REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY DETECTION METHOD FOR THE IDENTIFICATION AND DETECTION OF FAT-SOLUBLE VITAMINS IN PLASMA

A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

IN THE GRADUATE SCHOOL OF THE

TEXAS WOMAN'S UNIVERSITY

DEPARTMENT OF NUTRITION AND FOOD SCIENCE

COLLEGE OF HEALTH SCIENCES

BY SARAH TUCKER, BS

DENTON, TX

AUGUST 2017

ACKNOWLEDGEMENTS

I would first like to extend extreme gratitude towards my family. Thank you for every bit of support and encouragement you have provided me as I have worked on not only this project but also throughout my master's degree.

I would also like to thank my committee chair, Dr. K. Shane Broughton, and the rest of my committee, Dr. Nancy DiMarco and Dr. Mindy Maziarz, for their help and tireless efforts to help me finish this project when everything seemed to go wrong. I would particularly like to thank Dr. Broughton for patiently teaching me about the HPLC, giving me autonomy, and allowing me to test out my own ideas. Not only did you provide me the tools to succeed, but also the inspiration to love science. Dr. DiMarco, I would like to specially thank for offering emotional support and providing me a family within the IWH, as well as always coming through with the funding needed to complete this project. Finally, Dr. Maziarz, I would especially like for stepping in last minute and providing flexibility, encouragement, and support in my next step.

I would finally like to thank my friends and colleagues, Thomas Hoover, Sarah Deemer, Todd Castleberry, Marco Avalos, Pallavi Panth, Desiree Patterson, and Nathaniel Kerr. Without their help, data collection and analysis for this project would not have been possible. A very special thanks to Thomas Hoover for always standing by my side offering encouraging words and food.

ABSTRACT

SARAH TUCKER

REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY DETECTION METHOD FOR THE IDENTIFICATION AND QUANTIFICATION OF FAT-SOLUBLE VITAMINS IN PLASMA

AUGUST 2017

The purpose of this study was to establish a method for simultaneous evaluation and quantification of fat-soluble vitamins in human blood samples using reverse-phase high performance liquid chromatography coupled with photodiode array detection. The biochemical forms of fat-soluble vitamins analyzed include retinol, cholecalciferol, calcidiol, α-tocopherol, γ-tocopherol, and phylloquinone. This method is novel in the use of prostaglandin B_1 as an internal standard for quantification and utilizes a simple method of extraction without derivatization or saponification. The method had a high degree of reproducibility as evidenced by coefficients of variation 1.7% and 2.5% of the mean for retinal and cholecalciferol respectively.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS iii

ABSTRACT iv

LIST OF TABLES viii

LIST OF FIGURES ix

Chapter

I. INTRODUCTION 1

Problem Statement 1
Specific Aims 3
Definitions and Abbreviations 3
Assumption 4
Limitations 5
Purpose of Study 5

II. REVIEW OF LITERATURE 6

Fat-soluble vitamins 6
Vitamin A 6
Functions/forms 6
Retinol chemical characteristics 8
Methods for serum/plasma retinol analysis 9
Vitamin D 11
Functions/forms 11
Cholecalciferol chemical characteristics 13
Calcifidiol chemical characteristics 14
Methods for serum/plasma vitamin D analysis 15
Vitamin E 17

Functions/forms 17
Chemical characteristics for α-Tocopherol 19
Chemical characteristics for γ-Tocopherol 20
Methods for α-Tocopherol and γ-Tocopherol analysis 20
Vitamin K 21
Functions/forms 21
Phylloquinone chemical characteristics 22
Methods for phylloquinone analysis 23
High performance liquid chromatography 25
Chromatography 25
Elution 26
Migration rates 27
Band broadening 28
Optimization of performance 29
Detectors 31
Ideal detector 31
Types of detectors 32
Photodiode array detection 32
Quantitative Analysis 33
Inclusion of PGB_1 as a standard 34
Molar extinction coefficient 35
Extraction 35
Types of extraction 35
Single-phase extraction 36
Double-phase extraction 36
Saponification 37
Solid-phase extraction 37
Methodology overview 37

III. METHODOLOGY 38

Materials 38
Standards and solvents 38
Instrumentation 38
Methods 38
Plasma sample collection 38
Preparation of standards 40
Re-suspension solvent 41
Isocratic mobile phase 41
Gradient mobile phase 42
Mobile phase 42
Flow rate 43

Sample preparation 43
Final methodology 44
Validation 44
Reproducibility 44

IV. PRESENTATION OF FINDINGS 45

Re-suspension solvent 45
Mobile phase 45
Flow rate 47
Extraction 47
Saponification 47
Validation 48
Reproducibility 48

V. DISCUSSION AND SUMMARY 49

Analytes 49
PGB_1 as an internal standard 49
Isocratic vs. gradient mobile phase 51
Flow rate 51
Saponification 51
Reproducibility 52
Use of photodiode array compared to mass spectroscopy 54
Limitations 55
Future research 55
Summary 56

REFERENCES 57

APPENDICES

A. Texas Woman's University Institutional Review Board Human Subjects Review Committee Approval 63
B. Consent Form 65
C. Recruitment Flier 69
D. Questionnaire 71

LIST OF TABLES

Table Page

1: Standard Dilutions and Solvents 40

2: Molar Extinction Coefficients 41

3: Results of Saponification Shown as AUC Maximum Percent 47

LIST OF FIGURES

Figure Page

1. Retinol Structure 9

2. Cholecalciferol Structure 14

3. Calcifidiol Structure 15

4. D-α-Tocopherol Structure 19

5. D- λ-Tocopherol Structure 20

6. Phylloquinone Structure 23

7. (a) Diagram showing the separation of a mixture of components A and B by column elution chromatography. (b) The output of the signal detector at the various stages of elution shown in (a) 26

8. Prostaglandin B_1 Structure 34

9. Vitamin D_3 Chromatogram at 264nm of a [100:0] transition to [13:87] [MeOH:ACN] ramp 46

10. Graph of Vitamin D_3 [100:0] transition to [13:87] [MeOH:ACN] ramp at 264 nm 46

11. Flow Rate Effect on Peak Height: Width Ratio 47

12. The photochemical reaction in human epidermis of 7-DHC to $preD_3$. The $preD_3$ either thermally converts to vitamin D_3 or photoisomerizes to $lumisterol_3$ and $tachysterol_3$ 53

13. Ultraviolet absorption spectrums of (a) $preD_3$, (b) $tachysterol_3$, (c) 7-DHC, and (d) $lumisterol_3$, isolated from human epidermis 53

CHAPTER I

INTRODUCTION

Problem statement

Vitamins are carbon-containing compounds that serve as cofactors in a range of required physiologic processes. Vitamins can be divided into water- and fat-soluble, which include vitamins A, D, E, and K. The fat-soluble vitamins are essential and must be consumed in the diet because only small amounts of vitamins D and K, but no vitamin A and E, are endogenously synthesized. The fat-soluble vitamins, A, D, E, and K, are absorbed in chylomicrons with dietary fat and, in general, are stored in adipose tissue. Due to a longer duration of storage in the body, daily consumption of fat-soluble vitamins is not necessary, making them more prone to toxicity. Deficiency and toxicity of these vitamins can have serious repercussions for health, so an accurate method for evaluation is needed. There is no current method for a comprehensive evaluation of circulating forms for these vitamins simultaneously, rather, they are independently analyzed. This results in costly analysis that is time inefficient. A faster and more economical methodology to detect fat-soluble vitamins through high performance liquid chromatography (HPLC) coupled with photodiode array (PDA) detection will allow for a larger population to be tested in reduced time for potential deficiencies or toxicities, proving beneficial over the current methods for analysis of each fat-soluble vitamin.

