light scattering detector (Skoog et al., 1997).

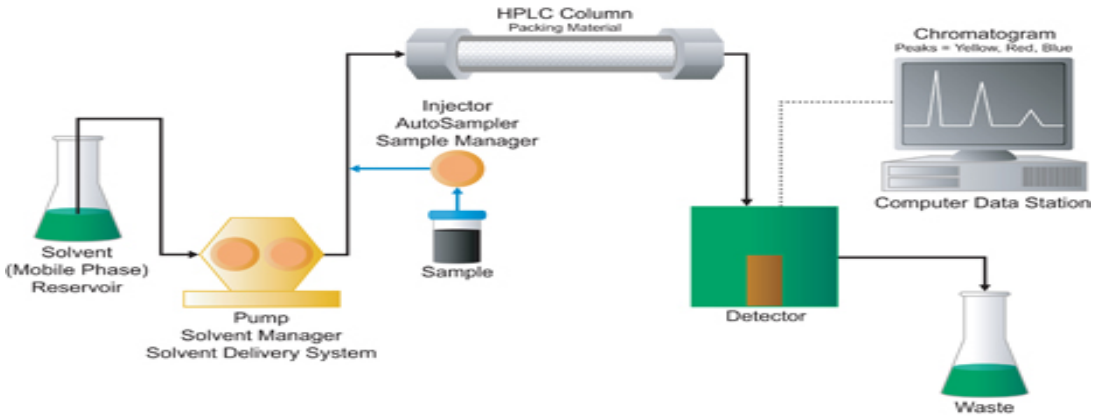


Figure 11: Schematic representation of the components of an HPLC system

Advantages of HPLC over Other Techniques of Analysis

HPLC can be used for any compound that is soluble in a liquid phase. Also, HPLC requires the use of lower temperatures, as compared to other techniques like gas chromatography. This prevents thermal degradation of the sample. Further, the retention of solutes depends on their interactions with both the mobile phase as well as the stationary phase. HPLC is also a non-destructive technique, as compared to other separation methods.

Applications of HPLC

HPLC is widely used for the analysis of vitamins, minerals, fats and oils, antioxidants, packaging materials, food components, food adulterants and pesticides (Skoog et al, 1997).

Mass Spectrometry

Mass spectrometry is a technique of separation that is used to measure the masses of atoms or molecules or fragments of molecules. It measures the atomic or molecular weight of an ion based on its charge to mass ratio. Mass spectrometry is widely used to determine elemental composition of matter, structures of organic, inorganic, and biological compounds, qualitative and quantitative determination of compounds, structure and composition of solid surfaces, and isotopic ratios of atoms in samples (Skoog et al., 1997).

Principle. Electrons with high energy are bombarded onto the analyte molecules in the sample. Once the electrons collide with the analyte molecule, they impart enough energy to the molecules to leave them in an excited state. Once these excited molecules return back to their ground states during relaxation, a part of the analyte molecule fragments, to produce ions of lower masses. Some other positively charged ions are also formed in smaller amounts during this process. These positive ions produced due to electron impact pass through the slit of the mass spectrometer, where they are sorted according to their charge to mass ratios, and displayed in the form of a mass spectrum (Skoog et al., 1997).

Instrumentation. Atomic mass spectrometery involves atomization, followed by conversion of atoms to ions. The ions are then separated based on charge to mass ratio. Measurement of the number of ions is then conducted. It is composed of the following components:

Inlet system. The main purpose of an inlet system is introduction of the sample to be analyzed into the ion source with minimal loss of vacuum (Figure 12). The sample is volatilized externally and then allowed to leak into the ionization region. Different types of inlets are used to accommodate different types of samples. These include batch inlets, direct probe inlets, chromatographic inlets, and electrophoretic inlets. Batch inlet system can be used for both solid as well as liquid analytes. It is the simplest inlet system where the sample is heated to 400°C in a small external oven, and the vapor is made to pass through the analyser. The direct probe inlet system is used for solids and non-volatile liquids, which can be introduced into the ionization source using a sample holder or probe. This probe can be heated or cooled. Less amount of sample is required and there is less waste. The sample vial is introduced into the ionization chamber where the sample is vaporized. Chromatographic inlets are used for liquid and gas chromatography. A jet separator is used for this purpose which is used for separating the analyte from the carrier gas. The heavier sample travels in a straight line in the jet stream, while the carrier gas gets deflected (Skoog et al., 1997).

Ion source. Ion sources ionize and fragment the molecules of the analyte to form gaseous ions, which are then focused into a beam of ions, which pass through the mass spectrometer. Hard sources produce fragment ions with charge to mass ratio

less than the molecular ion. Soft sources, on the other hand, cause little fragmentation. Ion sources are of two kinds – gas phase and desorption. The gas phase type of ionization source is used mainly for thermally stable compounds with a molecular weight less than 1000 daltons. The sample is first volatilized and then ionized. Examples include electron impact, chemical ionization, and field ionization. The desorption type of ionization sources are used for molecules with molecular weight greater than 10^5 daltons. These are used for solid or liquid molecules which are directly converted into gaseous ions. Examples include field desorption, electrospray ionization, matrix-assisted desorption ionization (MALDI), plasma desorption, fast atom bombardment, thermospray ionization (Skoog et al., 1997).

Mass analyzers. Mass analyzers use electric and magnetic fields to apply a force on charged particles. The function of a mass analyzer is similar to that of a monochromator, the only difference is that dispersion is dependent upon the charge to mass ratio, instead of wavelength. The commonly used mass analyzers include quadrupole mass analysers, time of flight mass analysers, and double focussing analysers.

Ion transducers. The commonly used transducers in mass spectrometry include electron multipliers, which are similar to photomultiplier tubes. They typically have 20 dynodes, and the gain of electrons is about 10^7. The continuous dynode multiplier is a trumpet shaped device which works on the same principle. Faraday cup is another type of transducer where the electrode is surrounded with a cage to prevent escape of electrons.

Pumps. Pumps are used to create a vacuum. They are of two types –oil diffusion pump and turbo pump.

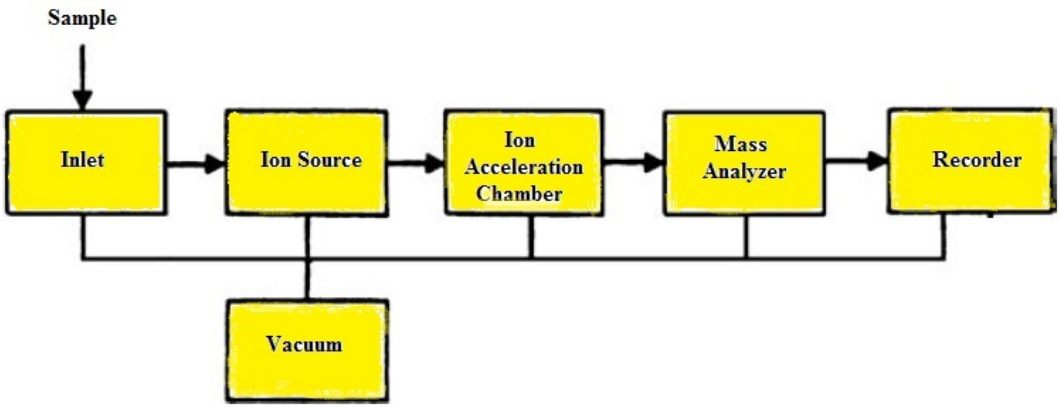


Figure 12: Components of a mass spectrometer

Applications. Mass spectrometry is widely used to determine the elemental composition of matter, structures of organic, inorganic, and biological compounds, qualitative and quantitative determination of compounds, structure and composition of solid surfaces, and isotopic ratios of atoms in samples.

Spectrophotometry

Spectrophotometry refers to the method of analysis based on the interaction, absorption, or production of light by matter. A spectrophotometer measures the intensity of light as a function of its wavelength. A lamp emits radiation, which then passes through a monochromator (a prism or grating) that selects the light of the desired wavelength. This beam of light of the desired wavelength then falls on the sample placed in a cuvette. The amount of light absorbed by the sample, and the

amount of light transmitted is recorded by the detector. Beer's law gives the relationship between absorbance of light and the concentration of the sample.

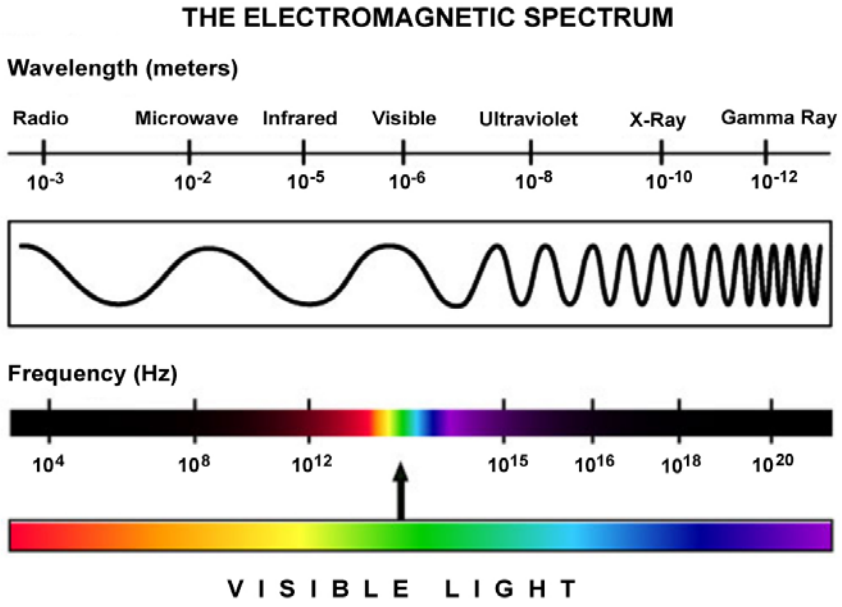


Figure 13: Electromegnetic spectrum

Beer's Law

Beer's law states that when a beam of parallel monochromatic radiation with power P_o, passes through a block of absorbing matter (solid, liquid or gas), its power is decreased to P when it is transmitted from the medium, as a result of absorption of light by the absorbing medium (Figure 14). For monochromatic radiation, the absorbance of light (A) is directly proportional to the path length (b) of the absorbing medium, and the concentration (c) of the analyte. The equation for absorbance is given as follows (Skoog et al., 1997):

A = εbc, where A = Absorbance, ε (epsilon) = extinction coefficient, b = path length of the absorbing medium, c= concentration of the analyte.

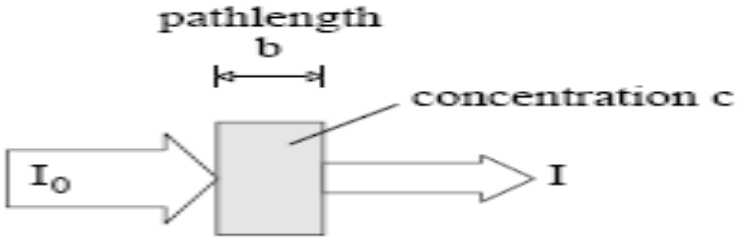


Figure 14: Beer's law

CHAPTER III

METHODOLOGY

Materials and Reagents

The standards for fourteen different forms of fat soluble vitamins (beta carotene, retinol, retinyl acetate, retinyl palmitate, vitamin D_2, vitamin D_3, alpha/beta/gamma/delta tocopherols, tocopheryl acetate, vitamins K_1, K_2, and K_3) were purchased from Sigma Aldrich (St Louis, MO). LC-MS grade solvents (hexane, methanol, chloroform, dimethyl sulfoxide) were purchased from Fisher Scientific (Pittsburg, PA). The solid phase extraction columns (C18 reverse phase columns) were purchased from Phenomenex (Torrance, CA). The food samples were provided by Analytical Food Laboratories (Grand Prairie, TX).

Development of SPE Method

The solid phase extraction method was first optimized to ensure complete extraction of the analytes from the samples. Several different reverse phase SPE cartridges were tested to determine which type and brand of cartridge would be most suitable for the extraction process. Different lengths and volumes of the cartridges were also tested. A standard solution of beta carotene was used for the development of the SPE procedure, as beta carotene is orange. Once this compound is retained on the column, it appears as a bright orange ring on the column. This colored compound

makes it easier for determining the amounts of conditioning, washing and elution solvents, as well as determining the amount of vitamin that would be loaded on the column.

The polarities of organic solvents were determined by experimentation using trial and error. This information was required since SPE requires the use of polar solvents for conditioning of the reverse phase column, and a non-polar solvent for elution of the non-polar fat soluble vitamins. A suitable solvent that had the ability of dissolving all 14 forms of fat soluble vitamins also had to be determined. The solubilities of different solvents such as hexane, methanol, acetonitrile, chloroform, benzene, diethyl acetate, ethyl ether, and dimethyl sulfoxide were tested. The polarities of the fourteen forms of fat soluble vitamins (including beta carotene, retinyl palmitate, retinyl acetate, retinol, tocopheryl acetate, alpha/beta/gamma/delta tocopherols, ergocalciferol, cholecalciferol, and vitamins K_1, K_2, K_3) were also determined by conducting a literature search on the polarities of different functional groups. The solubilities of all these functional groups were also tested by mixing them with different polar and non-polar solvents using trial and error.

Conditioning Stage

Various factors were considered while developing the SPE method. Firstly, during the conditioning stage of the reverse phase column, an appropriate conditioning solvent had to be determined to clean and activate the column. This conditioning solvent had to be slightly polar in nature to be passed through a non-polar reverse phase column. The polarity of this solvent had to be such that it would

dissolve all 14 forms of fat soluble vitamins. The specific amount of solvent to pass through the column was also determined.

Another factor studied was whether the solvent needed to drip from the column under the action of gravity, or whether some external vacuum pressure was required to pull the solvent out of the column. The time taken for the solvent to pass through the column was also standardized.

Loading Stage

The second stage involved loading the vitamin sample solution onto the column. A standard beta carotene solution (concentration: 1mcg/ml) was used to develop this method since it is clearly visible as an orange colored ring once it is loaded on the column. Moreover, beta carotene is also the most non-polar vitamin out of all the fourteen fat soluble vitamins that were being tested.

The beta carotene sample was dissolved in the same polar solvent that was used for conditioning the column. Various solvents were tested for this process, including methanol, dimethyl sulfoxide, acetonitrile, and chloroform. The amount and concentration of the beta carotene sample solution to be loaded on the column was also tested. Trial and error method was used to determine the appropriate concentration of the sample to be loaded onto the column, and the amount to be used for the loading stage (1 ml, 2ml, 3ml, 4ml, 5ml, 6ml, and 10ml). The amount of sample solution to be loaded onto the column was dependent upon the loading capacity of the column, which had to be considered while developing the SPE procedure.

Washing Stage

Once the analyte of interest (beta carotene) was retained on the column, the third stage involved washing the column with water to remove all the polar impurities from the sample. The amount of water required for this step was standardized using trial and error method. Different amounts of water were tested (including 6ml, 5ml, 4ml, 3ml, 2ml, and 1ml). During this step, it was ensured that no loss of analyte occurred from the column during.

Elution Stage

The beta carotene retained on the column was eluted using an appropriate solvent. Various types of solvents with different polarities ranging from polar to very non-polar were tested. Solvents such as hexane, chloroform, methanol, diethyl ether, dimethyl sulfoxide, iso-propanol, pentane, and benzene were tested for this stage (Chatzimichalakis, 2004; Iwase, 2002). The amount of solvent required for the elution process was also tested. Once the eluent was collected in a test tube, a small amount of sodium sulfate was added to the contents of the test tube to absorb any traces of water that could have been pushed out of the column during the elution stage.

Applications of the SPE Method

Recoveries of Vitamin Standard Solutions

The SPE method developed in this study was subsequently used for testing the percent recoveries of known concentrations of fourteen vitamin standard solutions, when each standard solution was loaded on the SPE column individually. After

elution from the column, the percent recovery of each of the analytes was determined using a Thermo Scientific Genesys 10S UV-Vis spectrophotometer. The wavelengths and the extinction coefficients for each of the vitamins were determined through extensive literature search. The readings were taken against a blank of methanol in quartz cuvettes in the spectrophotometer, by conducting a survey scan. The absorbance of the analyte was noted and the concentration of the standard solution was calculated using Beer's law. The percent recoveries of the standard solutions after SPE were then calculated.

Some factors that were considered during this experiment were the concentrations of the vitamin standard solutions (in accordance with the loading capacity of the column), the solubility of the vitamin standards in organic solvents, and the type of spectrophotometric method used (survey scan). The percent recoveries for each of these vitamins were measured using Beer's law: $A = \varepsilon bc$, where A = absorbance, ε = extinction coefficient, b = path length of the absorbing medium, c= concentration of the analyte.