Using HPLC with ultraviolet (UV) detection can be an ideal method to quantify the fat-soluble vitamins in human plasma or serum. Currently, the best indicator of vitamin A status is a liver biopsy as vitamin A is primarily stored in the liver. While this is acceptable for mouse or rat models, it is unsuitable in a human population as it is invasive [1]. The preferred method to measure serum or plasma retinol is HPLC with UV detection after separation from its protein carrier, retinol-binding protein. Enzyme-linked immunosorbent assay (ELISA) can also be used to determine plasma retinol concentrations, but at approximately $4.60 per assay, the HPLC may prove to be a more cost effective solution for multiple analyses [1]. Normal vitamin D assessment methods utilize antibody-based assays, such as ELISA, or HPLC with UV/Visible detection [2]. HPLC is the preferred method as antibody-based assays are not specific enough and are often criticized for overestimation[3]. They are sensitive to exogenous interference, and show variability in results. HPLC with mass spectrometry detection is emerging as a gold standard; however is often cost prohibitive [3]. Normal vitamin E analysis uses HPLC with fluorescence detection, or through gas chromatography with mass spectrometry detection [3]. Both of these methods have comparable time, cost, and reproduction of results. When contrasted with HPLC and PDA detection, PDA demonstrates less interference with higher sensitivity [4]. Vitamin K is normally assessed indirectly by the proteins in the Vitamin K absence (PIVKA) II Test [1]. PIVKA II are precursors of active prothrombin, which are normally converted to the active form by vitamin K, but accumulate in blood when vitamin K is deficient [3].

Use of HPLC with PDA detection will alleviate many limitations while allowing for more specific and reproducible testing of all fat-soluble vitamins. Furthermore, simultaneous examination of fat-soluble vitamins is beneficial as it allows for a quick comprehensive test for fat-soluble status. A comprehensive test could aid in diagnosis of deficiency or toxicity.

Specific aims

1. Establish an optimal extraction method.
2. Determine HPLC-PDA methodology for fat-soluble vitamin identification and quantification.

Definitions and abbreviations

Chromatography**:** A powerful separation method in which a chemical mixture is separated as a result of differential distribution of solutes as they interact with stationary and mobile phases, invented and named by the Russian botanist Mikhail Tswett shortly after the turn of the 20^{th} century [5].

Column Chromatography**:** A form of chromatography in which a solvent mixture is introduced to the top of column packed with a stationary phase, usually in the form of a silica gel, and passes through the column at a rate dependent on the relative affinity of the solute for the stationary or mobile phase.

High Performance Liquid Chromatography (HPLC)**:** A column chromatography method in which separation occurs induced by forcing a solvent through a column under pressure [5].

Solute-Property Detector**:** a detector that responds to a unique property of the solute, such as UV absorbance or fluorescence, that is not possessed by the mobile phase [5].

Photodiode Array (PDA) Detection: A detector in which a solute flows from a column, through a flow cell and is exposed to a beam of UV light. The light absorbance is measured and plotted as a chromatogram as a function of time. Many simultaneous wavelengths can be detected due to the scanning capability of the detector [5]**.**

Internal standard: The most precise method of quantification of an unknown quantity of a compound uses a carefully measured quantity of a known internal-standard. The standard is spiked into each sample, and uses the internal standard peak areas and known mathematical relationship to establish the unknown quantity of a known compound [5].

Prostaglandin B_1 (PGB_1): a synthetic prostaglandin used as an internal standard to improve accuracy in quantification.

Assumption

The assumption of this study will be

1. Prostaglandin B_1 will be used as an internal standard. PGB_1 will extract at a similar and proportional efficiency of extraction and spectrophotometric behavior as the fat-soluble vitamins due to its relatively similar structure and solubility.

Limitations

The limitations of this study will be:

1. The inability to identify cis-/trans-isomerization, which may result in peak isomer separation would reduce overall area under the curve (AUC) of a known peak.
2. This HPLC method is designed to only detect fat-soluble vitamins in the free form and not bound to proteins or other ligands.

Purpose of study

The purpose of this study is to establish a method for simultaneous evaluation and quantification of fat-soluble vitamins, including retinol, cholecalciferol, calcidiol, α-tocopherol, γ-tocopherol, and phylloquinone, in human blood samples using HPLC coupled with PDA detection.

CHAPTER II

REVIEW OF LITERATURE

Fat-Soluble vitamins

Vitamins are essential micronutrients required to assist in various metabolic processes in the human body. They are divided into two categories: fat-soluble and water-soluble. The fat-soluble vitamins, A, D, E, and K, differ from water-soluble vitamins, the B-complex and C, in absorption, transport, and storage. Fat-soluble vitamins are hydrophobic and require emulsification with polar bile salt micelles to cross the unstirred water layer into the enterocytes, where they are repackaged into chylomicrons and leave the enterocyte via exocytosis into the lymph. The vitamins travel in the lymph and enter blood circulation at the thoracic duct. They require carriers for transport throughout the blood, and are stored in body lipids, though the amount stored varies widely between the different vitamins. For example, vitamin A is primarily stored in the liver stellate cells. The differing functions, forms, chemical characteristics, and uses for plasma/serum analysis in recent research for each vitamin are discussed below.

Vitamin A

Functions/forms

Vitamin A refers to the group of retinoids, including retinol, retinal, and retinoic acid as preformed vitamin A, as well as proformed carotenoids. Most think of the

retinoids and their role in vision, where they are involved in eye adjustment to changes in light [6]. However, vitamin A is also required for cell differentiation in a role critical for embryonic development, fertilization, and aids healthy bone growth and immune function [6]. Vitamin A is normally reported as retinol activity equivalents (RAE). One RAE is equal to 1 μg of retinol, 12 μg of β-carotene, 24 μg of α-carotene, and 24 μg β-cryptoxanthin. The Institute of Medicine (IOM) established the recommended dietary allowance (RDA), or the daily sufficient intake for nearly all healthy people, for vitamin A at 900 μg/day for men and 700 μg/day for women with an upper limit (UL) of 3,000 μg/day [1]. Consuming less than the RDA can cause deficiency. Vitamin A deficiency (VAD) symptoms include anorexia, retarded growth, increased susceptibility to infection, obstruction and enlargement of hair follicles, keratinization of epithelial cells of the skin, and ocular events such as night blindness, xerophthalmia, corneal xerosis, Bitot's spots, or keratomalacia [6]. People who are most at risk for VAD are those with fat malabsorption disorders, chronic nephritis, acute protein VAD, intestinal parasites, and those with acute infections, such as measles [6]. Toxicity of vitamin A is termed hypervitaminosis A. Chronic intake of doses that are three to four times greater than the RDA or a single megadose such as 15,000 μg may result in acute hypervitaminosis A, with symptoms including nausea, vomiting, double or blurred vision, increased intracranial pressure, headache, dizziness, skin desquamation, and muscle incoordination [3]. Toxicity can be produced with roughly ten times the RDA and is characterized by anorexia, skin desquamation, alopecia and coarsening of the hair, ataxia, headache, bone

and muscle pain, conjunctivitis and eye pain, nausea, vomiting, abdominal pain, liver damage, and increased risk of bone fractures [3]. Additionally, toxicity has multiple detrimental effects on the liver, the primary storage site for vitamin A, including fat-storing cell hyperplasia and hypertrophy, fibrogenesis, sclerosis of veins, portal hypertension, and congestion in perisinusoid cells, which leads to hepatocellular damage and cirrhosis or a cirrhosis-like disorder [3]. In addition, vitamin A is a teratogen and can cause severe birth defects when high levels are consumed during pregnancy. People at risk for excessive intake of vitamin A include those who may take medications such as oral acne treatment medications Accutane and Roaccutane, or those who are improperly supplementing [6]. Plasma retinol levels between 20 to 50 μg/dL are normal, less than 10 μg/dL indicate VAD, and plasma retinol levels exceeding 100 μg/dL indicate hypervitaminosis A [1].

Retinol chemical characteristics

Retinol is the alcohol form of the retinoids, and is identified by the hydroxy group on the 15^{th} carbon. This is the form found most abundantly in circulation, which is typically bound to retinol binding protein. Retinol has a molecular weight of 286.459 g/mol, a boiling point of 137-138 degrees Celsius, and a melting point of 62-64 degrees Celsius [7]. Free alcohol is sensitive to air oxidation, but is stable in an oil matrix. Retinol is inactivated by UV light. It is soluble in absolute alcohol, methanol, chloroform, ether, acetone, and benzene. It has a UV max in ethanol of 325nm [7].

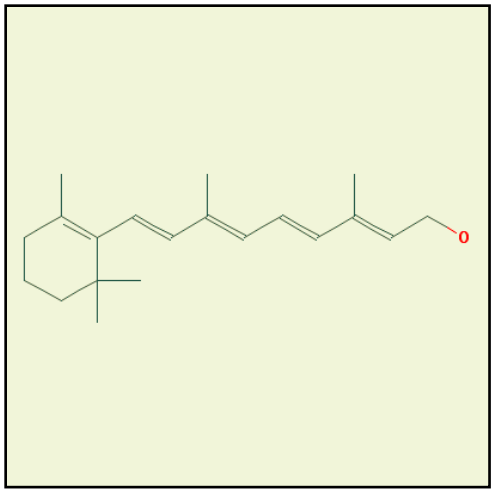

Fig. 1. Retinol structure [7]

Methods for serum/plasma retinol analysis

There are significant albeit limited reasons for evaluating serum or plasma retinol concentrations. The primary use of retinol analysis is as an assessment for VAD, an established public health concern in numerous developing countries. In 2015 Cediel et al., published a review that evaluate the changes in vitamin A status in Latin America and the Caribbean before and after 1998 [8]. This review identified ten national surveys and six representative studies documenting a significant decrease of VAD in children under 6 years of age in many Central American countries, but confirms VAD as a public health problem in a multitude of Latin America and Caribbean countries, particularly among disadvantaged and vulnerable groups. Limitations existed in the use of serum/plasma retinol as a biomarker for body stores and that results should be interpreted with caution. Spectrophotometry was used to assess serum retinol in two of the studies; the remaining

ten used HPLC for analysis. Proceedings from the XX International Vitamin A Consultative Group meeting discussed the limitation that Cediel et al., mentioned. Pee and Dary outline that serum retinol is homeostatically controlled and will not drop until body stores are significantly compromised. Furthermore, serum retinol can be influenced by factors that influence holo-RBP release from the liver, including infection, protein status, adequacy of other nutrients, and organ disease [9]. In two of the sixteen studies reviewed by Cediel et al., where there was an expected reduction in retinol, when adjusted for inflammation, levels were not as depressed as anticipated. This highlights a notable limitation in vitamin A research. Despite its limitations, serum and plasma retinol remains the preferred indicator for assessment of VAD because it is the best established biochemical indicator of vitamin A status and many labs can analyze for levels [9]. The usual method for retinol assessment is by HPLC or spectrophotometry, though fluoremetry can be used. While spectrophotometry is simpler and less costly, HPLC analysis is preferred because of its sensitivity. This is particularly significant as the primary use for serum and plasma retinol analysis is as an indicator for VAD. Fluoremetry is rarely used due to its poor specificity.

Serum Retinol Binding Protein (RBP) is a common substitute biomarker to assess vitamin A status since it occurs in a 1:1:1 M complex with retinol and transthyretin. There are many benefits to utilizing RBP as a biomarker. Because it is a protein, RBP can be detected immunologically, meaning it is simple and inexpensive. Furthermore, the serum protein is easier since RBP is more stable than retinol and is not influenced by

light and temperature, and only 10 to 20 μL is required to as opposed to 100μL minimum required for HPLC, the difference between a finger prick and venous blood [9]. These benefits however, are outweighed by their limitations. The 1:1:1 M ratio is theoretical, therefore there is no cut-off for serum RBP that has been accepted to determine deficiency. This is partially because immunologic assays cannot distinguish the difference between holo- and apo-RBP, and the variance between the two is heavily influenced by many acute-phase responses such as protein or energy malnutrition, liver disease, chronic renal failure, and acute-stress [9]. While there is a clear use for assessment of RPB in the field, serum or plasma retinol is the preferred method because of the limitations in RBP assessment, but the gap in difficulty of assessment needs to be lessened between the two biomarkers.

Vitamin D

Functions/forms

Vitamin D, also called calciferol, refers to a group of seco-sterols with the two main forms ergocalciferol (D_2) found in plants, and cholecalciferol (D_3) found in animals [6]. Vitamin D can be obtained through the diet or synthesized from 7-dehydrocholesterol following light activation in the skin from ultraviolet B (UVB) radiation exposure [3]. Calcidiol (25-OH D), the major form in circulation is bound to the plasma carrier protein, vitamin D binding protein (DBP), has a high affinity for calcitriol ($1,25\text{-}(OH)_2D_3$) in the kidney and other tissues [3]. Calcitriol is the biologically active hormone responsible for most of the roles of vitamin D. Beyond its role in regulation of serum calcium and

phosphorus that maintain bone health; vitamin D may reduce the risk of several cancers, diabetes mellitus, multiple sclerosis, tuberculosis, and susceptibility to infection [10]. The RDA for vitamin D, while highly debated, in 2010 was set at 600 international units (IU), or 15μg per day for ages 19 to 70, 800 IU (20μg) per day for adults over 70 years of age [2]. Reports suggest toxicity levels may occur at approximately 10,000 to 40,000 IU/day [2]. Vitamin D Deficiency (VDD), widespread across the United States and the world, results in rickets in children, and osteomalacia and/or osteoporosis in adults [3]. Sun exposure can promote endogenous synthesis of vitamin D and maintain adequate status for most people, however aging reduces synthesis of 7-dehydrocholesterol in the skin and reduces activity of renal 1-hydroxylase, so older individuals can be at risk for insufficiency [6]. People with inadequate sun exposure and those with certain diseases or conditions such as Crohn's disease, pancreatitis, and liver damage, may also be at risk for deficiency [6]. Enhanced breakdown resulting in deficiency is found in epileptics being treated with phenobarbital or Dilantin. Toxicity, while rare, can be the result of chronic intakes of 10,000 IU or more resulting in hypercalcemia and calcinosis, the calcification of soft tissues such as kidneys, heart, lungs, and blood vessels [2]. Additionally, toxicity can cause hyperphosphatemia, hypertension, anorexia, nausea, weakness, headache, renal dysfunction, and may even result in death [3]. Toxicity cannot be reached through diet alone; however, as many foods are vitamin D fortified, consumers are encouraged to take note of what they consume when considering supplementation. Controversy of deficiency levels exist between health agencies, each preferring to use different serum 25-OH D

levels to indicate vitamin D status [3]. The IOM defines nutritional categories of serum 25-OH D less than 20 ng/mL as deficient, 20-50 ng/mL as sufficient, and toxicity as >50 ng/mL [2]. Other investigators include an additional category of insufficiency, using 30 ng/mL as the difference between inadequacy and adequacy [11], while other groups identify normalcy between 20 to 60 ng/mL [2]. The differing definitions are founded on differing indicators of vitamin D function. For example, the IOM definitions are based on levels resulting in normal bone health in 97.5% of the population, while others use serum parathyroid hormone (PTH) as the indicator, which is normalized when serum 25-OH D is approximately 32 ng/mL [3]. The controversy extends to questioning the use of 25-OH D as a relevant biomarker of effect, as significant variation has produced an information gap [3].