Separation of Fat Soluble Vitamins from Food Matrices

Solvent extraction. The SPE methodology developed in this study was used to separate commonly used fourteen chemical forms of FSVs from different fortified food products and nutritional supplements, such as multivitamin pills, chewable multivitamin tablets, meal replacement shakes, and provitamin powders. 0.1- 0.5 g of the sample was placed in a mortar and pestle (8 inches diameter, 4 inches tall). The amount of sample taken for analysis was dependent on the concentration of fat soluble

vitamins in the sample, and the loading capacity of the SPE column. The sample was ground into a fine powder. A small amount of solvent (about 10 ml) was added to the powder to dissolve the contents and form a paste. The type of solvent required for this process was determined by studying the polarities and solubilities of all of the ingredients of the sample by conducting a literature search and also by experimentation in the laboratory. An appropriate solvent that had the ability of dissolving all the ingredients of the sample was selected and added to the sample to form a paste. This paste was then transferred to a volumetric flask, and the volume was made up using methanol.

Methanol had to be used to make up the volume in the volumetric flask as it is a slightly more polar solvent as compared to the other solvents that were used in this process. Moreover, polar solvents are required for performing reverse phase SPE, and methanol was also the solvent used for conditioning of the column and loading of the sample on the column. Therefore methanol was used to make up the volume in the volumetric flask. A 200 ml volumetric flask was used for most of the samples, as it diluted the concentration of the sample solution to 1mg/ml, which was the loading capacity of the SPE column. For samples such as pro-vitamin powders which were highly concentrated, higher amounts of dilutions had to be prepared to achieve a final concentration of 1 mg/ml for the SPE procedure.

SPE of fat soluble vitamins extracted from food matrices. SPE column was conditioned using the appropriate amount of the conditioning solvent selected through experimentation with different solvents. 1 ml of the sample solution was then loaded onto the column, and the solvent was allowed to drip under the action of gravity. The

time required for this process was standardized for all the vitamin standard solutions as well as the food sample solutions. Once the analyte was retained on the column, the column was washed with water to remove all the polar impurities. The amount of water required for this process was determined by trial and error. Finally, the analyte was allowed to dry, and was then eluted out with an appropriate solvent that had the ability to dissolve the analyte retained on the column. The type and amount of this solvent was determined by experimentation with different solvents (Table 4). Once the analyte was eluted out in an appropriate solvent, the solvent was evaporated under a steady stream of nitrogen, and the residue was reconstituted in methanol. This sample was then analyzed and quantified using a Thermo Scientific Genesys 10S UV-Vis spectrophotometer and a 4000QTrap liquid-chromatography-mass spectrometry system.

Table 4

SPE Conditions for the Separation of Fat Soluble Vitamins from Food Matrices

Sample	Amount	Amount of solvent used for extraction	Conditioning solvent	Loading concentration	Elution solvent
Provitamin Sample (retinyl palmitate)	0.5 g	200 ml (DMSO and methanol)	Methanol Water (5ml)	1mg/ml	Hexane (10 ml)
Provitamin Sample (tocopheryl acetate)	0.1 g	200 ml (DMSO and methanol)	Methanol Water (5ml)	1mg/ml	Hexane (10 ml)
Upcal D Drink Mix	1 packet (5 g)	200 ml (DMSO and methanol)	Methanol Water (5ml)	1mg/ml	Hexane (10 ml)
Chewable Nutrition Supplements	1 tablet	200 ml (DMSO and methanol)	Methanol Water (5ml)	1mg/ml	Hexane (10 ml)
Multiformula Capsules	6 capsules	200 ml (DMSO and methanol)	Methanol Water (5ml)	1mg/ml	Hexane (10 ml)

Quantification of Analytes after SPE

Spectrophotometric Analysis of Vitamin Standard Solutions

A Thermo Scientific Genesys 10S UV-Vis spectrophotometer was used for the measurement of the concentrations of the standard vitamin solutions. Each standard vitamin solution (in methanol) was taken in a glass cuvette and was read at the appropriate wavelength against a blank (methanol). The dilution of the standard vitamin solution in methanol was such that the absorbance reading in the spectrophotometer ranged from 0.5 - 2. Beer's law was used to calculate the concentration of the standard solutions using the equation: $A=\varepsilon lc$, where A= Absorbance, ε= extinction coefficient, l= path length, c= concentration.

LC-MS Analysis of Fat Soluble Vitamins Separated From Food Matrices

The fat soluble vitamins were extracted in triplicate from each of the food samples. The extraction solvent was evaporated and the residue was reconstituted in methanol for injection in a 4000QTrap LC-MS system. Each of the analytes in the samples had to be diluted with LC-MS grade methanol to achieve a concentration ranging between 0-300 ppb, which is the maximum amount that could be injected in the LC-MS column. 950 µL of the sample solution and 50 µL of internal standard (deuterated vitamin D_3) were transferred into a glass vial. A standard curve was prepared using 10 ppb, 25 ppb, 50 ppb, 100 ppb, 200 ppb, and 300 ppb solution of the vitamin to-be-analyzed in different LC-MS vials. 50 µL internal standard solution was

added to each vial. The volumes were made up to 1 ml by adding LC-MS grade methanol (940 µL, 925 µL, 850 µL, 750 µL, 650 µL). 10 µL of each of the standard solutions were injected first, followed by the sample solutions, in a Luna C8 LC-MS column (50* 2.00 mm), at 40°C column temperature

A combination of two mobile phases (acetonitrile/water/formic acid and methanol/formic acid) was used in the system for separation and quantification of the analytes. A quadrupole mass spectrometry system with an APCI (Atmospheric Pressure Chemical Ionization) method was used for ionization of the samples. Different dilutions of the standard solutions were prepared to obtain a standard curve on the LC-MS.

Statistical Analysis

Statistical analysis of the data was conducted using the IBM SPSS statistics software, version 19. One way repeated measures ANOVA was used to compare the mean of the total FSVs mentioned on the nutrition label of each product, with the means of the total FSVs recovered after solid phase extraction in triplicate. A p value ≤ 0.05 was considered to be significant. The internal consistency of the data (amount of FSV recovered in triplicate from each supplement), was analyzed using an internal-consistency-reliability test. Reliability was measured using cronbach's alpha ranging between infinity and one. A good internal consistency and reliability was considered with cronbach alpha value > 0.9. The relationship between the FSV recoveries after the three extractions, and the FSV amounts mentioned on the product labels were tested using regression analysis.

CHAPTER IV

RESULTS AND DISCUSSION

Development of the SPE Method

SPE Cartridges

Several different reverse phase SPE cartridges were tested to determine which type and brand of cartridge would be most suitable for the extraction process. Reverse phase Bond Elute cartridges with plungers, and reverse phase C18 Phenomenex cartridges of different volumes (2 ml, 6 ml, and 10 ml) were tested. It was observed that the Bond Elute cartridges used a plunger with a rubber tip to pass the sample through the column. Since the rubber tip on the plunger was reacting with the organic solvents, the C18 phenomenex cartridges were used for the extraction process, as they required vacuum to pull the solvent out of the cartridge, instead of a plunger. Moreover, phenemenex cartridges were made of polypropylene, which is resistant to most organic solvents.

Phenomenex cartridges with different volumes were also tested to determine which volume would give the best separation. It was observed that a 2ml cartridge was too small to efficiently separate beta carotene from the solution. This was because the column started bleeding every time a concentrated solution of beta carotene was loaded on to it. The most efficient separation was observed using a 6 ml cartridge.

Therefore, C18 phenomenex cartridges, with a loading capacity of 1000 mg / 6ml (maximum loading capacity: 5% of 1000 mg = 50 mg /6 ml), were used for the extraction process.

Polarities of Organic Solvents

The polarities of different organic solvents (including hexane, methanol, acetonitrile, chloroform, benzene, diethyl acetate, ethyl ether, and dimethyl sulfoxide) were determined by conducting a literature search for polarities and dielectric constants of the solvents, and also by trial and error. It was observed that chloroform, ether, ethyl acetate, acetonitrile, dimethyl sulfoxide, and methanol are slightly more polar in nature. However, solvents such as benzene and hexane are non-polar solvents because they are made of hydrocarbon chains and do not contain any oxygen in their structures. The presence of oxygen makes a compound polar because it is electronegative in nature.

The atomic number of oxygen is eight; it consists of eight protons, eight neutrons, and eight electrons. It has six valence electrons. As a result, the oxygen atom requires two more electrons to complete its valence of eight. It shares two of its own electrons with other atoms (example - water molecule) to complete its valence. However, even after sharing two electrons, the oxygen atom is left with two pairs of unpaired electrons. The presence of two pairs of unpaired electrons makes oxygen molecule reactive. It therefore pulls the electron density of the molecule towards itself and makes the molecule more polar (Pauling, 1932; IUPAC, 1997; Pauling, 1960; Greenwood and Earnshaw, 1984).

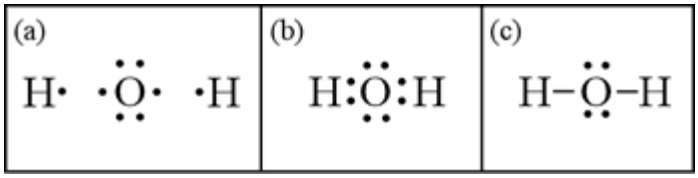


Figure 15: Lewis structures depicting electronegativity of oxygen atom

When two atoms combine to form a molecule by the formation of covalent bonds, the attraction exerted by both the atoms on the bonding pair of electrons determines the polarity of the molecule. In case there is equal attraction from both the atoms, the bond becomes non-polar. In case one atom exerts a stronger pull on the electrons as compared to the other atom, then it makes the bond more polar. This pull is determined by electro-negativity of the atoms. Electro-negativity is the tendency of an atom (when it is bound in a molecule) to attract a bonding pair of electrons (or electron density) towards itself. The electro-negativity scale ranges from 0.7 (francium) to 3.98 (for Fluorine). Oxygen has an electronegativity of 3.44. The higher the electronegativity number on the Pauling scale, the more the atom attracts electrons towards itself. Further, the more protons an atom has, the stronger pull it has on the negative electrons. In a carbon-oxygen bond, or a hydrogen-oxygen bond, the electrons are pulled towards oxygen as it is more electronegative. This results in the development of a partial negative charge on the oxygen atom, and a partial positive charge on the carbon or hydrogen atom. Such a molecule is polar in nature (Pauling, 1932; IUPAC, 1997; Pauling, 1960; Greenwood and Earnshaw, 1984).

Electronegativity creates a dipole moment, which refers to the distance between two opposite charges multiplied by the magnitude of the charge ($\mu=qr$, where $\mu$is the dipole moment, q is the magnitude of the charge, and r is the distance between

the charges). The dipole moment acts in the direction of the vector quantity. For example, the structure of the water molecule is asymmetric and bent due to the lone pair of electrons on the oxygen atom. The vectors do not cancel each other thus making the water molecule polar (figure 16).

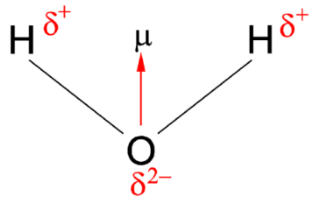


Figure 16: Dipole moment of water

Polarities of Fourteen Forms of Fat Soluble Vitamins

The polarities of the fourteen forms of fat soluble vitamins (including beta carotene, retinyl palmitate, retinyl acetate, retinol, tocopheryl acetate, alpha/beta/gamma/delta tocopherols, ergocalciferol, cholecalciferol, and vitamins K_1, K_2, K_3) were also determined by conducting a literature search on different functional groups. The polarities of the vitamins were determined by studying the chemical nature of their functional groups. All the fourteen forms of fat soluble vitamins are non-polar in nature; however, within this group of compounds, the polarity ranged from compounds that were very non-polar, to compounds that were relatively more polar.

The most non polar vitamin form was determined to be beta carotene, as it is a hydrocarbon molecule with no oxygen atom in its structure. The vitamin ester forms (retinyl palmitate, retinyl acetate, and tocopheryl acetate) were also found to be non polar, as the ester bond in their structures ties up the alcohol and fatty acid groups.

The alpha/beta/gamma/delta tocopherols contain an alcohol and ether group in their structures, and hence they are relatively more polar as compared to beta carotene and vitamin esters. The ketone and alcohol groups in vitamins D_2, D_3, K_1,K_2,K_3, and retinol have oxygen groups in their structures, and are relatively the most polar as compared to all the other vitamin forms that were tested.

All fourteen vitamin forms were found to be insoluble in water, as they all are non-polar in nature. All vitamins were found to be soluble in non polar solvents like hexane and benzene. However, the tocopherols, vitamin D_2, D_3, K_1, K_2, K_3, and retinol were also found to be slightly soluble in relatively more polar solvents such as methanol, ethanol, chloroform, and dimethyl sulfoxide. The order of increasing polarities of the fourteen forms was as follows:

Beta carotene < Retinyl palmitate / retinyl acetate / tocopheryl acetate < alpha / beta / gamma and delta tocopherols) < (vitamins D_2, D_3, K_1, K_2, K_3, and retinol)

Stage One – Conditioning of the SPE Column

For the conditioning stage of the reverse phase column, an appropriate polar conditioning solvent had to be used to clean and activate the column. Methanol was found to be a suitable solvent for this process as it is a more polar solvent and has been used previously for conditioning of reverse phase columns. Methanol followed by water in different amounts (1 ml, 2 ml, 3 ml, 4 ml, and 5 ml) was used for conditioning of the column. It was determined that conditioning the column with 5 ml of methanol, followed by 5 ml of water yielded good recoveries of the samples (figure 17).

Vacuum pressure was applied to the column to pull the solvent out of the column. However, the application of vacuum was found to result in inefficient absorption of the solvent in the stationary phase leading to drying of the column. Also, when the sample solution was loaded on the column, the vacuum did not allow sufficient time for interaction between the analyte and the stationary phase, leading to loss of sample. Therefore, the solvent was allowed to drip by gravity until all solvent dripped from the column and the analyte was retained on the column. It was observed that 5 ml of solvent completely dripped out of the column in less than 5 minutes.

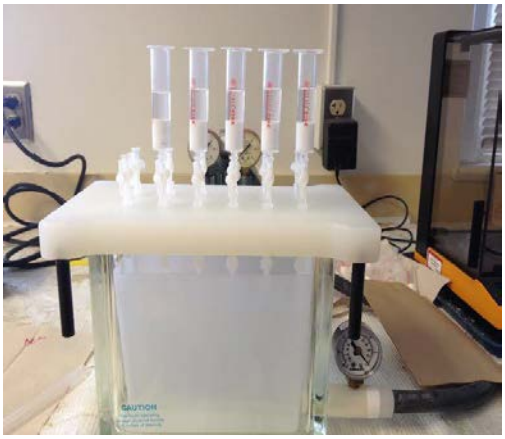


Figure 17: Conditioning of the SPE column

Stage Two– Loading of the Sample

The sample solution to be loaded on the column had to be dissolved in methanol, as methanol was the solvent used for conditioning of the column. However one major challenge with this step was that the more non-polar vitamins such as beta carotene and the vitamin esters were not soluble in methanol. Therefore, many trials were conducted to test the solubilities of these vitamins in different solvents. Dimethyl sulfoxide (DMSO) was found to dissolve all fourteen forms of fat soluble

vitamins. DMSO is also soluble in methanol. Therefore all samples were first dissolved in a small amount of DMSO, and their volumes were made to the required dilution with methanol. Once the sample solution was prepared in methanol, trial and error method was used to determine the appropriate concentration of sample solution to be loaded onto the column, and the amount to be used for the loading stage (1ml, 2ml, 3ml, 4ml, 5ml, 6ml, and 10ml). The amount of sample solution to be loaded onto the column was dependent upon the loading capacity of the column, which had to be considered while developing the SPE procedure (figure 18).

The loading capacity of the reverse phase Phenomenex column was 1000 mg/6ml (maximum loading capacity: 5% of 1000 mg = 50 mg/ml). However, 50 mg / ml was overloaded. Overloading the column resulted in bleeding of beta carotene from the column. The concentration that gave the maximum recovery was 1mg of sample in 1 ml of methanol.

Stage Three – Washing Off the Impurities from the Column

Once the analyte of interest (beta carotene) was retained on the column, the third stage involved washing of the column with water to remove all polar impurities from the sample. Larger amounts of water (3 ml to 6 ml) pushed some of the analyte out of the column, leading to a lower percent recovery. 1 ml of water did not cause any bleeding of the column.