Cholecalciferol chemical characteristics

Cholecalciferol is a derivative of 7-dehydroxycholesterol, formed in the skin by the UV ray induced breaking of the C9-C10 bond. Abbreviated as D3, it differs from ergocalciferol by having a single bond between C22 and C23, and lacking a methyl group at C24.

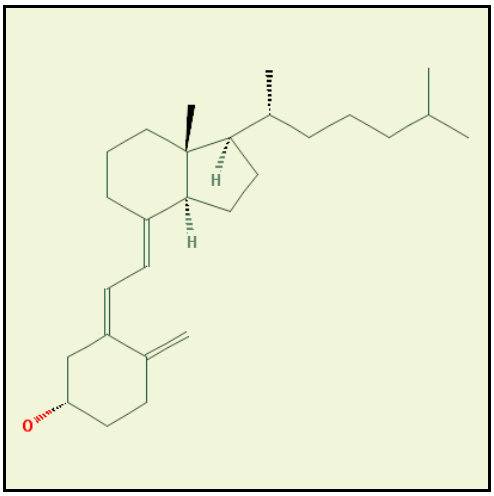

Fig. 2. Cholecalciferol structure [12]

D_3 is found in the blood, as part of a chylomicron or bound to Vitamin D Binding Protein (DBP). D_3 has a molecular weight of 384.64 g/mol, and a melting point of 84-85 degrees Celsius [12]. D_3 can be oxidized and inactivated by moist air within a few days, and is affected by light. Degradation, however, is negligible after storage in amber tubes at refrigerator temperatures after 1 year [12]. It is soluble in usual organic solvents and has a UV max in alcohol or hexane of 265nm [12].

Calcifidiol chemical characteristics

Calcifidiol the major circulating metabolite of vitamin D_3 and is abbreviated 25-OH D_3. It is hydroxylated on C25 in the liver by 25-hydroxylase, and serves as the best indicator of the body's vitamin D stores. 25-OH D has a molecular weight of 418.647 g/mol, and a melting point of 84-85 degrees Celsius [13].

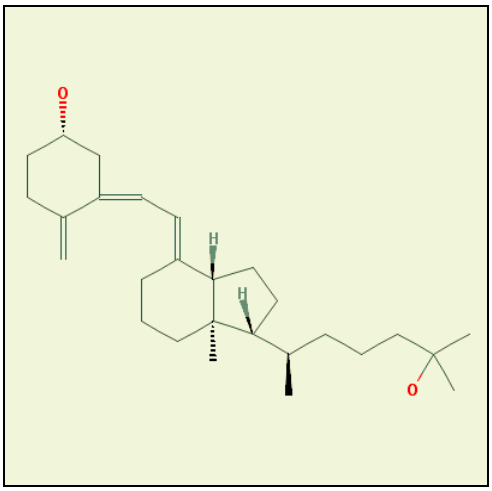

Fig. 3. Calcifidiol Structure [13]

Methods for serum/plasma vitamin D analysis

Vitamin D research spans a wide variety of subjects, but current trends primarily focus on the continuing evidence of widespread deficiency and the role of 1,25-$(OH)_2D_3$ in hormonal regulation and potential genetic polymorphisms in vitamin D receptor (VDR). Calcitriol is taken up by the cells in all tissues and enters the nucleus to bind to the nucleus receptor on DNA known as the VDR. At this point, the VDR can associate with other nuclear receptors bound with a ligand to form a Homodimer or heterodimer. Upon dimerization, gene transcription is either up- or down-regulated to increase or decrease the production of certain proteins. As a result of its many functions, vitamin D is being explored as a potential cause or contributor in many disease states. These include associations between vitamin D and insulin resistance and the consequential impact in metabolic syndrome, an increased risk of tuberculosis, reproductive dysfunction, functional decline, and multiple sclerosis [14–18]. More classic and direct roles of

vitamin D continue to be explored in areas including various cancers, autoimmune disorders, cardiovascular disease, muscle weakness and frailty [11,17].

The many metabolites of vitamin D differ in biological activity and availability for assessment. Herrmann et al., identified that 1,25-$(OH)_2D_3$ is significantly more active than the other metabolites of vitamin D, and the affinity is 900 times lower for 25-OHD and 5000 times lower for 24,25-$(OH)_2D_3$, the inactive form when 25-OHD is not needed, therefore resulting in dramatically different blood concentrations [14]. This sparks debate as to the best measure to assess vitamin D status.

Holick et al. recommends using circulating serum 25-OH D, measured by a reliable assay, to measure for VDD, but also notes that multiple measurement methodologies, including radioimmune assay, HPLC, and liquid chromatography tandem mass spectroscopy exist and are adequate for clinical evaluation of deficiency [19]. Circulating 1,25-$(OH)_2D_3$ has a half-life of approximately 4 hours, it circulates at approximately 1000 times lower concentration than 25(OH)D, and the blood level is tightly regulated by PTH, calcium, and phosphate, and therefore, is not a useful biomarker for vitamin D status. Additionally, 1,25-$(OH)_2D_3$ is often normal or elevated in those with VDD due to secondary hyperparathyroidism, so use as a measure for vitamin D status can produce type I or II error [19]. Herrmann et al. adds that serum 25-OHD is indicative of clinical outcomes associated with deficiency, such as bone mineralization, fracture risks, falls risk, all-cause mortality, and cardiovascular events, has little deviation within short periods of time due to its half-life of 2-3 weeks, and represents both dietary

intake and dermal production of vitamin D. This argument is juxtaposed with 1,25-(OH)-$_2D_3$, which shows a significant response to sun exposure, evidenced by seasonal variation, and to vitamin D supplementation [14]. Holick et al. argues that 1,25-$(OH)_2D_3$ is an appropriate measure for disorders in the metabolism of 25(OH)D and phosphate, such as chronic kidney disease, hereditary phosphate-losing disorders, oncogenic osteomalacia, pseudovitamin D-deficiency rickets, vitamin D-resistant rickets, as well as chronic granuloma-forming disorders such as sarcoidosis and some lymphomas [19].

Vitamin E

Functions/forms

Vitamin E, found primarily in plant oils, includes eight compounds containing a phenolic functional group on a chromoanol/chromane ring and an attached phytyl side chain, and is divided into two classes, tocopherols and tocotrienols [6]. Of the eight forms, only d-alpha tocopherol has functional biologic activity that can meet the body's requirement [6]. Therefore the RDA of 15 mg/day for men and women and the UL of 1,000 mg/day is based only on 2R-α-tocopherol [4]. The primary function of vitamin E is as an antioxidant in free radical termination, preventing the propagation of lipid peroxidation, and is therefore thought to aid in prevention and treatment of heart disease, cataracts, and age-related macular degeneration, improved immune function, and cancer prevention [3]. The different forms of vitamin E have other additional functions, notably its role in inhibition of protein kinase C [3]. Deficiency is rare with only a few population groups at risk, namely premature infants because of their impaired fat utilization, and

those with fat malabsorption disorders such as cystic fibrosis characterized by pancreatic lipase deficiency, and hepatobiliiary system disorders, characterized by decreased bile production [4]. More recently, vitamin E deficiency has been documented in women with repeated liposuction surgery [4]. Deficiency symptoms include myopathy and muscle weakness, hemolytic anemia, and degenerative neurological problems such as peripheral neuropathy, ataxia, loss of vibratory sense, and loss of coordination of limbs [6]. Toxicity is rare, as vitamin E appears to be the least toxic of the fat-soluble vitamins [6]. Large doses between 200 mg and 800 mg may cause mild gastrointestinal problems that are thought to be due to increased tendency for bleeding due to squelching of free radicals necessary in the process of thrombus formation [4]. Doses of 1,000 mg or greater can cause nausea, diarrhea, flatulence, impaired blood coagulation, exacerbation of respiratory infections, muscle weakness, fatigue, and double vision [4]. The IOM defines a plasma concentration lower than 12μmol α-tocopherol/L as deficient, and found plasma levels of approximately 20μmol α-tocopherol in physiologically normal subjects [4]. When assessing vitamin E score, however, blood lipids must be considered because even normal levels indicated in plasma may not be sufficient to protect from oxidative stress. Therefore, an additional value exists that divides the plasma α-tocopherol by the sum of plasma total cholesterol and triglycerides, where normal is >0.8 mg α-tocopherol/g total lipid is considered normal [3].