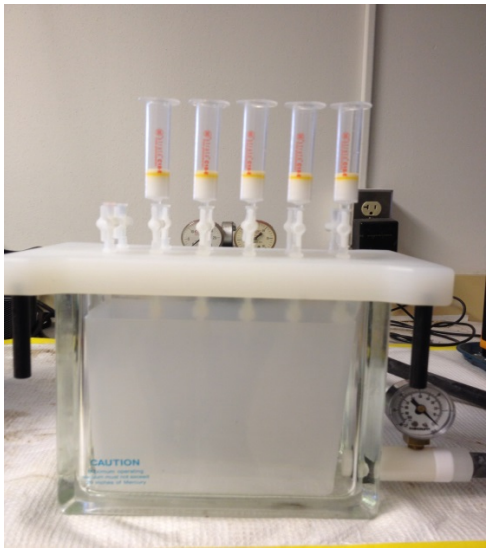


Figure 18: Loading of beta carotene sample on the SPE column

Stage Four – Elution of the Analyte

Elution with hexane resulted in maximum recovery of beta carotene when measured with the spectrophotometer, as hexane is a non-polar solvent. Methanol gave poor recoveries of beta carotene as it is relatively more polar as compared to hexane. All other solvents tested led to losses in beta carotene as it is the most non-polar of all the vitamins. Beta carotene was therefore eluted with hexane, a non-polar solvent. Hexane also yielded good recoveries on other thirteen forms of fat soluble vitamins. Elution was carried out using 1 ml to 10 ml of hexane. The use of 10 ml of hexane yielded good recoveries of all the fourteen vitamin forms (figure 19).

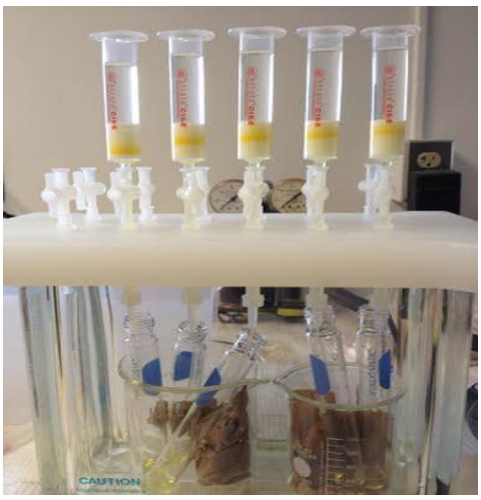


Figure 19: Elution of beta carotene from the SPE column with hexane

Applications of the SPE Methodology

Recoveries of Vitamin Standard Solutions

The SPE method developed in this study was subsequently used for testing the percent recoveries of known concentrations of fourteen vitamin standard solutions, when each standard solution was loaded on the SPE column individually. The wavelengths and extinction coefficients of each of the fourteen vitamin forms was determined and the concentrations of the standard solutions were calculated using Beer's law (Table 5).

The percent recoveries of the fourteen forms of fat soluble vitamins were found to vary from 82% to 100%. This can be attributed to the different polarities and solubilities of the fourteen vitamin forms (Table 5).

Table 5

Wavelengths, Extinction Coefficients, and Percent Recoveries of Vitamin Standard Solutions

Fat soluble vitamins	Solvent	Wavelength	Extinction coefficient	Percent recovery
1. Beta carotene	Hexane	450	2500	99.2
2. Retinyl acetate	Ethanol	325	1550	97.2
3. Retinyl palmitate	Ethanol	324	975	87.6
4. Retinol	Ethanol	325	1835	100
5. Alpha tocopherol	Ethanol	292	75.8	87.4
6. Beta tocopherol	Ethanol	296	89.4	86.7
7. Gamma tcopherol	Ethanol	298	91.4	100
8. Delta tocopherol	Ethanol	298	87.3	100
9. Tocopheryl acetate	Ethanol	298	43.6	95.8
10. Ergocalciferol	Ethanol	263	18000	82.5
11. Cholecalciferol	Ethanol	263	18000	98.7
12. Vitamin K_1	Ethanol	240-280	18900	99.5
13. Vitamin K_2	Ethanol	240 - 280	18900	99.2
14. Vitamin K_3	Ethanol	240 - 280	18900	84.3

Separation of Fat Soluble Vitamins from Food Matrices

Solvent extraction of fat soluble vitamins from food matrices. 0.1- 0.5 g of the sample was placed in a mortar and pestle and ground to a fine powder. A small amount of DMSO was added to the powder to form a paste, as all the fat soluble vitamins are soluble in DMSO. This paste was then transferred to a volumetric flask, and the volume was made up using methanol.

Methanol had to be used to make up the volume in the volumetric flask as it is a slightly more polar solvent as compared to the other solvents that were used in this process. Moreover, polar solvents are required for performing reverse phase SPE, and methanol was also the solvent used for conditioning of the column and loading of the sample on the column. Therefore methanol was used to make up the volume in the volumetric flask. A 200 ml volumetric flask was used for most of the samples, as it diluted the concentration of the sample solution to 1mg/ml, which was the loading capacity of the SPE column. For samples such as provitamin powders, which were highly concentrated, greater dilutions had to be prepared to achieve a final concentration of 1 mg/ml for the SPE procedure.

SPE of fat soluble vitamins extracted from food matrices. An SPE column was conditioned using 5 ml of methanol, followed by 5 ml of water. 1 ml of the sample solution (concentration: 1 mg/ml) was then loaded onto the column, and the solvent was allowed to drip under the action of gravity until all the solvent passed through. The column was then washed with 1 ml of water, as 1 ml water did not lead to any losses in the analyte from the column. For the elution process, 10 ml of hexane

was used to elute the analyte completely from the column. Quantification of fat soluble vitamin recoveries was conducted using the LC-MS (Table 6). A standard curve was prepared using 10 ppb, 25 ppb, 50 ppb, 100 ppb, 200 ppb, and 300 ppb vitamin standard solution in different LC-MS vials. 50 µL internal standard solution was added to each vial. The volumes were made up to 1 ml by adding LC-MS grade methanol (940 µL, 925 µL, 850 µL, 750 µL, 650 µL). Standard solutions were injected first, followed by the sample solutions.

The provitamin samples 1 and 2 consisted of corn starch fortified with retinyl palmitate and tocopheryl acetate respectively. The mean recoveries of retinyl palmitate from provitamin sample 1 (95.3%), and tocopheryl acetate from provitamin sample 2 (87%) after solid phase extraction, were close to the amount that was mentioned on the nutrition label of the products (Table 6). This indicates that the solvent extraction method using DMSO and methanol, and the SPE method were able to efficiently separate the retinyl palmitate and tocopheryl acetate from corn starch in the two samples. This was possible because the corn starch is water soluble and retinyl palmitate and tocopheryl acetate are hydrophobic and fat soluble. As a result, corn starch could be washed off during the washing stage and retinyl palmitate and tocopheryl acetate could be eluted from the sample using hexane, without any interference from corn starch.

Table 6

Recoveries of Fat Soluble Vitamins from Food Matrices after Three SPE Extractions

Supplement sample	Fat Soluble Vitamin	Expected value	Mean of 3 extractions ± S.D.	Percent Recovery
1. Provitamin Sample 1	Retinyl palmitate (mg)	0.9152	0.87 ± 0.05	95.3
2. Provitamin sample 2	Tocopheryl acetate (mg)	0.77	0.67 ±0.05	87
3. Upcal D (drink mix)	Vitamin D (mcg)	12.5	32.68 ± 0.76	261.5
4. Chewable supplements	Beta carotene (mcg)	1800	1459.81 ± 125.79	81
	Retinyl acetate (mcg)	688	1002.71 ± 12.53	145.7
	Total vitamin A (mcg)	2488	2462.52 ± 123.58	99
	Vitamin D (mcg)	16.68	19.81 ± 0.73	118.8
	Vitamin K (mcg)	60	30.10 ± 1.13	50.2
5. Multiformula capsules	Beta carotene(mcg)	4500	2674.2 ± 128.97	59.4
	Retinyl acetate (mcg)	860	755.02 ± 47.74	87.8
	Total vitamin A (mcg)	5360	3429.22 ± 175.80	64
	Vitamin D (mcg)	25	29.78 ± 0.07	119.1

The percent recovery of vitamin D_3 from the Upcal D drink mix sample after solid phase extraction was 261.5% (Table 6). A possible explanation for a higher percent recovery could be the extraction of other ingredients along with vitamin D_3 during the extraction process. The other ingredients in this drink mix were calcium citrate and dextrose. Dextrose and citric acid are soluble in DMSO (www.medical-library.net). Therefore, it is very likely these compounds were extracted along with the fat soluble vitamins during the solvent extraction process. It is very unlikely that the column material as well as the stationary phase dissolved in hexane and came out into the solution with vitamin D_3. This is because the column is made of polypropylene which does not react with methanol and hexane. Also the column bed is made up of silica which does not dissolve in hexane.

In the chewable vitamin tablets, it was observed that the values of beta carotene, and vitamin K recovered after solid phase extraction were lower as compared to the amounts mentioned on the nutrition label of this product. On the other hand, the recovery of retinyl acetate and vitamin D_3 were higher as compared to the amount mentioned on the nutrition label. This sample had 22 ingredients in one tablet, and therefore there was a lot of interference from other ingredients during the extraction process. Some of the other ingredients such as sodium ascorbate, chromium picolinate, and some flavonoids, are soluble in DMSO (Vincent, 2013; Anderson & Markham, 2006), and were therefore extracted during the solvent extraction process. Citric acid is also soluble in DMSO and therefore some metal salts of citric acid could have also been extracted along with the FSVs (www.medical-library.net). The higher amounts of retinyl acetate and vitamin D_3 recovered after SPE indicate that there

could have been other compounds that eluted along with the fat soluble vitamins in hexane.

It can therefore be concluded that the presence of other ingredients such as metal citrates, ascorbates, picolinates, and flavonoids can interfere with the SPE process as they are soluble in DMSO and may elute in the solvent along with FSVs.

In the multiformula capsules, the recoveries of total vitamin A in the sample ranged from 60-70%. The losses in the total content of vitamin A during the extraction process could be attributed to interferences from the other ingredients in the capsules. There were 31 ingredients present in every capsule, including various vitamins, minerals, other bound nutrients, flavors and gelatin. All these compounds have different physical properties and the polarities which range widely from polar to non-polar. Since DMSO and methanol were used for extraction of fat soluble vitamins from the capsules, many other ingredients could have also been extracted out in the solvent along with fat soluble vitamins. Further, solid phase extraction might not have been able to separate the fat soluble vitamins from the other ingredients, thereby leading to poor recoveries.

Other compounds such as lipoic acid, magnesium stearate, and flavonoids are soluble in hexane and were therefore competing with the FSVs during the elution stage. The higher amounts of vitamin D_3 recovered after SPE indicate that there were other compounds that were eluting along with the fat soluble vitamins in hexane.

It can therefore be concluded that the presence of other food ingredients such as dextrose, citric acid, metal citrates, picolinates and ascorbates, and some flavonoids

can be extracted from food matrices along with FSVs as they are soluble in DMSO. Further, some compounds such as magnesium stearate, lipoic acid, and some flavonoids can also interfere with the SPE separation process as they are soluble in hexane. Interference from all these other ingredients in the FSV extraction process can lead to poor recoveries of FSVs. Therefore, the solvent extraction and SPE methods developed in this study can only be applied to food matrices that do not contain ingredients which are soluble in the extraction solvents. This methodology can yield good recoveries of FSVs from samples such as provitamin powders, which contain few other ingredients that do not interfere with the extraction process and are mostly water soluble in nature.

Statistical Analysis

Statistical analysis of the data was conducted using the IBM SPSS statistics software, version 19. One way repeated measures ANOVA was used to compare the mean of the total FSVs mentioned on the nutrition label of each product, with the means of the total FSVs recovered after solid phase extraction in triplicate. A p value ≤ 0.05 was considered to be statistically significant.

In the case of the chewable nutrition supplement sample, no significant difference was observed between the mean value of total FSV from the label and the mean value of total FSVs from the three extractions (Table 7 and 8). The p value was determined to be greater than 0.05. There was a lot of interference in the extraction process from the other 22 ingredients, and as a result other compounds seemed to have eluted along with the fat soluble vitamins during SPE of this sample. As a result,

the recovery values of most of the FSVs were skewed. The recoveries of beta carotene, and vitamin K were lower than expected, while the recoveries of retinyl acetate and vitamin D_3 were higher than expected. Therefore it can be concluded that even though statistically there seems to be no significant difference between the label values and the values obtained post SPE, the results are skewed due to the elution of other nutrients along with the FSVs.

Another statistical test was used to determine the internal consistency of the data (amount of FSV recovered in triplicate from each supplement). The consistency of results within the three SPE extractions was determined using an internal-consistency reliability test (Table 7 and 8). Reliability was measured using cronbach's alpha ranging between negative infinity and one. A good internal consistency and reliability was considered with cronbach alpha value > 0.9. The analysis indicated that there was a strong reliability in the FSV values from the three extractions in the chewable nutrition supplements. The cronbach alpha value was 0.996 which indicated stability in the extraction process. The relationship between the FSV recoveries after the three extractions, and the FSV amounts mentioned on the product labels was tested using regression analysis (Table 7 and 8). Regression analysis between the FSV values of the three extractions showed that there was a strong correlation between the three SPE extraction processes of the chewable supplement sample.

Therefore, it can be concluded that the SPE methodology developed in this study consistently yielded similar recoveries on each of the FSVs in the chewable supplement samples. However, even though the means of the FSVs from the three SPE extractions were not significantly different from the FSV means from the

nutrition label, there was elution of other ingredients along with the FSVs in hexane. Therefore, it can be concluded that the SPE method developed in this study can only be applied to products which have distinct fat soluble and water soluble ingredients which can be separated from each other easily. In case there are many ingredients with ranging polarities, then they would interfere with the extraction process and either compete with the FSVs or elute along with them.

The statistical analysis of the FSVs in the multiformula capsules also yielded similar results. The mean of the total FSVs mentioned on the label was found to be significantly different from the means of the FSVs from the three SPE extractions. The p value was 0.069. It can be therefore concluded that even though the p value was slightly greater than 0.05, there was a difference between the mean of the FSVs of the label and the mean of the FSVs after SPE. This difference in the mean can be attributed to poor recoveries of the FSVs due to interference from other ingredients. The internal consistency reliability test and regression analysis yielded that the values from the three extractions had strong correlation and reliability. The cronbach's alpha value was 0.979. Therefore it can be concluded that the SPE methodology developed in this study consistently yielded similar recoveries on each of the FSVs in the chewable supplement samples. However, there was a significant difference between the means of the FSVs from the label and the means of FSVs from the three extractions. Therefore it can be concluded that there was a lot of interference from the other ingredients in the sample during the extraction process.

A reliability test was conducted on the FSVs of all the 5 samples (provitamin powders, chewable supplements, multiformula tablets, and upcal D). The cronbach's

alpha value was determined to be 0.999. This indicates that there was strong reliability between the three SPE extractions, as they gave similar values for each vitamin during each extraction.

Table 7

Statistical Analysis of Food Samples

	Repeated Measures ANOVA			
	Chewable nutrition tablets		Multiformula capsules	
	Means	P value	Means	P value
Mean of total FSVs (on nutrition label)	1010.536	0.688	2686.250	0.069
Mean of total FSVs (extraction 1)	1003.672	0.545	1817.431	0.164
Mean of total FSVs (extraction 2)	942.064	0.572	1704.455	0.164
Mean of total FSVs (extraction 3)	1039.233	0.518	1644.280	0.165
Reliability Test (Cronbach's Alpha)	0.996		0.979	
Regression Analysis (R square value)	1.0		0.994	

Table 8

Statistical Analysis of Fat Soluble Vitamins from All Five Food Samples

	Chewable nutrition tablets, Multiformula capsules, Upcal D, Provitamin sample 1, Provitamin sample 2			
	Mean of total FSVs of all 5 food samples (on nutrition label)	Mean of total FSVs of all 5 food samples (extraction 1)	Mean of total FSVs of all 5 food samples (extraction 2)	Mean of total FSVs of all 5 food samples (extraction 3)
Repeated Measures ANOVA	1317.655	1026.806	963.525	984.018
P value	0.175	0.175	0.175	0.175
Reliability Test (Cronbach's Alpha)	0.982			
Regression Analysis (R square value)	0.993			

CHAPTER V

SUMMARY AND CONCLUSION

A solid phase extraction method was developed to separate and quantify fourteen chemical forms of fat soluble vitamins in vitamin fortified food products and nutritional supplements. A reverse phase C18 phenomenex cartridge, with a volume of 6 ml and a loading capacity of 1000 mg (maximum loading capacity 5% of 1000 mg = 50 mg) provided the most efficient separation of fat soluble vitamins from food matrices.