Chemical characteristics of α-Tocopherol

α-Tocopherol is a naturally-occurring form of vitamin E and the most active form. It serves as an antioxidant as a result of the phenolic hydrogen that reacts with oxygen, thereby neutralizing free radicals and protecting against oxidation. The saturated 16 carbon phytyl side chain classifies this form of vitamin E as a tocopherol. It is the most abundant form in a blood matrix and the best characterized in current research [20]. α-Tocopherol has a molecular weight of 430.717 g/mol, and a melting point of 3 degrees Celsius [21]. It is unstable in UV light, alkalies, and oxidation, and soluble in alcohol, ether, acetone, and chloroform [21]. In methanol, α-tocopherol has a uv max of 292 nm [21].

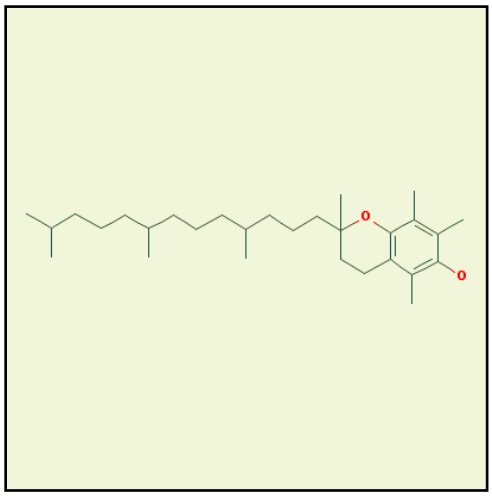

Fig 4. D-α-Tocopherol Structure [21]

Chemical characteristics of λ-Tocopherol

λ-Tocopherol is a naturally occurring and bioavailable tocopherol with less active antioxidant properties than α-tocopherol, but structurally also contains the active phenolic functional hydrogen as well as the saturated 16 carbon phytyl side chain. It is the second most abundant form of vitamin E in a blood matrix [20].

λ-Tocopherol has a molecular weight of 416.69 g/mol [22].

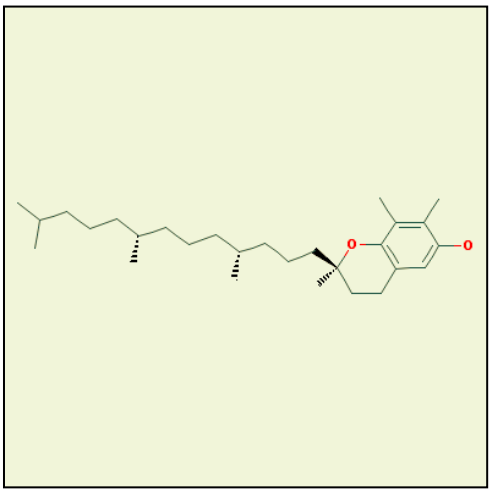

Fig. 5. D- λ-Tocopherol Structure [22]

Methods for α-Tocopherol and λ-Tocopherol analysis

Vitamin E, then called factor X, was discovered in 1922 by Evans and Bishop [23]. Since then, the unique roles of each isomer has been explored, yet not fully understood. Current applications in vitamin E research include its potential use to slow cognitive decline in Alzheimer's disease (AD). Farina et al., concluded in a 2017 review that, while there is no evidence that alpha-tocopherol given to patients with mild

cognitive impairment (MCI) prevents progression to dementia or causes improvement in cognitive function in MCI or AD, there is moderate evidence to suggest that it slows functional decline in AD without any increased risk of adverse events or mortality [24]. This is in contrast with Grimm et al., who have more modest conclusions. While they do acknowledge beneficial effects of supplementation on plaque load reduction, they note that vitamin E supplementation has caused more rapid cognitive decline in AD- and MCI-Patients in some studies [25]. Overall it is noted that more research in understanding the molecular mechanisms of the disease is needed to understand potential influences. In their review of current vitamin E research, Galli et al., point out other studies that evaluated vitamin E and understanding it's application in Atherosclerosis and associated cardiovascular complications, Immunomodulation in allergic disease and non-alcoholic fatty liver and steatohepatitis [26].

Torquato et al., notes that vitamin E is primarily analyzed by liquid or gas chromatography. In liquid chromatography, detection commonly includes electrochemistry, fluorescence, ultraviolet absorbance, and evaporative light-scattering, respectively decreasing in sensitivity [20].

Vitamin K

Functions/forms

Vitamin K occurs naturally as phylloquinone (K_1), the plant form, and menaquinone (K_2), produced by bacteria in the lower bowel [6]. However, the amount of vitamin K produced by bacteria is unknown and is not enough to meet the RDA. The

primary function of vitamin K is its role in blood coagulation, specifically in posttranslational conversion of glutamyl residues to γ-carboxyglutamyl (Gla) residues in prothrombin and in the plasma procoagulants, factors VII, IX, and X [3]. The adequate intake (AI), based on dietary intake data from healthy individuals, is set at 120 μg per day for men older than 19 years of age and 90 μg per day for women older than 19 years of age [1]. Vitamin K deficiency results in a vitamin K-responsive increase in prothrombin activation time, or in severe cases a hemorrhagic event [1]. Those at risk for deficiency include newborn infants, those using chronic antibiotics, those with severe fat malabsorption disorders, and the elderly [3]. Toxicity has not been shown with phylloquinone, however the synthetic menadione can cause hemolytic anemia and liver damage attributable to combining with sulfhydryl groups leading to glutathione oxidation and membrane damage induced by phospholipid oxidation [3]. Deficiency is identified by plasma concentrations less than 0.5 μg/L while a UL limit remains undefined [3].

Phylloquinone chemical characteristics

Phylloquinone, often referred to as vitamin K1, is part of a family of phylloquinones characterized by a ring of 2-methyl-1,4-naphthoquinone and an isoprenoid side chain with only one double bond. It is the primary circulating form of vitamin K and has been successfully used to measure vitamin K status on clinical and population levels [27]. Phylloquinone has a molecular weight of 450.707 g/mol, a boiling point of 140-145 degrees Celsius, and a melting point of -20 degrees Celsius [28]. It is not stable and will decompose in sunlight. It is moderately soluble in methanol, and

soluble in acetone, benzene, petroleum ether, hexane, dioxane, and chloroform. It has a UV max in petroleum ether of 242, 248, 260, 269, or 325 nm [28].