The polarities of various organic solvents were determined; methanol was found to be a slightly polar solvent (due to the presence of an alcohol group in its structure) and hexane was determined to be a non-polar solvent (due to the absence of oxygen atoms in its structure). As a result, methanol was used for conditioning of the SPE column, and hexane was used for elution of the fat soluble vitamins from the column. The polarities of the fourteen different chemical forms of fat soluble vitamins were also determined. All fourteen fat soluble vitamins were non-polar; however within this group of compounds, the polarity ranged from non-polar to slightly more polar. The most non polar vitamin was beta carotene, as it is a hydrocarbon molecule with no oxygen atom in its structure. The vitamin ester forms (retinyl palmitate, retinyl acetate, and tocopheryl acetate) were non polar, as the ester bond in their structures ties up the alcohol and fatty acid groups. The alpha/beta/gamma/delta tocopherols contain an alcohol and ether group, and hence are relatively more polar as

compared to beta carotene and vitamin esters. The ketone and alcohol groups in vitamins D_2, D_3, K_1, K_2, K_3, and retinol have oxygen groups. They were the most polar as compared to all vitamins. All fourteen vitamins were insoluble in water as they are non-polar in nature, and soluble in non-polar solvents, like hexane and benzene. However, the tocopherols, vitamin D_2, D_3, K_1, K_2, K_3, and retinol were also slightly soluble in relatively more polar solvents such as methanol, ethanol, chloroform, and dimethyl sulfoxide.

The SPE column was conditioned with 5 ml of methanol, followed by 5 ml of water. The conditioning solvents were allowed to drip by gravity. The FSVs were extracted from the food matrices by dissolving the food sample in a small amount of DMSO, and making up the volume to the required dilution with methanol. A concentration of 1 mg/ml of the sample was determined to be a suitable amount for loading of the sample onto the column. 1 ml of water was used for washing all polar impurities from the column, and non-polar fat soluble vitamins were eluted with 10 ml of hexane. This SPE methodology yielded good percent recoveries (82 – 100%) when known concentrations of vitamin standard solutions were loaded on the columns.

The SPE methodology was then applied to separate and quantify FSVs from different fortified food products and nutritional supplements. The SPE methodology provided good recoveries (80-100%) of FSVs in the two provitamin samples, each of which contained corn starch fortified with one vitamin form: retinyl palmitate and tocopheryl acetate. The recoveries of retinyl palmitate/tocopheryl acetate after solid phase extraction of these two provitamin samples were close to amounts on the

nutrition label of the product. The solvent extraction method using DMSO and methanol, and the SPE method were able to efficiently separate the FSVs from the corn starch in the samples. Corn starch is water soluble and retinyl palmitate/tocopheryl acetate is hydrophobic and fat soluble. As a result, corn starch was washed during the washing stage and retinyl palmitate/tocopheryl acetate was eluted from the sample using hexane, without any interference from corn starch.

The SPE methodology was also applied to more complex food samples such as multivitamin capsules, vitamin fortified drink mixes, and chewable nutrition tablets, which consisted of a variety of different ingredients including fat and water soluble vitamins, minerals, carbohydrates, proteins, fats, added flavors, and gelatin. All these ingredients interfered with separation of FSVs as they have different physical properties and polarities which range from polar to non-polar. Since DMSO and methanol were used for extraction of fat soluble vitamins from the samples, many other ingredients such as dextrose, citric acid, metal citrates, picolinates and ascorbates, and some flavonoids were extracted in the solvent along with fat soluble vitamins as they are soluble in DMSO (Vincent, 2013; Anderson & Markham, 2006). Further, some compounds such as magnesium stearate, lipoic acid, and some flavonoids interfered with the SPE separation process as they are soluble in hexane (www.medical-library.net). This resulted in poor recoveries of fat soluble vitamins as these compounds competed with FSVs during SPE elution stage.

Amounts of beta carotene and vitamin K in chewable nutrition tablets were lower than expected, while amounts of vitamin D_3 and retinyl acetate were higher than expected. The vitamin D_3 levels were much higher in the vitamin fortified drink

mix than the amount mentioned on the label. Further, in the multiformula capsules, the recoveries of retinyl acetate and vitamin D_3 were higher than expected, and recovery of beta carotene was lower than expected.

Statistical analysis of results indicated that there was a strong reliability between the FSV values determined from the three extractions in all five food samples. Regression analysis between the three extractions from each sample showed that there was a strong correlation between the three SPE extraction processes. Therefore, it can be concluded that the SPE methodology developed in this study provides consistent separation and recoveries of FSVs with strong internal reliability. It was also observed that the means of the total FSVs from the three SPE extractions were not significantly different from the FSV means from the nutrition label of each sample. Even then, it is likely that some of the other ingredients were eluted in the hexane along with the FSVs, as the FSV values obtained after SPE were different from those mentioned on the nutrition label of the products.

Therefore, it may be concluded that presence of many other ingredients with different polarities can interfere with the SPE process, and this could lead to the extraction of other compounds in DMSO and hexane along with FSVs. The SPE methodology developed in this study can only be applied to products which do not have compounds that interfere with the extraction process. In case there are other ingredients with differing polarities, there is a possibility that these ingredients might interfere with the extraction process and either compete with the FSVs or elute with them, thereby affecting the percent recoveries of FSVs after the SPE process. However, if this SPE methodology is applied to food products such as provitamin

powders which contain few ingredients that have distinct polarities, it could lead to efficient separations of the fat soluble vitamins from the food matrices and yield good recoveries when quantified using the LC-MS system.

Future research in this area needs to focus on using a mixture of solvents and different ratios of solvents for the extraction of FSVs from food matrices. Further, the use of multicolumn solid phase extraction, and the use of SPE columns with bigger volumes will help in better separation of FSVs from complex food ingredients.

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APPENDIX A

Calculations of FSV Recoveries after SPE extraction

Analysis of Food Samples and Supplements

Provitamin Powder – Corn Starch Fortified With Retinyl Palmitate

Ingredients: Retinyl palmitate and corn starch

Expected concentration (as indicated on the label): 256,000 IU of retinyl palmitate / g of powder (140.8 mg/g).

Sample taken: 0.5 g (Expected concentration: 70.4 mg retinyl palmitate)

Extinction coefficient for retinyl palmitate: 975 M^{-1} cm^{-1}

Wavelength (λmax): 325 nm

Extraction solvent: 10 ml (1 ml DMSO + 9 ml methanol), concentration: 70.4 mg/10 ml = 7.04 mg/ml. This was diluted to 10 ml with Methanol.
Therefore final concentration of solution = 0.704 mg/ml

Sample loaded on SPE column: 1.3 ml (0. 92 mg retinyl palmitate)

Elution: 10 ml hexane. Hexane was evaporated and residue was reconstituted in 1 ml methanol (Expected concentration: 0.92 mg/ml). This was diluted using 48 ml methanol to achieve the desired concentration for spectrophotometer. Reading was made in a spectrophotometer against a blank of methanol at 325 nm wavelength.

Sample 1. Absorbance = 1.806**,** concentration = A/ε= 1.806/975 = 0.002% = 18.52 ppm. This was 48 X dilution, therefore, original concentration = 18.52 * 48 = 889.11 ppm.

- ⇨ In 1000 ml, concentration is 889.11 mg retinyl palmitate
- ⇨ In 1 ml, concentration is 0.90 mg.
- ⇨ Expected concentration: 0.92 mg/ml
- ⇨ Therefore percent recovery = 0.90/0.92 * 100 = 97.2 %

Sample 2. Absorbance = 1.854, concentration = A/ε= 1.854/975 = 0.00190154% = 19.0154 ppm. This was 48 X dilution, therefore, original concentration = 19.0154 * 48 = 912.7392 ppm.

- ⇨ In 1000 ml, concentration is 912.7392 mg
- ⇨ In 1 ml, concentration is 0.9127392 mg.
- ⇨ Expected concentration: 0.9152 mg/ml
- ⇨ Therefore percent recovery = 0.9127392/0.9152 * 100 = 99.7 %

Sample 3. Absorbance = 1.655, concentration = A/ε= 1.655/975 = 0.00169744% = 16.9744 ppm. This was 48 X dilution, therefore, original concentration = 16.9744 * 48 = 814.7712 ppm.

- ⇨ In 1000 ml, concentration is 814.7712 mg
- ⇨ In 1 ml, concentration is 0.8147712 mg.
- ⇨ Expected concentration: 0.9152 mg/ml

⇨ Therefore percent recovery = 0.8147712/0.9152 * 100 = 89.03 %

Provitamin Powder – Corn Starch Fortified with Tocopheryl Acetate

Ingredients. Tocopheryl acetate and corn starch

Expected concentration (as indicated on the label). 770 IU tocopheryl acetate/g of powder (770 mg/g powder). This value of tocopheryl acetate mentioned on the product label is an approximate value, as ingredients are usually added in amounts which fall within an acceptable range of values.

Sample taken. 0.1 g (Expected concentration: 77 mg tocopheryl acetate)

Extinction coefficient for tocopheryl acetate. 43.6 M^{-1} cm^{-1}

Wavelength (λmax). 288 nm

Extraction solvent. 10 ml (1 ml DMSO + 9 ml methanol). Concentration: 77 mg/10 ml = 7.7 mg/ml. This was diluted to 10 ml with Methanol.
Therefore final concentration of solution = 0.77 mg/ml

Sample loaded on SPE column. 1 ml (0.77 mg tocopheryl acetate)

Elution. 10 ml hexane. Hexane was evaporated and residue was reconstituted in 10 ml methanol. Reading was taken in a spectrophotometer against a blank of methanol at 288 nm wavelength.

Sample 1. Absorbance = 0.313, concentration = A/ε= 0.313/43.6 = 0.0071789% = 71.789 ppm. This was 10 X dilution, therefore, original concentration = 71.789 * 10 =717.819 ppm.

- ⇨ In 1000 ml, concentration is 717.819 mg
- ⇨ In 1 ml, concentration is 0.717819 mg.
- ⇨ Expected concentration: 0.77mg/ml
- ⇨ Therefore percent recovery = 0.717819/0.77 * 100 = 93.2%

Sample 2. Absorbance = 0.195, concentration = A/ε= 0.195/43.6 = 0.00447248% = 44.7248 ppm. This was 15 X dilution, therefore, original concentration = 44.7248 * 15 =670.872 ppm

- ⇨ In 1000 ml, concentration is 670.872 mg
- ⇨ In 1 ml, concentration is 0.670872 mg.
- ⇨ Expected concentration: 0.77mg/ml
- ⇨ Therefore percent recovery = 0.670872/0.77 * 100 = 87.13%

Sample 3. Absorbance = 0.272, concentration = A/ε= 0.272/43.6 = 0.00623853% = 62.3853 ppm. This was 10 X dilution, therefore, original concentration = 62.3853 * 10 =623.853 ppm.

- ⇨ In 1000 ml, concentration is 623.853 mg
- ⇨ In 1 ml, concentration is 0.623853 mg.
- ⇨ Expected concentration: 0.77mg/ml

⇨ Therefore percent recovery = 0.623853/0.77 * 100 = 81%

UpCal D – Vitamin D3 Fortified Drink Mix

Serving size. 1 packet (5g).

Ingredients. Calcium citrate (500 mg elemental): 2500 mg, vitamin D3: 500 IU, dextrose: 3g

Expected concentration (as indicated on the label). 120% DV - 500 IU of vitamin D3 /serving (12.5 mcg /serving).

Sample taken. 1 packet = 5 g drink mix (expected concentration: 12.5 mcg vitamin D3)

Extraction solvent. 100 ml (10 ml DMSO + 90 ml methanol). Concentration: 12.5 mcg/100 ml = 0.125 mcg/ml = 125 ppb (concentration required for LCMS quantification = 0-300 ppb)

Sample loaded on SPE column. 1 ml (0.125 mcg vitamin D3)

Elution. 10 ml hexane was used for elution. Hexane was evaporated and residue was reconstituted in 10 ml LCMS grade methanol. 950 µL of this sample was taken in a LCMS vial and 50 µL of internal standard was added to it. This was injected in the LCMS system along with the standards for vitamin D3.

Standard curve. Vitamin D3 standard solution was prepared: concentration = 184 ppm.

This was diluted to achieve a concentration of 1ppm

M1V1=M2V2= 184*V1 = 1*25 = V1= 0.138 ml

- ⇨ 136 µL of standard solution was dissolved in 25 ml LCMS grade methanol to achieve a concentration of 1ppm.

A standard curve was prepared using 10 ppb, 25 ppb, 50 ppb, 100 ppb, 200 ppb, and 300 ppb vitamin D3 solution in different LCMS vials. 50 µL internal standard solution was added to each vial. The volumes were made up to 1 ml by adding LCMS grade methanol (940 µL, 925 µL, 850 µL, 750 µL, 650 µL). Standard solutions were injected first, followed by the sample solutions.

Sample 1.

Concentration = 31.979 ppb = 0.031979 ppm

This was 10 X dilution in LCMS grade methanol, therefore, original concentration = 0.031979* 10 =0. 31979 ppm

Expected concentration= 0.125 ppm

- ⇨ Therefore percent recovery >100 %.

Sample 2.

Concentration = 32.579 ppb = 0.032579 ppm

This was 10 X dilution in LCMS grade methanol, therefore, original concentration = 0.032579* 10 =0. 32579 ppm

Expected concentration= 0.125 ppm

- ⇨ Therefore percent recovery >100 %.

Sample 3.

Concentration = 33.496 ppb = 0.033496 ppm

This was 10 X dilution in LCMS grade methanol, therefore, original concentration = 0.033496* 10 =0. 33496 ppm

Expected concentration= 0.125 ppm

- ⇨ Therefore percent recovery >100 %.

Bariatric Advantage– Chewable Nutritional Supplements

- ⇨ **Serving size**. 1 chewable tablet.
- ⇨ **Servings per container**. 180
- ⇨ **Total Ingredients**. 22, including vitamin A (beta carotene and vitamin A acetate): 5000 IU, vitamin C (from sodium ascorbate and ascorbic acid): 60 mg, vitamin D3 (as cholecalciferol): 667 IU, vitamin E (As d-α-tocopheryl succinate): 250 IU, vitamin K (as phytonadione): 60 mcg, vitamin B1 (from thiamine mononitrate): 3mg, vitamin B2 (as riboflavin): 1.7 mg, niacin (as

niacinamide): 25 mg, vitamin B6 (from pyridoxine HCl): 2mg, folic acid: 400 mcg, vitamin B12 (as cyanocobalamin): 50 mcg, biotin: 150 mcg, pantothenic acid (from calcium D-pantothenate): 5 mg, calcium (from calcium citrate): 50 mg, magnesium (from magnesium citrate): 25 mg, zinc (from zinc citrate): 7.5 mg, selenium (from L-selenomethionine): 67 mcg, copper (from copper citrate): 0.5 mg, manganese (From manganese sulfate): 1 mg, chromium (From chromium picolinate): 50 mcg, molybdenum (from sodium molybdate): 37.5 mcg, citrus bioflavonoids (from citrus fruit peel bioflavanoid complex): 6.25 mg.

- ⇨ **Expected concentration (as indicated on the label)**. Vitamin A (Beta carotene and retinyl palmitate): 5000 IU/tablet (1.71 mg/tablet), 100% daily value (DV), vitamin D3 (Cholecalciferol) = 667 IU/ tablet (16.68 mcg / tablet), 167% DV, vitamin E (d-α-tocopheryl succinate) = 250 IU/tablet = 206.61157 mg/ tablet, vitamin K (Phytonadione) = 60 mcg/ tablet.
- ⇨ (Tocopheryl succinate was not included in the analysis as it is not commonly used for vitamin E fortification. As a result, the Analytical Food Labs did not have standard solutions and a standardized method for tocopheryl succinate analysis.
- ⇨ **Sample taken**. 1 tablet
- ⇨ **Extraction solvent**. 200 ml (50 ml DMSO + 150 ml methanol).

The tablet was first ground using a mortar and pestle into a very fine powder. A small amount of DMSO was added to the powder to form a paste. This paste was transferred to a 200 ml volumetric flask and volume was made up with methanol. The mortar and pestle were repeatedly rinsed with methanol and the contents were transferred to the flask, to ensure complete transfer of all the contents. About 3 ml of this solution was taken and solvent was evaporated, and reconstituted in 1 ml of methanol. This 1 ml solution was loaded onto a conditioned SPE column. Elution was conducted with 10 ml hexane. The solvent was evaporated and the residue was reconstituted in 3 ml of hexane. 1ml of 10% sodium sulphate was added to the solution to absorb any traces of water. The solution was vortexed and then centrifuged to separate the hexane layer from the water layer.

For vitamin D3 and vitamin K: 1 ml of the hexane layer was collected and the solvent was evaporated. The residue was reconstituted in 200 µL DMSO and 800 µL methanol.

For beta carotene and retinyl acetate, 1ml of the hexane layer was diluted to 200 ml with methanol.

950 µL of each sample was taken in a glass vial and 50 µL of internal standard was added to it. This was injected in the LCMS along with the vitamin standards.

Standard curve. A standard curve was prepared for all of the vitamins.

Beta carotene. The concentration of standard beta carotene solution was checked using a UV-Vis spectrophotometer. A 1 ppm solution was prepared with standard beta carotene solution in LCMS grade methanol.

Concentration of beta carotene solution: 350.687 ppm

M1V1=M2V2

- ⇨ 350.687*V1= 1*25
- ⇨ V1 = 71.289 µL
- ⇨ 71.289 µL of beta carotene standard solution was dissolved in 25 ml of LCMS grade methanol to achieve a concentration of 1 ppm.

Retinyl acetate. The concentration of standard retinyl acetate solution was checked using a UV-Vis spectrophotometer. A 1 ppm solution was prepared with standard beta carotene solution in LCMS grade methanol.

Concentration of retinyl palmitate solution: 772.658 ppm

M1V1=M2V2

- ⇨ 772.658*V1= 1*25
- ⇨ V1 = 32.356 µL
- ⇨ 32.356 µL of retinyl palmitate standard solution was dissolved in 25 ml of LCMS grade methanol to achieve a concentration of 1 ppm.

Vitamin D3. The concentration of standard Vitamin D3 solution was checked using a UV-Vis spectrophotometer. A 1 ppm solution was prepared with standard beta carotene solution in LCMS grade methanol.

Concentration of Vitamin D3 solution: 184 ppm.

M1V1=M2V2

- ⇨ 184*V1= 1*25
- ⇨ V1 = 136 µL
- ⇨ 136 µL of retinyl palmitate standard solution was dissolved in 25 ml of LCMS grade methanol to achieve a concentration of 1 ppm.

Vitamin K. The concentration of standard Vitamin K solution was checked using a UV-Vis spectrophotometer. A 1 ppm solution was prepared with standard beta carotene solution in LCMS grade methanol.

Concentration of Vitamin K solution: 84 ppm.

M1V1=M2V2

- ⇨ 84*V1= 1*25
- ⇨ V1 = 298 µL
- ⇨ 298 µL of retinyl palmitate standard solution was dissolved in 25 ml of LCMS grade methanol to achieve a concentration of 1 ppm.

A standard solution was prepared using the above mentioned amounts of beta carotene, retinyl palmitate, vitamin D3, and vitamin K solutions (concentration of each vitamin = 1ppm) in 25 ml LCMS grade methanol. A standard curve was prepared using 10 ppb, 25 ppb, 50 ppb, 100 ppb, 200 ppb, and 300 ppb concentrations of the standard solution in different glass vials. 50 µL internal standard solution (deuterated vitamin D3)

was added to each vial. The volumes were made up to 1 ml by adding LCMS grade methanol (940 µL, 925 µL, 850 µL, 750 µL, 650 µL). Standard solutions were injected first, followed by the sample solutions.

LCMS analysis of chewable nutritional supplements

Sample 1.

Beta carotene concentration. 37.384 ppb = 0.037384 ppm

This was 200 times dilution => 0.037384*200 = 7.4768 ppm

This was also 200 times dilution => 7.4768 *200 = 1495.36 ppm

Retinyl acetate concentration. 24.706 ppb = 0.024706 ppm

This was 200 times dilution => 0.024706*200 = 4.9412 ppm

This was also 200 times dilution => 4.9412 *200 = 988.24 ppm

Total vitamin A. 1495.36 + 988.24 = 2483.6 ppm

Beta carotene concentration / reinyl pamitate concentration = 1686.04 / 1134.76 = 1.5

⇨ Beta carotene / retinyl pamitate ratio in the tablet is approximately 60% / 40%.

Since 1 tablet contains 5000 IU vitamin A (beta carotene (60%) and retinyl palmitate 40%),

Concentration of beta carotene in one tablet: 60%*5000 = 3000 IU = 1800 µg

Concentration of retinyl palmitate in one tablet: 40%*5000 = 2000 IU =688 µg

Therefore total vitamin A in 1 tablet: 1800+688 = 2488 µg

Percent recovery: 2483.6/2488 * 100 = 99.8%

Vitamin D3 concentration. 101.29 ppb = 0.10129 ppm

This was 200 times dilution => 0.10129*200 = 20.258 ppm

Original concentration: 16.68 ppm.

Therefore percent recovery >100%

Vitamin K concentration: 154.51 ppb = 0.15451 ppm

This was 200 times dilution => 0.154.51*200 = 30.902 ppm

Percent recovery = 30.902/60 * 100 = 51.5%

Sample 2.

Beta carotene concentration. 33.002 ppb = 0.033002 ppm

This was 200 times dilution => 0.033002*200 = 6.6004 ppm

This was also 200 times dilution => 6.6004 *200 = 1320.08 ppm

Retinyl acetate concentration. 25.242 ppb = 0.025242 ppm

This was 200 times dilution => 0.025242*200 = 5.0484 ppm

This was also 200 times dilution => 5.0484 *200 = 1009.68 ppm

Total vitamin A. 1320.08 + 1009.68 = 2329.76 ppm

Percent recovery: 2329.76/2488 = 93.6%

Vitamin D3 concentration. 101.04 ppb = 0.10104 ppm

This was 200 times dilution => 0.10104*200 = 20.208 ppm

Original concentration: 16.68 ppm.

Therefore percent recovery >100%

Vitamin K concentration. 152.96 ppb = 0.15296 ppm

This was 200 times dilution => 0.15296*200 = 30.592 ppm

Percent recovery = 30.592/60 * 100 = 51 %

Sample 3.

Beta carotene concentration. 39.100 ppb = 0.039100 ppm

This was 200 times dilution => 0.039100*200 = 7.82 ppm

This was also 200 times dilution => 7.82 *200 = 1564 ppm

Retinyl acetate concentration. 25.255 ppb = 0.025255 ppm

This was 200 times dilution => 0.025255*200 = 5.051 ppm

This was also 200 times dilution => 5.051 *200 = 1010.2 ppm

Total vitamin A. 1564 + 1010.2 = 2574.2 ppm

Percent recovery: 2574.2/2488 = 103%

Vitamin D3 concentration. 94.818 ppb = 0.094818 ppm

This was 200 times dilution => 0.094818*200 = 18.9636 ppm

Original concentration: 16.68 ppm

Therefore percent recovery >100%

Vitamin K concentration. 143.95 ppb = 0.14395 ppm

This was 200 times dilution => 0.14395*200 = 28.79 ppm

Percent recovery = 28.8/60 * 100 = 48%

Bariatric Advantage– Multiformula Capsules

Serving size. 6 capsules

Servings per container. 30

Total Ingredients. 31, including vitamin A (75% beta carotene and 25% vitamin A acetate): 10,000 IU, vitamin C (as ascorbic acid): 120 mg, vitamin D3 (As cholecalciferol): 1000 IU, vitamin E (As d-α-tocopheryl acetate): 60 IU, vitamin B1 (from thiamine mononitrate): 6 mg, vitamin B2 (as riboflavin): 3.4 mg, niacin (as

niacinamide): 40 mg, vitamin B6 (from pyridoxine HCl): 4 mg, folic acid: 800 mcg, vitamin B12 (as cyanocobalamin): 4 mcg, biotin: 600 mcg, pantothenic acid (from calcium D-pantothenate): 20 mg, calcium (from calcium citrate): 200 mg, magnesium (from magnesium citrate): 100 mg, zinc (from zinc citrate): 15 mg, selenium (from selenomethionine): 100 mcg, copper (from copper citrate): 2 mg, manganese (From manganese citrate): 2 mg, chromium (From chromium picolinate): 120 mcg, molybdenum (as molybdenum aspartate): 75 mcg, potassium (as potassium chloride): 99 mg, choline: 10 mg, inositol: 25 mg, boron: 3mg, vanadium: 25 mcg, n-acetyl-L-cysteine (NAC): 100 mg, alpha lipoic acid: 25 mg, capsule (gelatin), microcrystalline cellulose, silicon dioxide, and magnesium stearate (vegetable).

Expected concentration (as indicated on the label). Vitamin A (75% Beta carotene and 25% retinyl acetate): 10,000 IU/serving

Beta carotene. 75% * 10,000 = 7500 IU = 4500 mcg/serving

Retinyl acetate. 25%* 10,000 = 2500 IU = 860 mcg/serving

Total vitamin A. 4500 + 860 = 5360 mcg/serving

Vitamin D3 (Cholecalciferol). 1000 IU/ serving = 25 mcg / serving

Vitamin E (d-α-tocopheryl acetate). 60 IU/tablet = 44117.647 mcg/ serving

Sample taken. 1 serving (6 capsules)

Extraction solvent. 200 ml (50 ml DMSO + 150 ml methanol).

The capsules were opened up and the contents of the capsule were ground using a mortar and pestle into a very fine powder. A small amount of DMSO was added to the powder to form a paste. This paste was transferred to a 200 ml volumetric flask and volume was made up with methanol. The mortar and pestle were repeatedly rinsed with methanol and the contents were transferred to the flask, to ensure complete transfer of all the contents. About 2 ml of this solution was taken and solvent was evaporated, and reconstituted in 1 ml of methanol. This 1 ml solution was loaded onto a conditioned SPE column. Elution was conducted with 10 ml hexane. The solvent was evaporated and the residue was reconstituted in 2 ml of hexane. 2 ml of 10% sodium sulphate was added to the solution to absorb any traces of water. The solution was vortexed and then centrifuged to separate the hexane layer from the water layer.

For beta carotene and retinyl acetate: 0.5 ml of the hexane layer was taken in a 50 ml volumetric flask, and the volume was made up using LCMS grade methanol. 950 µL of sample was taken in a glass vial and 50 µL of internal standard was added to it. This was injected in the LCMS along with the vitamin standards for analysis.

For vitamin E acetate: The analysis of vitamin E acetate was done using a GC-FID system. Concentration of sample was made up to 1 mg tocopheryl acetate/ ml of hexane. This sample was injected in the GC-FID system. No internal standard was used.

For vitamin D3: 1 ml of the hexane layer was collected and the solvent was evaporated. The residue was reconstituted in 200 µL DMSO and 800 µL methanol. For beta carotene and retinyl acetate, 1ml of the hexane layer was diluted to 200 ml with

methanol. 950 µL of sample was taken in a glass vial and 50 µL of internal standard was added to it. This was injected in the LCMS along with the vitamin standards for analysis.

Standard curve. A standard curve was prepared for all of the vitamins.

Beta carotene. The concentration of standard beta carotene solution was checked using a UV-Vis spectrophotometer. A 1 ppm solution was prepared with standard beta carotene solution in LCMS grade methanol.

Concentration of beta carotene solution: 294.496 ppm

M1V1=M2V2

- ⇨ 294.496*V1= 1*25
- ⇨ V1 = 84.89 µL
- ⇨ 84.89 µL of beta carotene standard solution was dissolved in 25 ml of LCMS grade methanol to achieve a concentration of 1 ppm.

Retinyl acetate. The concentration of standard retinyl acetate solution was checked using a UV-Vis spectrophotometer. A 1 ppm solution was prepared with standard beta carotene solution in LCMS grade methanol.

Concentration of retinyl palmitate solution: 756.656 ppm

M1V1=M2V2

- ⇨ 756.656*V1= 1*25
- ⇨ V1 = 33.04 µL

- ⇨ 33.04 µL of retinyl palmitate standard solution was dissolved in 25 ml of LCMS grade methanol to achieve a concentration of 1 ppm.

Vitamin D3. The concentration of standard Vitamin D3 solution was checked using a UV-Vis spectrophotometer. A 1 ppm solution was prepared with standard beta carotene solution in LCMS grade methanol.

Concentration of Vitamin D3 solution: 184 ppm.

M1V1=M2V2

- ⇨ 184*V1= 1*25
- ⇨ V1 = 136 µL
- ⇨ 136 µL of retinyl palmitate standard solution was dissolved in 25 ml of LCMS grade methanol to achieve a concentration of 1 ppm.

A standard solution was prepared using the above mentioned amounts of beta carotene, retinyl palmitate, and vitamin D3 solutions (concentration of each vitamin = 1ppm) in 25 ml LCMS grade methanol. A standard curve was prepared using 10 ppb, 25 ppb, 50 ppb, 100 ppb, 200 ppb, and 300 ppb concentrations of the standard solution in different glass vials. 50 µL internal standard solution (deuterated vitamin D3) was added to each vial. The volumes were made up to 1 ml by adding LCMS grade methanol (940 µL, 925 µL, 850 µL, 750 µL, 650 µL). Standard solutions were injected first, followed by the sample solutions.

LCMS Analysis of Multiformula Capsules

Sample 1.

Beta carotene concentration. 140.85 ppb = 0.14085 ppm

This was 100 times dilution => 0.14085*100 = 14.085 ppm

This was also 200 times dilution => 14.085 *200 = 2817 ppm

Retinyl acetate concentration: 40.147 ppb = 0.040147 ppm

This was 100 times dilution => 0.040147*100 = 4.0147 ppm

This was also 200 times dilution => 4.0147*200 = 802.94 ppm

Total vitamin A. 2817 + 802.94 = 3619.94 ppm

Expected concentration of total vitamin A: 5360 µg / serving

Percent recovery: 3619.94/5360 * 100 = 67.5 %

Vitamin D3 concentration. 149.22 ppb = 0.14922 ppm

This was 200 times dilution => 0.14922*200 = 29.844 ppm

Original concentration: 25 µg /serving.

Therefore percent recovery: 29.844 / 25 = 119 %

Sample 2.

Beta carotene concentration. 131.97 ppb = 0.13197 ppm

This was 100 times dilution => 0.13197*100 = 13.197 ppm

This was 200 times dilution => 13.197*200 = 2639.40 ppm

Retinyl acetate concentration. 37.733 ppb = 0.037733 ppm

This was 100 times dilution => 0.037733*100 = 3.7733 ppm

This was 200 times dilution => 3.7733*200 = 754.66 ppm

Total vitamin A. 2639.40 + 754.66 = 3394.06 ppm

Percent recovery: 3394.06/5360 = 63.3 %

Vitamin D3 concentration. 148.92 ppb = 0.14892 ppm

This was 200 times dilution => 0.14892*200 = 29.784 ppm

Original concentration: 25 µg / serving.

Therefore percent recovery = 119 %

Sample 3.