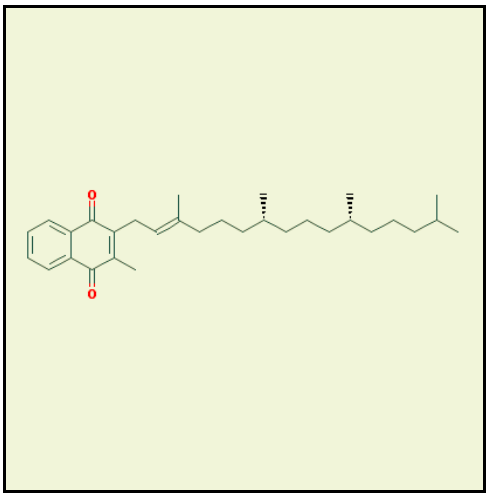

Fig. 6. Phylloquinone structure [28]

Methods for phylloquinone analysis

Serum and plasma phylloquinone research has a strong focus in the complex interaction of vitamin K with many anticoagulants. There is an ongoing challenge of managing medications for the differing biochemistry within each patient, coupled with controlling the fine balance between diet and medication that accompanies diseases such as atrial fibrillation. Managing the risk of myocardial infarction and stroke progression becomes a balancing act requiring constant evaluation of risk/benefit analysis for specific drugs and their mechanisms. The current research using serum/plasma phylloquinone is reflective of these interactions that are in need of constant evaluation.

Research generally uses food frequency questionnaires and 24-hour diet recalls to assess dietary vitamin K intake, most recently in association with bone health and the risk of fracture, along with mortality risk in people with chronic kidney disease [29,30]. Shea and Booth found that higher phylloquinone intake has been associated with higher bone mineral density and lower fracture risk, lower cardiovascular disease risk, improved insulin sensitivity, and lower mortality risk, while other research found no associations [27]. This highlights the existing conflict in vitamin K research. Shea and Booth also add that interpretation of phylloquinone intake should be made with caution as adequate vitamin K levels also reflect a healthy diet and lifestyle, and that a confounding effect may not be eliminated completely with adjustments [27]. This can also partially be due to the over-reporting of vegetable intake that is a known limitation of food frequency questionnaires and 24-hour diet recalls [31].

Because of the low circulating levels of phylloquinone, HPLC and mass spectrometry assays are used to assess circulating phylloquinone due to higher sensitivity [27]. The limitations of vitamin K analysis in research must be considered. There is a considerable amount of variation that has lead to inaccurate reporting, requiring considerations to account for when reporting. The changes in circulating phylloquinone due to diet and meals accounts for a large amount of deviation in the literature. The concentrations peak 6-10 hours post-prandial, with the response varying according to the type of meal [32,33]. Furthermore, since phylloquinone is transported in circulation within triglyceride-rich lipoproteins, it should be measured in fasting samples and

corrected for triglycerides to better reflect nutritional status. Furthermore, there is no established threshold of plasma/serum phylloquinone that indicates deficiency. When the AI of phylloquinone is met, circulating phylloquinone concentrations are approximately 1.0 nM, however there is debate whether the AI is sufficient to meet all physiologic needs [27] . Menaquinone is not generally used to assess vitamin K because it generally not detected in circulation unless supplements are taken or there is a very high intake of menaquinone-rich foods [27].

High performance liquid chromatography

Chromatography

Invented by Russian botanist Mikhail Tswett in 1900, chromatography encompasses a broad group of methods that allow for separation, identification, and determination of components in a mixture. The broad chromatographic method consists of two phases: a mobile phase and a stationary phase. The sample is dissolved into the mobile phase, which can be a gas, liquid, or supercritical fluid. Mobile phase is then forced through an immiscible stationary phase, which is either fixed in a column or on a solid surface. Components of the sample then scatter between the two phases based on affinity. Those that have a higher affinity for the stationary phase move slowly with the mobile phase, while those with a higher affinity for the mobile phase move quickly with the mobile phase. Consequently, the components separate into bands that can be analyzed, shown in Figure 7.

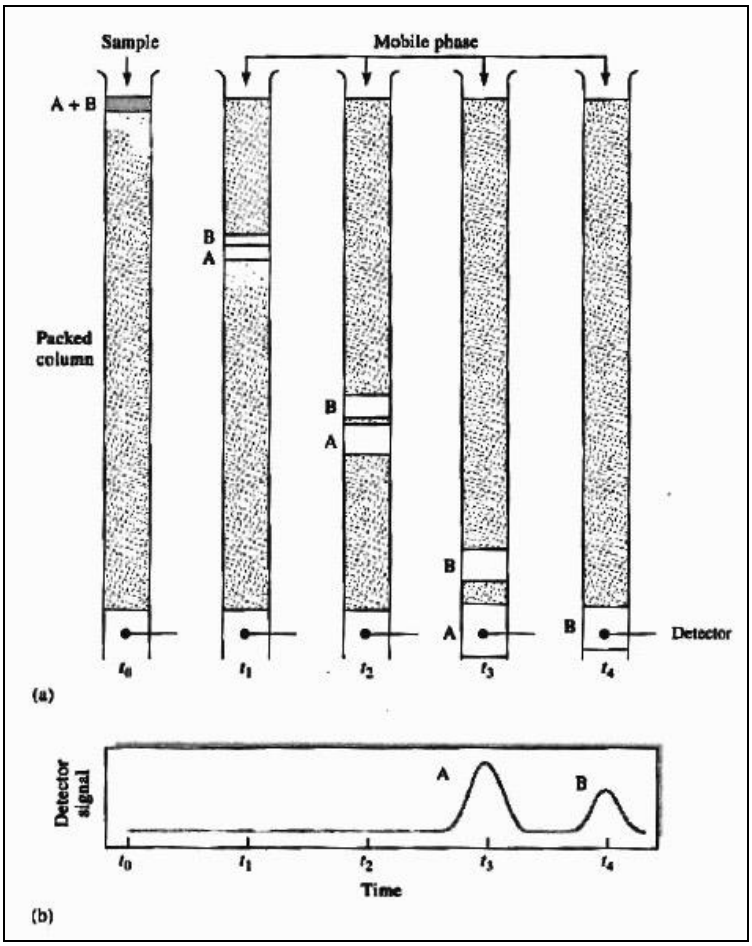

Fig. 7. (a) Diagram showing the separation of a mixture of components A and B by column elution chromatography. (b) The output of the signal detector at the various stages of elution shown in (a) [5]

Liquid chromatography is categorized by its liquid mobile phase. It is the only type of chromatography where the stationary phase can be packed into a column, or held by a planar surface.

Elution

Each fat-soluble vitamin has a molecular weight of less than 5000 g/mol and is generally most soluble in methanol over hexane. Therefore, a reverse phase column is

used. This indicates that the stationary phase packed inside the column is a hydrophobic resin. The use of organic, non-polar solvents reduce the hydrophobic interactions and allow the hydrophobic molecules to elute off the column based on relative hydrophobicity. The vitamins that are more structurally similar to the relatively hydrophilic solvent methanol, such as retinol, will elute off the column first, followed by the vitamins that are more structurally similar to the hydrophobic stationary phase.

Migration rates

Initially, mixtures of the vitamins in a sample are introduced, at time 0, to the top of the column. The pump then continuously adds fresh mobile phase to the column. At this point, based on hydrophobic interactions, the solutes distribute themselves between the mobile and stationary phase. Partitioning occurs with time. Movement of the solutes can only occur as a result of continuous transfer between the stationary phase to the mobile phase. Therefore, the average rate that a solute migrates through the column depends on the fraction of time it spends in the mobile phase. For solutes that move quickly as a result of having a higher affinity towards methanol, there is a high fraction. For solutes that move slowly, with a higher affinity towards the stationary phase, the fraction is smaller. The result of the differing rates is a separation of the solutes in the column into bands. These bands pass through and are eluted off the column then travel to the detector. The detector then responds to the solute concentration and is plotted as a function of time on a chromatogram.

Band broadening

The theory of why band broadening as a measure of decreasing column efficiency begins in the theoretical study by Martin and Synge, in which they described a column being made up of separate but adjacent layers called theoretical plates [34]. At each plate, an assumed equilibrium of solute between mobile and stationary phases is achieved, so migration down the column is theorized as a step-by-step transfer of equilibrated mobile phase from each plate to the next. The plate theory accounts for the ideal Gaussian shape shown in a chromatogram, but is limited as the state of equilibrium is a myth. In actuality, no equilibrium state can be reached when the mobile phase is in constant motion. This theory, while still used as a manufacturing method to describe column efficiency, has been abandoned and replaced with rate theory.