Beta carotene concentration. 128.31 ppb = 0.12831 ppm

This was 100 times dilution => 0.12831*100 = 12.831 ppm

This was 200 times dilution => 12.831 *200 = 2566.2 ppm

Retinyl acetate concentration: 35.373 ppb = 0.035373 ppm

This was 100 times dilution => 0.035373*100 = 3.5373 ppm

This was 200 times dilution => 3.5373 *200 = 707.46 ppm

Total vitamin A. 2566.2 + 707.46 = 3273.66 ppm

Percent recovery: 3273.66/5360 = 61.1 %

Vitamin D3 concentration. 149 ppb = 0.149 ppm

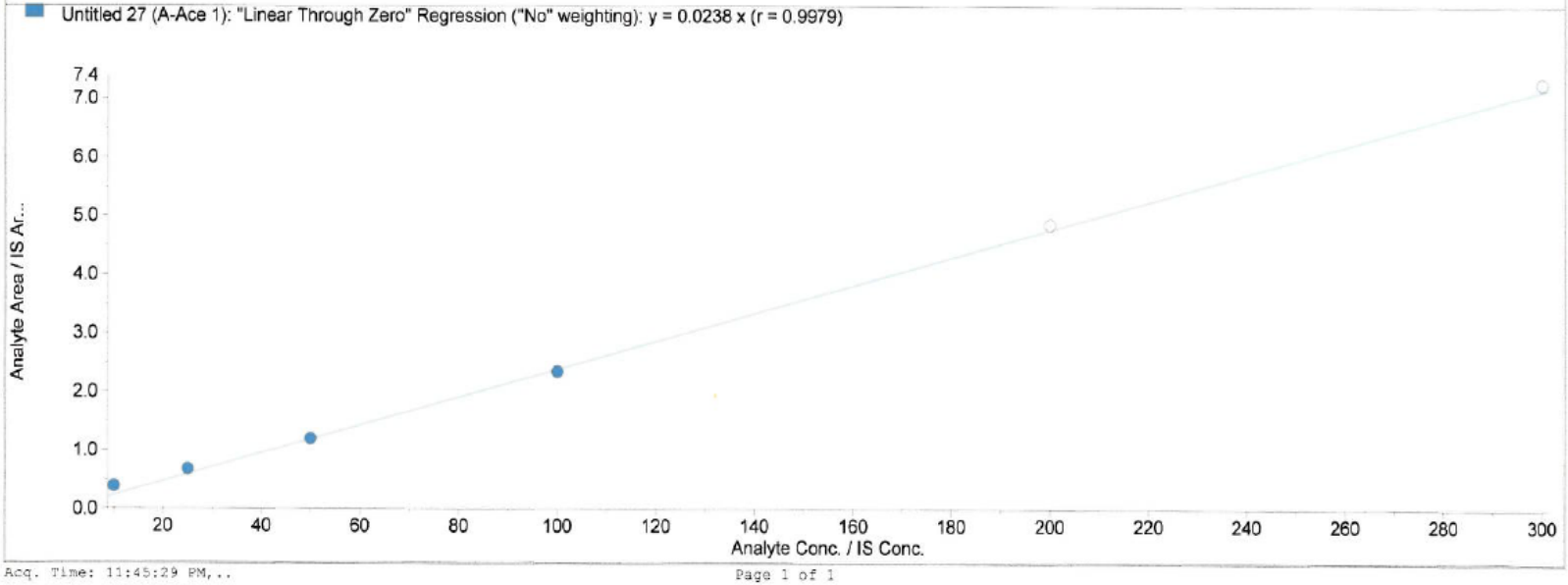
This was 200 times dilution => 0.149*200 = 29.8 ppm

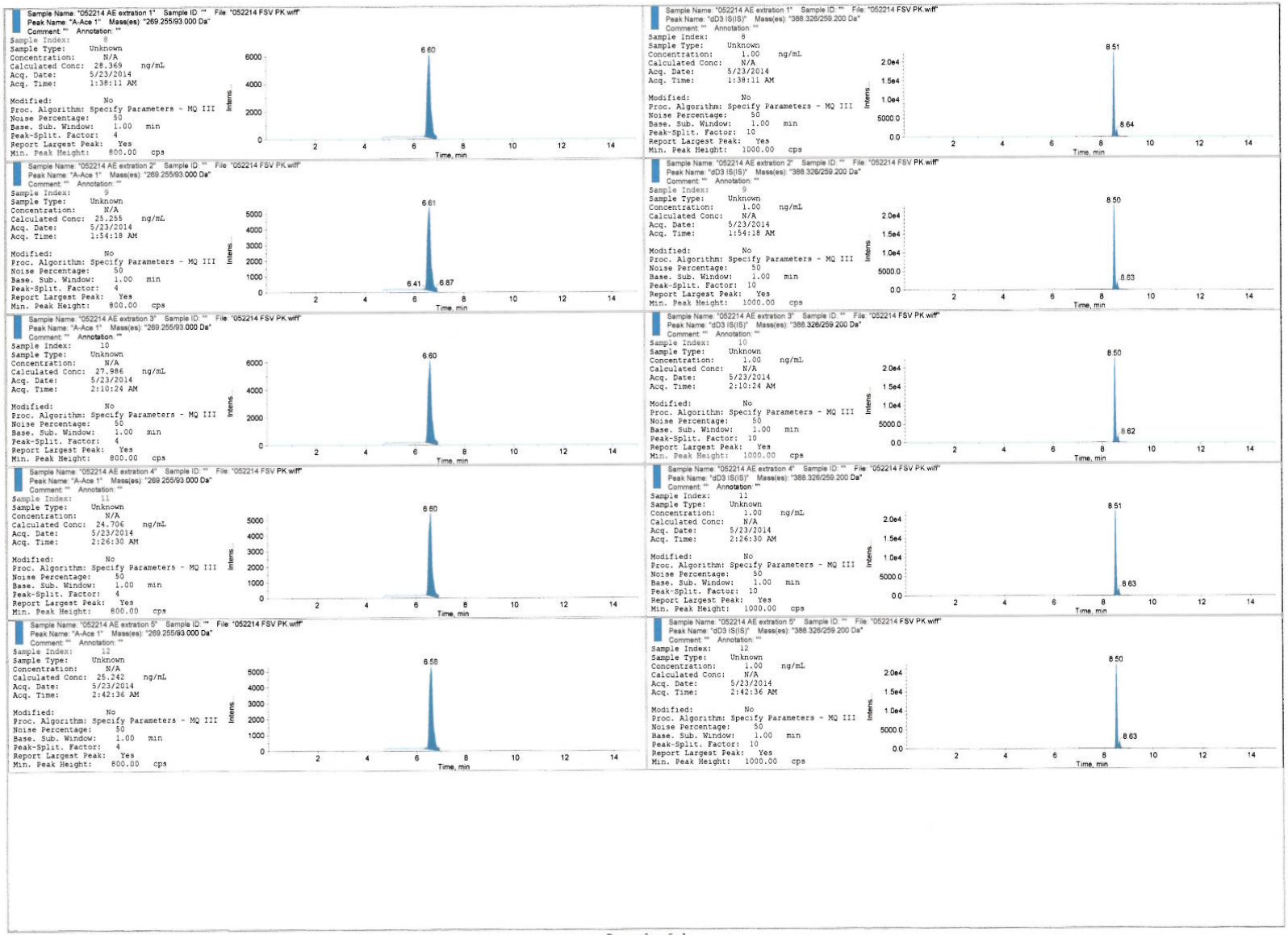
Original concentration: 25 µg / serving. Therefore percent recovery = 119 %.

APPENDIX B

LC-MS Analysis of Chewable Nutrition Supplement

	Sample Name	Sample Type	File Name	Dilution Factor	Analyte Peak Area (counts)	Analyte Peak Height (cps)	Analyte Concentration (ng/mL)	IS Peak Area (counts)	IS Peak Height (cps)	Use Record	Record Modified	Calculated Concentration (ng/mL)	Accuracy (%)
1	052214 PK DKAE 10ppb	Standard	052214 FSV PK.wiff	1.0000	3.43e+004	3.41e+003	10.0	8.79e+004	2.11e+004	☑	☐	16.351	164.
2	052214 PK DKAE 25ppb	Standard	052214 FSV PK.wiff	1.0000	6.81e+004	7.18e+003	25.0	1.01e+005	2.46e+004	☑	☐	28.373	113.
3	052214 PK DKAE 50ppb	Standard	052214 FSV PK.wiff	1.0000	1.20e+005	1.31e+004	50.0	1.00e+005	2.49e+004	☑	☐	50.275	101.
4	052214 PK DKAE 100ppb	Standard	052214 FSV PK.wiff	1.0000	2.43e+005	2.65e+004	100.	1.04e+005	2.55e+004	☑	☐	98.384	98.4
5	052214 PK DKAE 200ppb	Standard	052214 FSV PK.wiff	1.0000	4.99e+005	5.46e+004	200.	1.03e+005	2.53e+004	☐	☐	203.92	102.
6	052214 PK DKAE 300ppb	Standard	052214 FSV PK.wiff	1.0000	7.65e+005	8.55e+004	300.	1.05e+005	2.60e+004	☐	☐	304.91	102.
7	052214 AE extration 1	Unknown	052214 FSV PK.wiff	1.0000	6.34e+004	6.05e+003	N/A	9.37e+004	2.27e+004		☐	28.369	N/A
8	052214 AE extration 2	Unknown	052214 FSV PK.wiff	1.0000	5.68e+004	5.35e+003	N/A	9.44e+004	2.29e+004		☐	25.255	N/A
9	052214 AE extration 3	Unknown	052214 FSV PK.wiff	1.0000	6.29e+004	6.02e+003	N/A	9.44e+004	2.28e+004		☐	27.986	N/A
10	052214 AE extration 4	Unknown	052214 FSV PK.wiff	1.0000	5.49e+004	5.33e+003	N/A	9.32e+004	2.24e+004		☐	24.706	N/A
11	052214 AE extration 5	Unknown	052214 FSV PK.wiff	1.0000	5.61e+004	5.27e+003	N/A	9.33e+004	2.23e+004		☐	25.242	N/A

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Comment: "" Annotation: ""
Sample Index: 8
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 28.369 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 1:38:11 AM
Modified: No
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Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.60
Intens.
Time, min
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Comment: "" Annotation: ""
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Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 1:38:11 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.51
8.64
Intens.
Time, min
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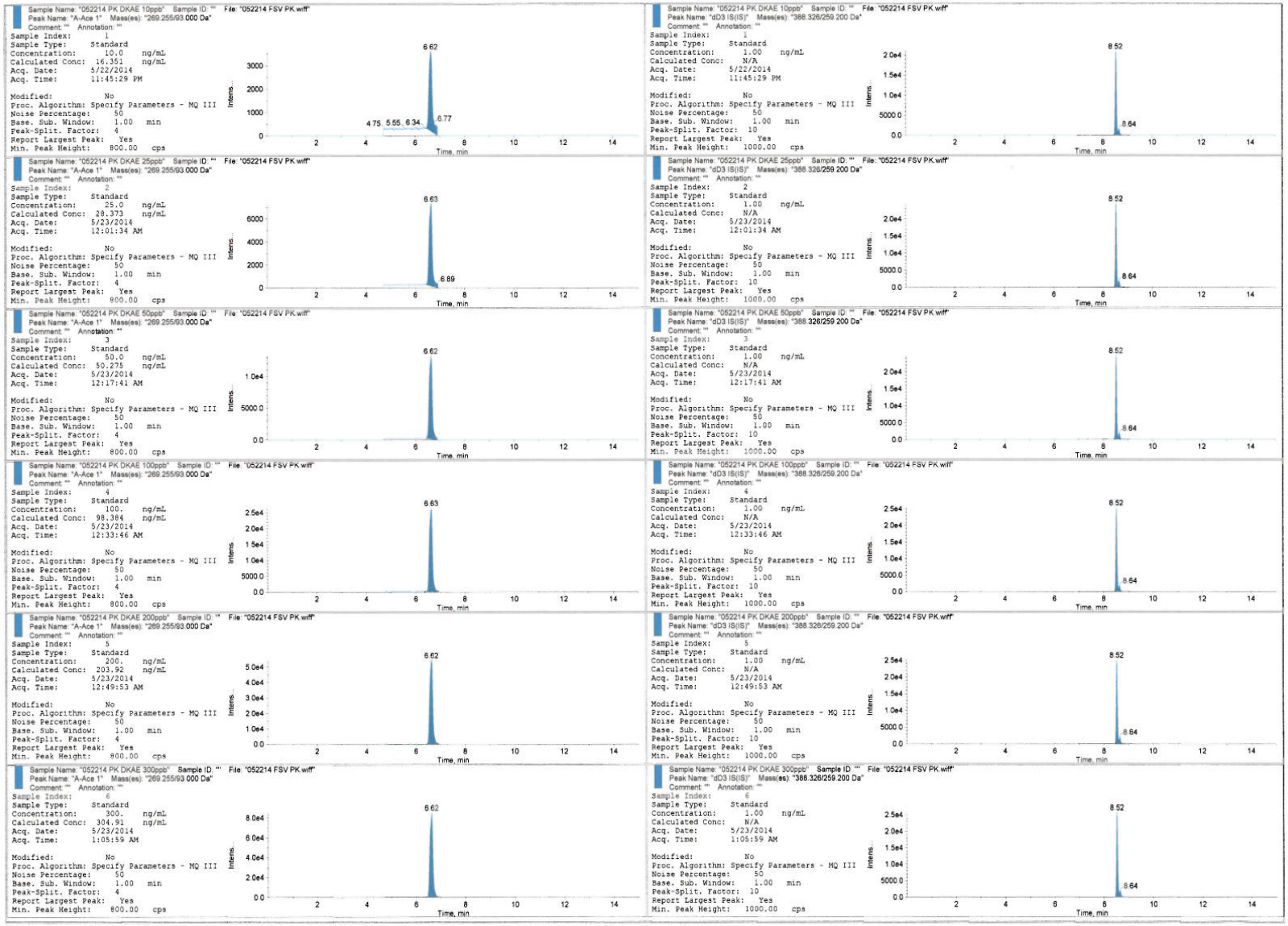
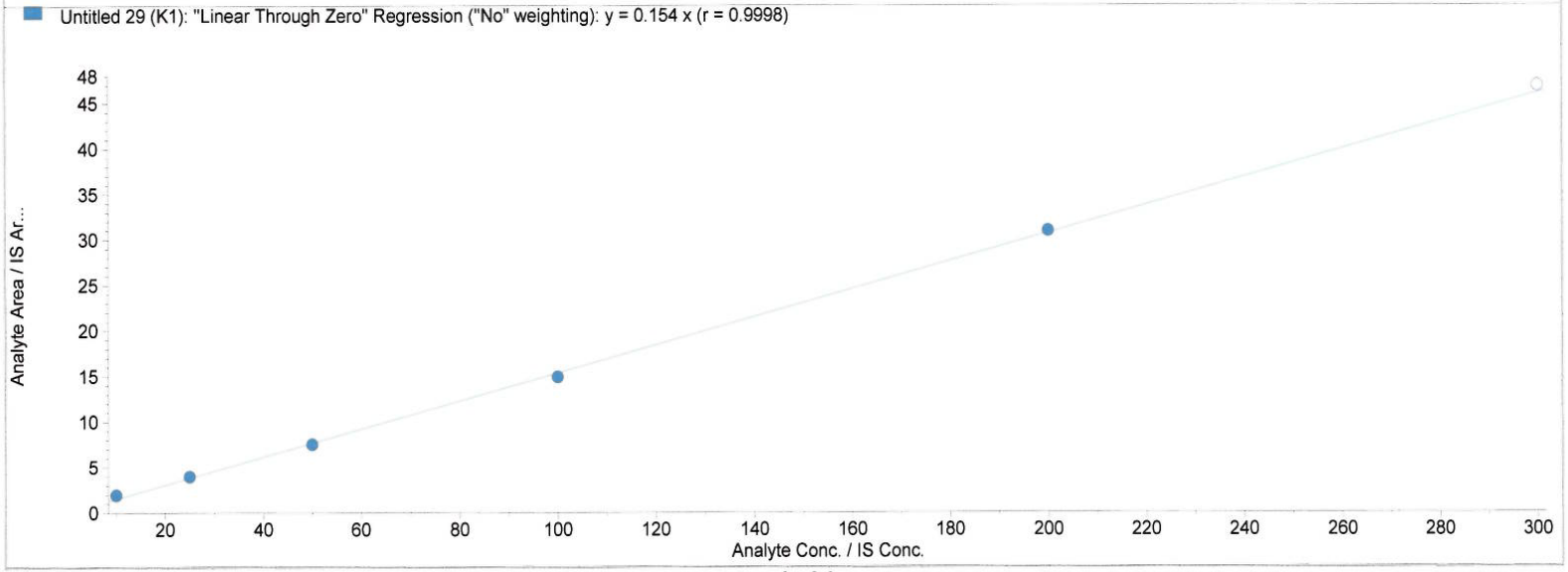
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 9
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 21.255 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 1:54:18 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.61
6.41
6.87
Intens.
Time, min
Sample Name: "052214 AE extration 2" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 9
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 1:54:18 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.50
8.63
Intens.
Time, min
Sample Name: "052214 AE extration 3" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 10
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 27.986 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 2:10:24 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.60
Intens.
Time, min
Sample Name: "052214 AE extration 3" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 10
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 2:10:24 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.50
8.62
Intens.
Time, min
Sample Name: "052214 AE extration 4" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 11
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 24.706 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 2:26:30 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.60
Intens.
Time, min
Sample Name: "052214 AE extration 4" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 11
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 2:26:30 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.51
8.63
Intens.
Time, min
Sample Name: "052214 AE extration 5" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 12
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 25.242 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 2:42:36 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.58
Intens.
Time, min
Sample Name: "052214 AE extration 5" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 12
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 2:42:36 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.50
8.63
Intens.
Time, min

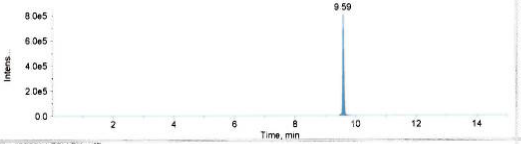

Sample Name: "052214 PK DKAE 10ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 1
Sample Type: Standard
Concentration: 10.0 ng/mL
Calculated Conc: 16.351 ng/mL
Acq. Date: 5/22/2014
Acq. Time: 11:45:29 PM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.62
4.75 5.55 6.34 6.77
Intens.
Time, min
Sample Name: "052214 PK DKAE 10ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 1
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/22/2014
Acq. Time: 11:45:29 PM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Intens.
Time, min
Sample Name: "052214 PK DKAE 25ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 2
Sample Type: Standard
Concentration: 25.0 ng/mL
Calculated Conc: 28.373 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 12:01:34 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.63
6.89
Intens.
Time, min
Sample Name: "052214 PK DKAE 25ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 2
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 12:01:34 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Intens.
Time, min
Sample Name: "052214 PK DKAE 50ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 3
Sample Type: Standard
Concentration: 50.0 ng/mL
Calculated Conc: 50.275 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 12:17:41 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.62
Intens.
Time, min
Sample Name: "052214 PK DKAE 50ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 3
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 12:17:41 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Intens.
Time, min
Sample Name: "052214 PK DKAE 100ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 4
Sample Type: Standard
Concentration: 100. ng/mL
Calculated Conc: 98.384 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 12:33:46 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.63
Intens.
Time, min
Sample Name: "052214 PK DKAE 100ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 4
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 12:33:46 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Intens.
Time, min
Sample Name: "052214 PK DKAE 200ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 5
Sample Type: Standard
Concentration: 200. ng/mL
Calculated Conc: 203.92 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 12:49:53 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.62
Intens.
Time, min
Sample Name: "052214 PK DKAE 200ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 5
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 12:49:53 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Intens.
Time, min
Sample Name: "052214 PK DKAE 300ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "A-Ace 1" Mass(es): "269.255/93.000 Da"
Comment: "" Annotation: ""
Sample Index: 6
Sample Type: Standard
Concentration: 300. ng/mL
Calculated Conc: 304.91 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 1:05:59 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 4
Report Largest Peak: Yes
Min. Peak Height: 800.00 cps
6.62
Intens.
Time, min
Sample Name: "052214 PK DKAE 300ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 6
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 1:05:59 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 10
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Intens.
Time, min

	Sample Name	Sample Type	File Name	Dilution Factor	Analyte Peak Area (counts)	Analyte Peak Height (cps)	Analyte Concentration (ng/mL)	IS Peak Area (counts)	IS Peak Height (cps)	Use Record	Record Modified	Calculated Concentration (ng/mL)	Accuracy (%)
1	052214 PK DKAE 10ppb	Standard	052214 FSV PK.wiff	1.0000	1.71e+005	5.30e+004	10.0	8.79e+004	2.11e+004	☑	☐	12.676	127.
2	052214 PK DKAE 25ppb	Standard	052214 FSV PK.wiff	1.0000	4.00e+005	1.26e+005	25.0	1.01e+005	2.46e+004	☑	☐	25.847	103.
3	052214 PK DKAE 50ppb	Standard	052214 FSV PK.wiff	1.0000	7.52e+005	2.30e+005	50.0	1.00e+005	2.49e+004	☑	☐	48.960	97.9
4	052214 PK DKAE 100ppb	Standard	052214 FSV PK.wiff	1.0000	1.54e+006	4.77e+005	100.	1.04e+005	2.55e+004	☑	☐	97.101	97.1
5	052214 PK DKAE 200ppb	Standard	052214 FSV PK.wiff	1.0000	3.18e+006	9.66e+005	200.	1.03e+005	2.53e+004	☑	☐	201.47	101.
6	052214 PK DKAE 300ppb	Standard	052214 FSV PK.wiff	1.0000	4.93e+006	1.49e+006	300.	1.05e+005	2.60e+004	☐	☐	305.03	102.
7	052214 DK Extraction 1	Unknown	052214 FSV PK.wiff	1.0000	2.75e+006	8.24e+005	N/A	1.01e+005	2.47e+004		☐	176.50	N/A
8	052214 DK Extraction 2	Unknown	052214 FSV PK.wiff	1.0000	2.37e+006	7.26e+005	N/A	1.07e+005	2.65e+004		☐	143.95	N/A
9	052214 DK Extraction 3	Unknown	052214 FSV PK.wiff	1.0000	2.32e+006	7.04e+005	N/A	9.89e+004	2.43e+004		☐	152.44	N/A
10	052214 DK Extraction 4	Unknown	052214 FSV PK.wiff	1.0000	2.56e+006	7.75e+005	N/A	1.08e+005	2.64e+004		☐	154.51	N/A
11	052214 DK Extraction 5	Unknown	052214 FSV PK.wiff	1.0000	2.44e+006	7.25e+005	N/A	1.04e+005	2.55e+004		☐	152.96	N/A



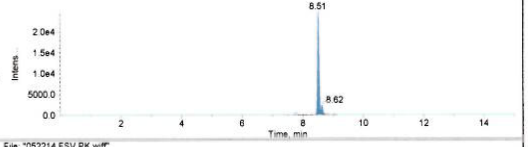
Sample Name: "052214 DK Extraction 1" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 14
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 176.50 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 3:14:49 AM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps



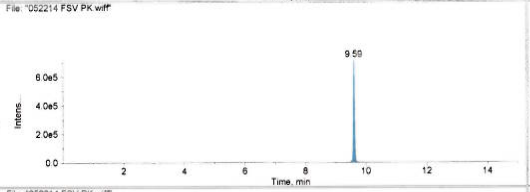
Sample Name: "052214 DK Extraction 1" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 14
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 3:14:49 AM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps



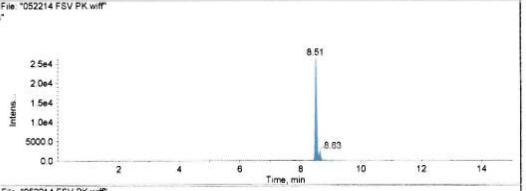
Sample Name: "052214 DK Extraction 2" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 15
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 143.95 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 3:30:55 AM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps



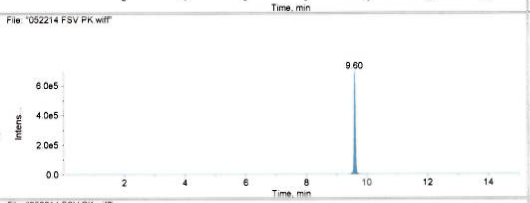
Sample Name: "052214 DK Extraction 2" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 15
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 3:30:55 AM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps



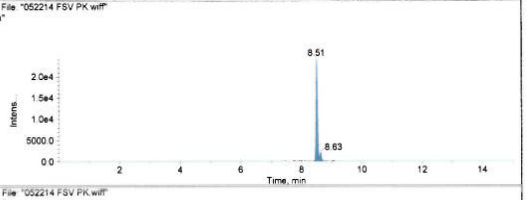
Sample Name: "052214 DK Extraction 3" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 16
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 152.44 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 3:47:02 AM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps



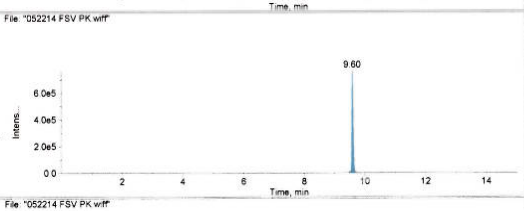
Sample Name: "052214 DK Extraction 3" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 16
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 3:47:02 AM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps



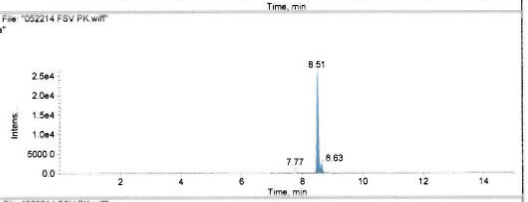
Sample Name: "052214 DK Extraction 4" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 17
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 154.51 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 4:03:08 AM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps



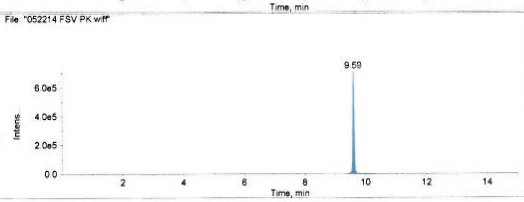
Sample Name: "052214 DK Extraction 4" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 17
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 4:03:08 AM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps



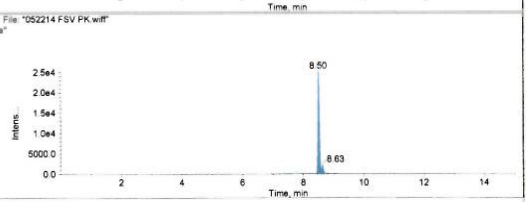
Sample Name: "052214 DK Extraction 5" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 18
Sample Type: Unknown
Concentration: N/A
Calculated Conc: 152.96 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 4:19:13 AM

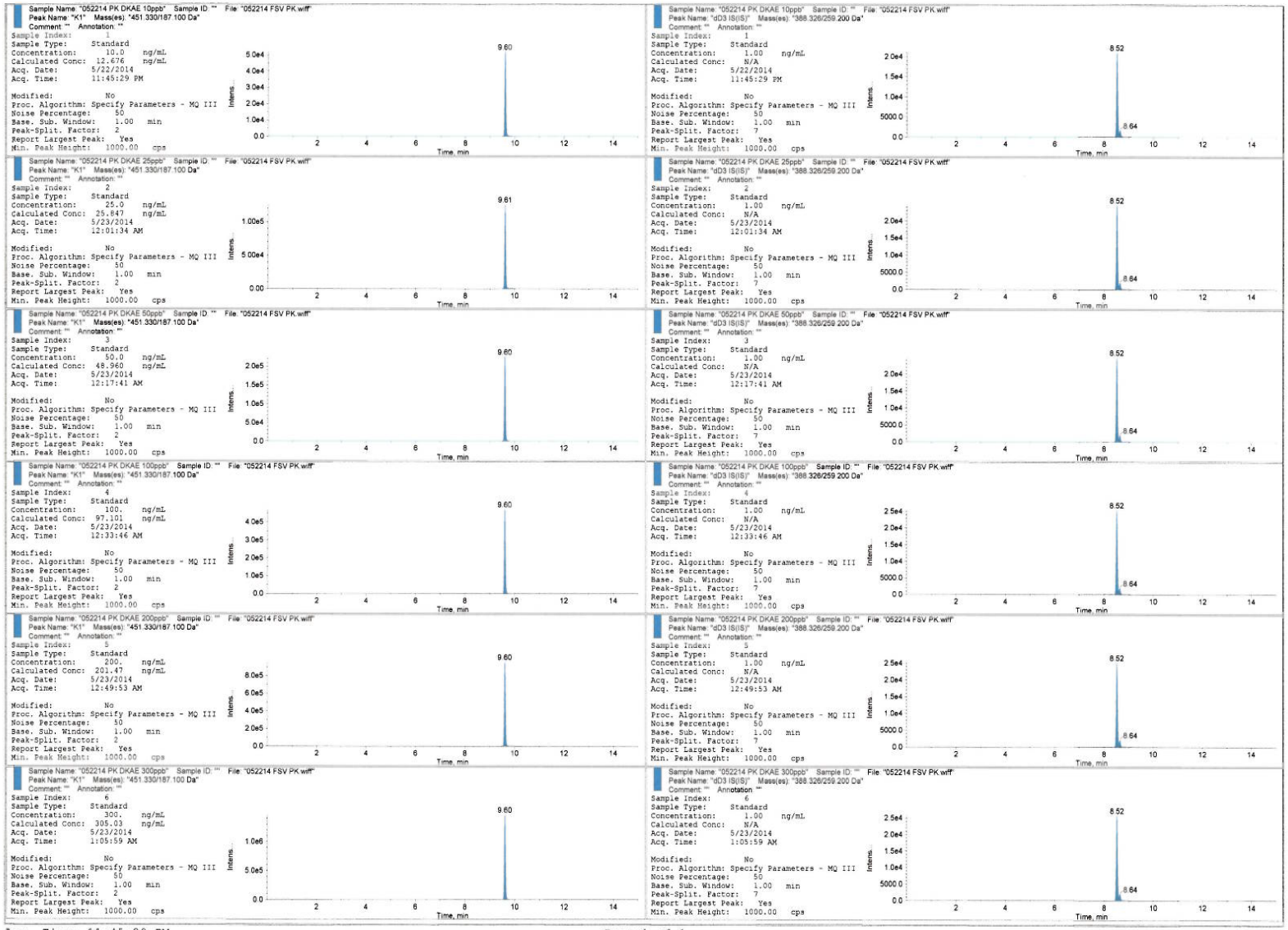
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps



Sample Name: "052214 DK Extraction 5" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 18
Sample Type: Unknown
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 4:19:13 AM

Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps




Sample Name: "052214 PK DKAE 10ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 1
Sample Type: Standard
Concentration: 10.0 ng/mL
Calculated Conc: 12.676 ng/mL
Acq. Date: 5/22/2014
Acq. Time: 11:45:29 PM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
9.60
Sample Name: "052214 PK DKAE 10ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 1
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/22/2014
Acq. Time: 11:45:29 PM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Sample Name: "052214 PK DKAE 25ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 2
Sample Type: Standard
Concentration: 25.0 ng/mL
Calculated Conc: 25.847 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 12:01:34 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
9.61
Sample Name: "052214 PK DKAE 25ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 2
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 12:01:34 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Sample Name: "052214 PK DKAE 50ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 3
Sample Type: Standard
Concentration: 50.0 ng/mL
Calculated Conc: 48.960 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 12:17:41 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
9.60
Sample Name: "052214 PK DKAE 50ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 3
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 12:17:41 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Sample Name: "052214 PK DKAE 100ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 4
Sample Type: Standard
Concentration: 100. ng/mL
Calculated Conc: 97.101 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 12:33:46 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
9.60
Sample Name: "052214 PK DKAE 100ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 4
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 12:33:46 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Sample Name: "052214 PK DKAE 200ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 5
Sample Type: Standard
Concentration: 200. ng/mL
Calculated Conc: 201.47 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 12:49:53 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
9.60
Sample Name: "052214 PK DKAE 200ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 5
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 12:49:53 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Sample Name: "052214 PK DKAE 300ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "K1" Mass(es): "451.330/187.100 Da"
Comment: "" Annotation: ""
Sample Index: 6
Sample Type: Standard
Concentration: 300. ng/mL
Calculated Conc: 305.03 ng/mL
Acq. Date: 5/23/2014
Acq. Time: 1:05:59 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 2
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
9.60
Sample Name: "052214 PK DKAE 300ppb" Sample ID: "" File: "052214 FSV PK.wiff"
Peak Name: "dD3 IS(IS)" Mass(es): "388.326/259.200 Da"
Comment: "" Annotation: ""
Sample Index: 6
Sample Type: Standard
Concentration: 1.00 ng/mL
Calculated Conc: N/A
Acq. Date: 5/23/2014
Acq. Time: 1:05:59 AM
Modified: No
Proc. Algorithm: Specify Parameters - MQ III
Noise Percentage: 50
Base. Sub. Window: 1.00 min
Peak-Split. Factor: 7
Report Largest Peak: Yes
Min. Peak Height: 1000.00 cps
8.52
8.64
Intens.
Time, min

APPENDIX C

Statistical Analysis

ANOVA The products and samples do not differ on average

Within-Subjects Factors
Measure:MEASURE_1

assays	Dependent Variable
1	Product
2	Sample1
3	Sample2
4	Sample3

Descriptive Statistics

	Mean	Std. Deviation	N
Product	1317.655433	1877.9282301	12
Sample 1	1026.805828	1293.9184143	12
Sample 2	963.525217	1210.1146748	12
Sample 3	984.018219	1216.5351487	12

Tests of Within-Subjects Effects

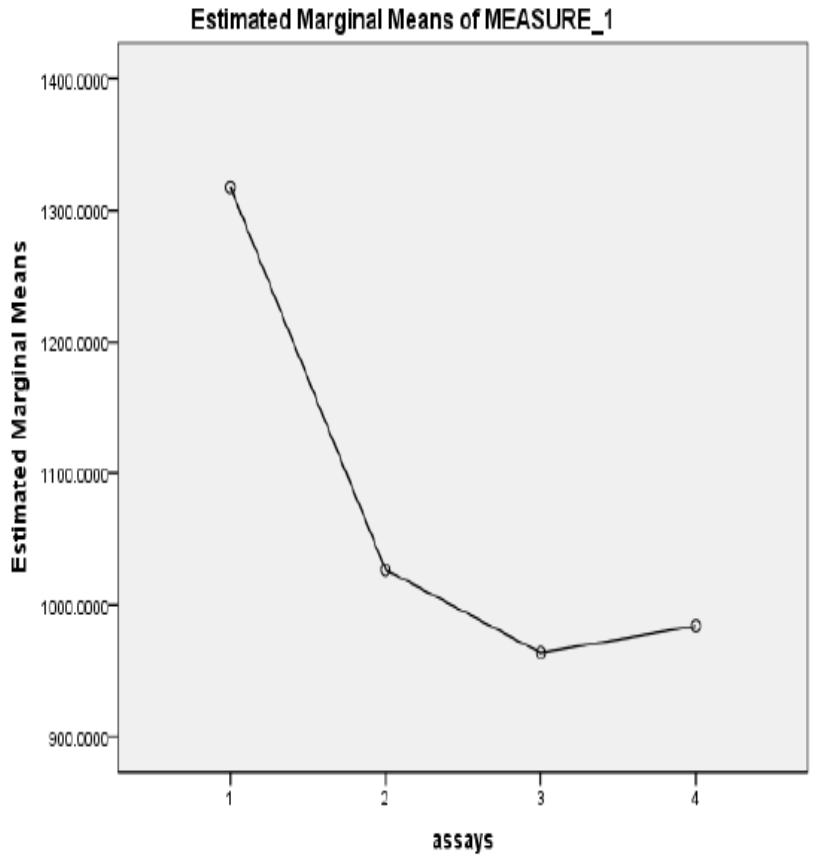
Measure:MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
assays	Sphericity Assumed	982712.015	3	327570.672	2.327	.093	.175
	Greenhouse-Geisser	982712.015	1.026	957473.521	2.327	.155	.175
	Huynh-Feldt	982712.015	1.034	950069.640	2.327	.154	.175
	Lower-bound	982712.015	1.000	982712.015	2.327	.155	.175
Error(assays)	Sphericity Assumed	4646348.685	33	140798.445			
	Greenhouse-Geisser	4646348.685	11.290	411547.170			
	Huynh-Feldt	4646348.685	11.378	408364.788			
	Lower-bound	4646348.685	11.000	422395.335			

Estimates

Measure:MEASURE_1

assays	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
1	1317.655	542.111	124.477	2510.834
2	1026.806	373.522	204.689	1848.922
3	963.525	349.330	194.655	1732.395
4	984.018	351.183	211.069	1756.968



RELIABILITY The products and samples appear to be stable estimates of vitamin/mineral content

Case Processing Summary

		N	%
Cases	Valid	12	100.0
	Excluded[a]	0	.0
	Total	12	100.0

a. Listwise deletion based on all variables in the procedure.