Rate theory is a description of the shapes and breadths of elution bands based on the mechanism that the solutes migrate through the column, and is used to help explain band broadening as well as peak irregularities such as fronting and tailing. This can be explained by considering the thousands of transfers between stationary and mobile phases a single molecule undergoes while migrating through a column. The transfer between the mobile and stationary phases requires energy that the molecule acquires from its surroundings, making the molecules residency in each phase irregular, with some transfers taking place faster than others. Since the molecule can only move while in the mobile phase, some molecules transfer faster than others as a result of accidental and random inclusion into the mobile phase, while others lag as a result of incorporation in

the stationary phase. Ideally, this is a symmetric spread that results in a mean peak value that can be used to describe the behavior of the average solute molecule. Band broadening occurs as a result of the increased amount of spreading allowed with increased time in the column and decreases optimal separation.

Additional causes of band broadening include dilution caused by the continual addition of mobile phase and a decrease in solute concentration at a specific time point. Therefore, the differing rates of solute movement cause separation between solutes, but also band broadening. This lowers the efficiency of separation of the column and is a reason for the high sensitivity seen in HPLC detectors. Other factors that can cause irregularities in peaks include too large a sample injection volume being introduced onto the column, which can cause band broadening with the initial introduction into the column, or fronting. Ideally, conditions can be found where band broadening is caused more slowly than band separation.

Optimization of performance

The efficiency of chromatographic columns has been approximated and can be explained by the factors found in the van Deemter equation [5]:

$$H = A + \frac{B}{\mu} + (C_s + C_m) \times \mu$$

where *H* is the plate height in centimeters, *A* is a coefficient that represents eddy diffusion, or multiple path effects, *u* is the linear velocity of the mobile phase in

centimeters per second, *B* is the longitudinal diffusion coefficient, and C_s and C_m are mass-transfer coefficients for the stationary and mobile phases respectively.

Eddy diffusion, or the multipath effect, *A*, refers to the many pathways a molecule can migrate through the packing, or stationary phase, of the column. The length of the pathways can differ due to the individual interactions between each molecule of the solute and the stationary phase. This is impacted by solvent velocity due to ordinary diffusion, or the dispersion of molecules following multiple pathways. With slow solvent flow rate, the molecule transfer occurs rapidly, spending brief periods in each path minimizing band broadening because the molecules approach the average rate. With increased solvent flow rate, the amount of transfers of molecules following different pathways does not occur and band broadening will be seen as a result. Thus, with sufficiently high velocities, eddy diffusion becomes independent of flow rate. This process is further complicated when a porous stationary phase is used, which can cause static pools of mobile phase. In this case, solutes have to diffuse across the pools of motionless mobile phase to transfer into the moving mobile phase which slows the transfer process. This occurs in direct proportion to the plate height and mobile phase velocity, and inversely proportional to the diffusion coefficient for the solute in the mobile phase.

The longitudinal diffusion $\left(\frac{B}{u}\right)$ is a constant that accounts for the concentration differences between the more and less dilute regions, as well as the back and forth

mobility of a solute in a given medium. This bears little significance in LC because diffusion rates are small, but a greater effect will be noticed with a large amount of eddy diffusion.

The stationary-phase mass-transfer term and the mobile-phase mass transfer term are both constants specific to the solute attraction to the column and the solute solubility in the solvent.

Optimization of Column Performance can be achieved by reducing band broadening and adjusting the migration rates of the solutes to optimize column resolution, or clean separation in a minimum amount of time. This can be accomplished through management of plate number, column height, type and particle size of the stationary phase, mobile phase composition, and mobile phase flow rate.

Detectors

Ideal detector

Ideal detectors for LC should have the following characteristics identified by Skoog et al. [5]:

1. Adequate sensitivity
2. Stability and reproducibility
3. Linear response to solutes extending over several orders of magnitude
4. Short response time independent of flow rate
5. High reliability and user friendly
6. Similarity in response towards similar solutes

7. Nondestructive
8. Compatible with liquid flow
9. Minimal internal volume to reduce band broadening

These characteristics allow for correct identification and quantification of solutes.

Types of detectors

There are two basic types of detectors available that achieve the characteristics of an ideal detector. Bulk-property detectors, which respond to a mobile phase bulk property such as refractive index or density modulated by the presence of analytes, or solute-property detectors, which respond to properties of the solute, such as UV absorbance or fluorescence. The most used detectors for HPLC is the solute-property detectors measuring UV or Visible absorbance.

Photodiode array detection

The biggest advantage of the PDA detector is that it can scan several wavelengths at once, making it useful for identifying mixtures of compounds by taking advantage of the differences of the UV spectra of each compound. While UV detection has been critiqued due to its lack of specificity, the ability to scan several wavelengths allows for a large storage of chromatographic data allowing wavelength differences to contribute in identification thereby diminishing this limitation. Improvements in data bunching, smoothing, and noise reduction software allow for recent generations of PDA detectors to perform well at high sensitivity.

Quantitative analysis

Quantitative analysis of the chromatogram is based on either on peak height/area of the analyte peak in comparison to a standard peak height/area. The measurement of peak height can be made with high precision, but is limited as peak height will only be accurate if the peak width of the analyte and the standard are not altered. Since peak area is independent of band broadening, it is preferred over peak height.

Quantification of the analyte must be achieved through either external- or internal-standards. The most commonly used is an external-standard, where a series of external-standard solutions in varying concentrations are used to plot a calibration curve where peak height/area is plotted as a function of concentration. Limitations of an external standard in liquid chromatography include the uncertainty in injection volume, often alleviated by use of an auto sampler, the inability to account for evaporation of the solvent used to reconstitute the sample, as well as the inability to account for sample recovery in extraction. The use of an internal-standard can control for these limitations. This is achieved by the addition of a carefully measured quantity of standard to the sample before extraction. The ratio of the peak areas is then used as the analytical variable for quantification. Success depends on complete separation of the standard peak from the sample peaks. An ideal internal standard should be structurally similar to the analytes, have comparable polarity, extractability, detectability, be absent in the sample, and be stable during sample preparation [35]. Prostaglandin B_1 (PGB1) serves as an ideal internal standard in fat-soluble vitamin analysis based on these characteristics.

Inclusion of PGB_1 as an internal standard

Prostaglandins are hormone-like lipids derived from arachidonic acid that serve as chemical messengers, most notably in inflammatory pathways. PGB1 is a synthetic prostaglandin used as an internal standard to improve accuracy in quantification and can account for variations within each run, primarily caused by fluctuations in temperature.

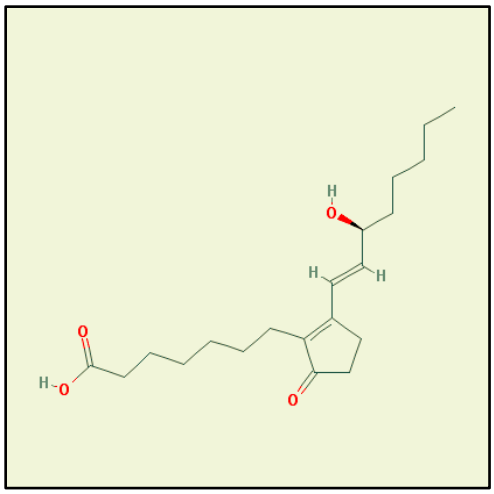

Fig. 8. Prostaglandin B_1 Structure [36]

PGB_1 has a molecular weight of 336.5 g/mol, is fat soluble, and can therefore be extracted with the other fat-soluble vitamins [36]. 0.04 µg of PGB1 will be added to each 100 µL sample before extraction. This amount has been calculated based on the approximate expected amount and corresponding peak height in a chromatogram for the fat-soluble vitamins found in plasma, and the amount of PGB1 necessary to obtain a comparable peak height. The peak height of the PGB1 needs to be a comparable to the

vitamin peak heights for ease of identification, but should not mask the vitamin peaks or skew the results.