Reliability Statistics

Cronbach's Alpha	N of Items
.982	4

Intraclass Correlation Coefficient

	Intraclass Correlation[a]	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.931[b]	.845	.977	54.850	11	33	.000
Average Measures	.982[c]	.956	.994	54.850	11	33	.000

Two-way mixed effects model where people effects are random and measures effects are fixed.

a. Type C intraclass correlation coefficients using a consistency definition-the between-measure variance is excluded from the denominator variance.

b. The estimator is the same, whether the interaction effect is present or not.

c. This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.

REGRESSION OF PRODUCT ON SAMPLES A nearly perfect correlation of products on samples is seen, while samples are quite redundant

Variables Entered/Removed[b]

Model	Variables Entered	Variables Removed	Method
1	Sample 3, Sample 2, Sample 1	.	Enter

a. All requested variables entered.
b. Dependent Variable: Product

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.997[a]	.993	.990	183.3432678

a. Predictors: (Constant), Sample 3, Sample 2, Sample 1

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	38523840.780	3	12841280.260	382.013	.000[a]
	Residual	268918.031	8	33614.754		
	Total	38792758.811	11			

a. Predictors: (Constant), Sample 3, Sample 2, Sample 1
b. Dependent Variable: Product

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Collinearity Statistics	
		B	Std. Error	Beta			Tolerance	VIF
1	(Constant)	-40.168	70.229		-.572	.583		
	Sample 1	8.879	1.662	6.118	5.342	.001	.001	1513.728
	Sample 2	-4.262	1.599	-2.747	-2.666	.029	.001	1224.998
	Sample 3	-3.712	.559	-2.404	-6.635	.000	.007	151.545

a. Dependent Variable: Product

Collinearity Diagnostics[a]

Model	Dimension	Eigenvalue	Condition Index	Variance Proportions			
				(Constant)	Sample 1	Sample 2	Sample 3
1	1	3.492	1.000	.03	.00	.00	.00
	2	.505	2.628	.94	.00	.00	.00
	3	.003	34.636	.02	.01	.04	.88
	4	.000	125.923	.02	.99	.96	.12

ANOVA CHEWABLE

Within-Subjects Factors

Measure:MEASURE_1

assay	Dependent Variable
1	Product
2	Sample1
3	Sample2
4	Sample3

Tests of Within-Subjects Effects

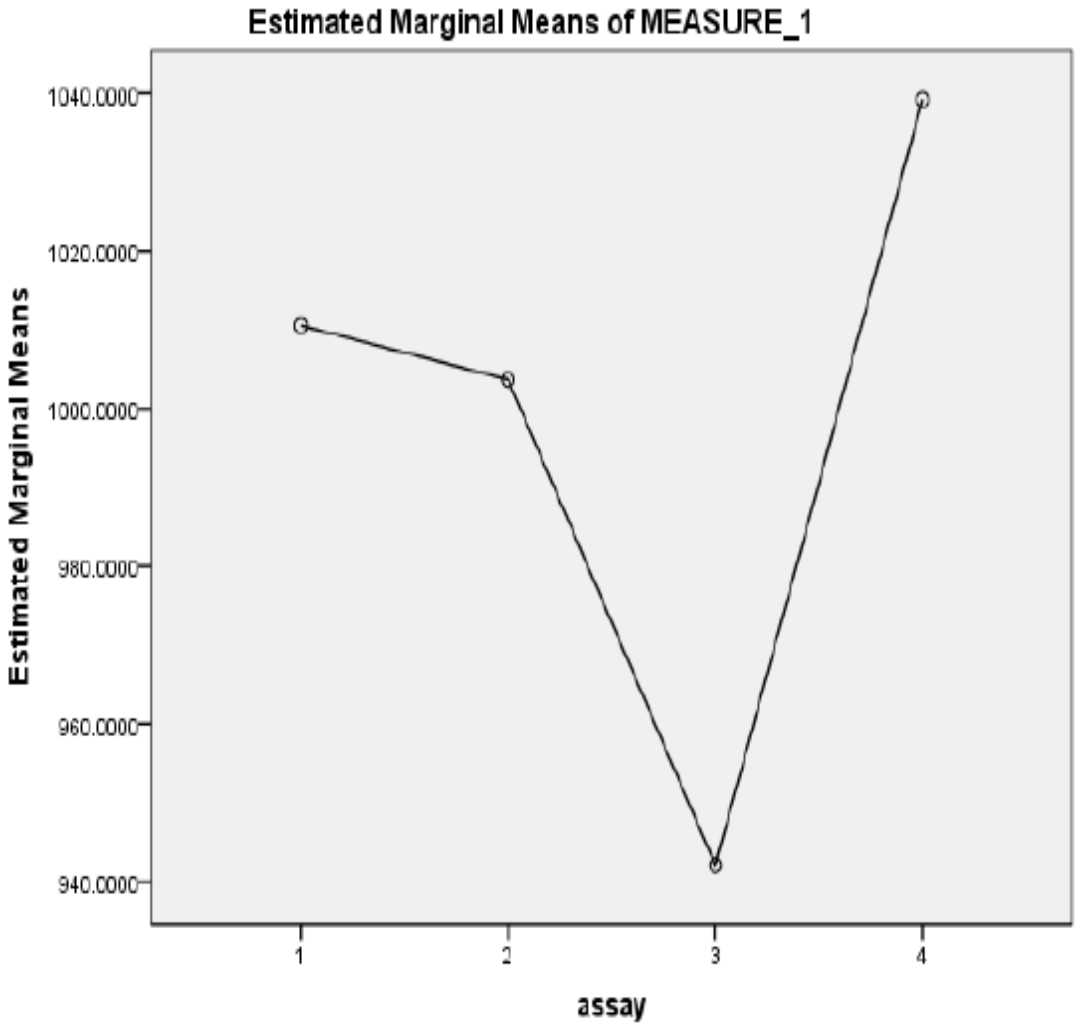
Measure:MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
assay	Sphericity Assumed	25076.159	3	8358.720	.502	.688
	Greenhouse-Geisser	25076.159	1.206	20793.778	.502	.545
	Huynh-Feldt	25076.159	1.442	17386.827	.502	.572
	Lower-bound	25076.159	1.000	25076.159	.502	.518
Error(assay)	Sphericity Assumed	199667.699	12	16638.975		
	Greenhouse-Geisser	199667.699	4.824	41392.362		
	Huynh-Feldt	199667.699	5.769	34610.442		
	Lower-bound	199667.699	4.000	49916.925		

Estimates

Measure:MEASURE_1

assay	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
1	1010.536	489.972	-349.844	2370.916
2	1003.672	466.131	-290.516	2297.860
3	942.064	433.230	-260.776	2144.904
4	1039.233	484.472	-305.878	2384.344


Estimated Marginal Means of MEASURE_1
Estimated Marginal Means
1040.0000
1020.0000
1000.0000
980.0000
960.0000
940.0000
1
2
3
4
assay

ANOVA MULTIFORMULA

Within-Subjects Factors

Measure:MEASURE_1

assay	Dependent Variable
1	Product
2	Sample1
3	Sample2
4	Sample3

Tests of Within-Subjects Effects

Measure:MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
assay	Sphericity Assumed	2850835.234	3	950278.411	3.350	.069
	Greenhouse-Geisser	2850835.234	1.003	2843673.766	3.350	.164
	Huynh-Feldt	2850835.234	1.006	2832976.428	3.350	.164
	Lower-bound	2850835.234	1.000	2850835.234	3.350	.165
Error(assay)	Sphericity Assumed	2553174.857	9	283686.095		
	Greenhouse-Geisser	2553174.857	3.008	848920.377		
	Huynh-Feldt	2553174.857	3.019	845726.906		
	Lower-bound	2553174.857	3.000	851058.286		

Estimates

Measure:MEASURE_1

assay	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
1	2686.250	1318.334	-1509.279	6881.779
2	1817.431	840.280	-856.713	4491.575
3	1704.455	787.179	-800.699	4209.609
4	1644.280	763.162	-784.442	4073.002

Estimated Marginal Means of MEASURE_1
Estimated Marginal Means
2750.0000
2500.0000
2250.0000
2000.0000
1750.0000
1
2
3
4
assay

RELIABILITY MULTIFORMULA

Case Processing Summary

		N	%
Cases	Valid	4	100.0
	Excluded[a]	0	.0
	Total	4	100.0

a. Listwise deletion based on all variables in the procedure.

Reliability Statistics

Cronbach's Alpha	N of Items
.979	4

Intraclass Correlation Coefficient

	Intraclass Correlation[a]	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.922[b]	.681	.994	48.411	3	9	.000
Average Measures	.979[c]	.895	.999	48.411	3	9	.000

Two-way mixed effects model where people effects are random and measures effects are fixed.

a. Type C intraclass correlation coefficients using a consistency definition-the between-measure variance is excluded from the denominator variance.

b. The estimator is the same, whether the interaction effect is present or not.

c. This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.

RELIABILITY CHEWABLE

Case Processing Summary

		N	%
Cases	Valid	5	100.0
	Excluded[a]	0	.0
	Total	5	100.0

a. Listwise deletion based on all variables in the procedure.

Reliability Statistics

Cronbach's Alpha	N of Items
.996	4

Intraclass Correlation Coefficient

	Intraclass Correlation[a]	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.985[b]	.940	.998	261.365	4	12	.000
Average Measures	.996[c]	.984	1.000	261.365	4	12	.000

Two-way mixed effects model where people effects are random and measures effects are fixed.

a. Type C intraclass correlation coefficients using a consistency definition-the between-measure variance is excluded from the denominator variance.

b. The estimator is the same, whether the interaction effect is present or not.

c. This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.

Excluded Variables[b]

Model		Beta In	t	Sig.	Partial Correlation	Collinearity Statistics		
						Tolerance	VIF	Minimum Tolerance
1	Sample 1	19.853[a]	1.729	.334	.866	3.760E-7	2659272.978	3.760E-7

a. Predictors in the Model: (Constant), Sample 3, Sample 2

b. Dependent Variable: Product

Collinearity Diagnostics[a]

Model	Dimension	Eigenvalue	Condition Index	Variance Proportions		
				(Constant)	Sample 2	Sample 3
1	1	2.651	1.000	.05	.00	.00
	2	.348	2.760	.93	.00	.00
	3	.001	49.689	.02	1.00	1.00

a. Dependent Variable: Product

REGRESSION CHEWABLE

Variables Entered/Removed[b]

Model	Variables Entered	Variables Removed	Method
1	Sample 3, Sample 2	.	Enter

a. Tolerance = .000 limits reached.

b. Dependent Variable: Product

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	1.000[a]	1.000	1.000	21.7898499

a. Predictors: (Constant), Sample 3, Sample 2

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	4800501.591	2	2400250.795	5055.314	.000[a]
	Residual	949.595	2	474.798		
	Total	4801451.186	4			

a. Predictors: (Constant), Sample 3, Sample 2

b. Dependent Variable: Product

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Collinearity Statistics	
		B	Std. Error	Beta			Tolerance	VIF
1	(Constant)	23.110	14.500		1.594	.252		
	Sample 2	-3.054	.165	-2.700	-18.510	.003	.005	215.204
	Sample 3	3.719	.148	3.677	25.204	.002	.005	215.204

a. Dependent Variable: Product

REGRESSION MULTIFORMULA

Variables Entered/Removed[b]

Model	Variables Entered	Variables Removed	Method
1	Sample 3[a]	.	Enter

a. Tolerance = .000 limits reached.
b. Dependent Variable: Product

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.997[a]	.994	.991	253.1059953

a. Predictors: (Constant), Sample 3

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	20727943.460	1	20727943.460	323.557	.003[a]
	Residual	128125.290	2	64062.645		
	Total	20856068.750	3			

a. Predictors: (Constant), Sample 3
b. Dependent Variable: Product

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Collinearity Statistics	
		B	Std. Error	Beta			Tolerance	VIF
1	(Constant)	-145.446	201.985		-.720	.546		
	Sample 3	1.722	.096	.997	17.988	.003	1.000	1.000

a. Dependent Variable: Product

RELIABILITY ALL ITEMS

Case Processing Summary

		N	%
Cases	Valid	12	100.0
	Excluded[a]	0	.0
	Total	12	100.0

a. Listwise deletion based on all variables in the procedure.

Reliability Statistics

Cronbach's Alpha	N of Items
.999	3

Intraclass Correlation Coefficient

	Intraclass Correlation[a]	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.996[c]	.989	.999	739.025	11	22	.000
Average Measures	.999[c]	.996	1.000	739.025	11	22	.000

Two-way mixed effects model where people effects are random and measures effects are fixed.
a. Type C intraclass correlation coefficients using a consistency definition-the between-measure variance is excluded from the denominator variance.
b. The estimator is the same, whether the interaction effect is present or not.
c. This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.

RELIABILITY CHEWABLE

Case Processing Summary

		N	%
Cases	Valid	5	100.0
	Excluded[a]	0	.0
	Total	5	100.0

a. Listwise deletion based on all variables in the procedure.

Reliability Statistics

Cronbach's Alpha	N of Items
.998	3

Intraclass Correlation Coefficient

	Intraclass Correlation[a]	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.996[b]	.978	.999	665.486	4	8	.000
Average Measures	.998[c]	.992	1.000	665.486	4	8	.000

Two-way mixed effects model where people effects are random and measures effects are fixed.
a. Type C intraclass correlation coefficients using a consistency definition-the between-measure variance is excluded from the denominator variance.
b. The estimator is the same, whether the interaction effect is present or not.
c. This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.

RELIABILITY MULTIFORMULA

Case Processing Summary

		N	%
Cases	Valid	4	100.0
	Excluded[a]	0	.0
	Total	4	100.0

a. Listwise deletion based on all variables in the procedure.

Reliability Statistics

Cronbach's Alpha	N of Items
.999	3

Intraclass Correlation Coefficient

	Intraclass Correlation[a]	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.998[b]	.984	1.000	1209.086	3	6	.000
Average Measures	.999[c]	.995	1.000	1209.086	3	6	.000

Two-way mixed effects model where people effects are random and measures effects are fixed.
a. Type C intraclass correlation coefficients using a consistency definition-the between-measure variance is excluded from the denominator variance.
b. The estimator is the same, whether the interaction effect is present or not.
c. This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.