Molar extinction coefficients

When each sample is run, the area under the curve (AUC) of the PGB1 allows for the calculation of vitamin amounts using the following example calculation:

$$[Vitamin\ in\ sample] = \left(\frac{AUC\ of\ Vitamin}{AUC\ of\ PGB_1}\right)([\mu g\ of\ PGB_1])\left(\frac{\varepsilon\ PGB_1}{\varepsilon\ Vitamin}\right)$$

In this calculation, epsilon refers to the molar extinction coefficients of the respective compounds. If there were a dilution or unit change, additional modification would be required in this equation.

The molar extinction coefficient is a measure of how strongly a chemical attenuates light at a given wavelength. As the vitamins and PGB_1 do not have the same weights or absorb light at the same wavelength, using the ratio of AUC and the molar extinction coefficients allows for vitamin quantification based on area when adjusted for by standardizing for light absorption capabilities.

Extraction

Types of extraction

Extraction is necessary for the isolation of fat-soluble compounds from aqueous blood biological samples. Homogenous samples, including plasma and serum, do not require any preparation prior to extraction. Solid samples, such as liver, require homogenization in an aqueous medium or directly in a organic extracting solvent. The

types of extraction include single-phase, double-phase, saponification, solid-phase extraction. Each are discussed below.

Single-phase extraction

In single-phase extraction, a sample is dissolved or diluted using a miscible solvent, such as hexane. In aqueous samples, such as plasma, proteins are separated through centrifugation. An aliquot of the clarified organic phase is then injected.

Double-phase extraction

Double-phase extraction includes the separation of the analyte between an aqueous or semiaqueous phase and a water-immiscible organic solvent. Protein precipitation using a water-miscible organic solvent, such as an alcohol, usually accompanies this technique. Treatment with SDS can sometimes precede the addition of the alcohol for cleaner extracts. The organic layer is isolated and evaporated to dryness under a vacuum or a stream of nitrogen. The solute is reconstituted into a compatible solvent and analyzed.

The most classical method for fat-soluble compound extraction is the Bligh and Dyer method, a type of double-phase extraction [37]. In this method, 200μL Plasma was combined with 3x volume 2:1 MeOH:$CHCl_3$. Samples are then vortexed regularly for 30 min. 200 μL saline and $CHCl_3$ is added, and the mixture is blended intermittently for another 30 min. The bottom $CHCl_3$ phase is isolated. The aqueous phase is re-extracted with an additional 200μL $CHCl_3$ and the process is repeated. The $CHCL_3$ phases are combined and evaporated to dryness under a nitrogen stream. This method is often

adapted for extractions of a specific compound or group of compounds. It is not atypical to repeat the $CHCl_3$ extraction phase and pool the extracts before evaporation.

Saponification

Saponification involves the heating of a sample with alcoholic potassium hydroxide, usually in the presence of an antioxidant, followed by a double-phase extraction. This process hydrolyzes triglycerides, phospholipids, and esters. The liberated potassium salt of the fatty acids and glycerol remain in the aqueous phase, decreasing the organic load in the extract and improving selectivity. This can only be performed if the desired extract is heat stable.

Solid-phase extraction

In solid-phase extraction, the analytes are collected from the sample on a disposable cartridge packed with microparticulate adsorbent or bonded phases. The cartridge is washed with a solvent to remove matrix components, then the analyte is displaced using a strong solvent.

Methodology Overview

The method used for sample analysis utilized a flow rate of 0.5 ml min^{-1} and a mobile phase of 87:33 ACN:MeOH. A diethyl ether method of extraction was used.

CHAPTER III

METHODOLOGY

Materials

Standards and solvents

Standards Retinol, Retinal, Retinoic Acid, cholecalciferol , 25-OH-D_3, α-Tocopherol, γ-Tocopherol, and Phylloquinone were purchase from Milipore-Sigma (St. Louis, MO USA). Internal Standard Prostaglandin B_1 was purchased from Cayman Chemical (Ann Arbor MI, USA). Solvents Acetonitrile, Methanol, Diethyl Ether, and Chloroform were HPLC grade (Fisher Scientific, Waltham MA, USA).

Instrumentation

HPLC-PDA was carried out on a Shimadzu SIL-20AD HPLC system (Kyoto, Japan) with a binary pump coupled to a SPD-M30A PDA detector. Separation was performed by reversed phase high performance liquid chromatography (RP-HPLC) using a Supelco Kromasil Eternity C18, 4.6 x 150 mm column, (Millipore Sigma St. Louis, MO USA) maintained at room temperature.

Methods

Plasma sample collection

This study was approved by the Texas Woman's University Institutional Review Board Human Subjects Review Committee. (Appendix A). All participants signed the

Informed Consent (Appendix B) prior to participation. Method of recruitment included a flier (Appendix C) presented in TWU anatomy and physiology courses. An instructor provided participants extra credit in their anatomy and physiology lab course for their participation in this study. A total of two-hundred and fifty-two interested participants responded by email or phone to the initial flyer. Fifty-two participants were randomly accepted, screened for eligibility, and completed the study. Fifty-one participants were able to donate useable fasting blood samples. Data were collected in November 2016 between 7:00 and 10:00 A.M.

Inclusion criteria included persons over the age of 18 willing to give consent. Exclusion criteria included persons under the age of 18. A questionnaire (Appendix D) assessed preexisting health conditions, medications or supplement use, and abnormal dietary fat-soluble vitamin intake. Each 13 ml blood sample was centrifuged at 3,000 rpm for 15 minutes to isolate plasma. Plasma was aliquoted and stored at -80°C for subsequent analysis.

Preparation of standards

Standards were diluted according to the final concentrations used in Table 1.

Table 1
Standard dilutions and solvents

Standard	**Final Concentration**	**Storage Reconstitution Solvent**
Retinol	[1 mg/ml]	$CHCl_3$
Cholecalciferol	[1 mg/ml]	Hexane
25-OH-D_3	[1 mg/ml]	Hexane
α-Tocopherol	[1 mg/ml]	Hexane
γ-Tocopherol	[1 mg/ml]	Hexane
Phylloquinone	[1 mg/ml]	Hexane
Prostaglandin B_1	[1 mg/ml]	Hexane

Concentrations were established following evaporation under a steady stream of nitrogen and reconstitution to the appropriate volume in the reconstitution solvent in amber vials before analysis. All standards were manipulated under yellow light to reduce light-induced isomeration and photo degradation. Molar Extinction Coefficients shown in Table 2 were obtained from De, Leenheer et al., 2000 [35], Suttie, 2009 [38], and Lianos, 1999 [39].

Table 2
Molar Extinction Coefficients.

Vitamin	**ε / L mol^{-1} cm^{-1}**	**Source**
All trans Retinol	52700	[35]
Cholecalciferol	18300	[35]
Calcifidiol	18300	[35]
α-tocopherol	3015	[35]
γ-tocopherol	3672	[35]
Phylloquinone	19,000	[38]
Prostaglandin B1	30,000	[39]

Re-suspension solvent

Standards were stored suspended in their re-suspension solvent for HPLC, standards were evaporated to dryness and assessed using Hexane as the re-suspension agent. The following solvents or mix of solvents were used to test efficacy of vitamins transfer to the column: MeOH:ACN (13:87), $CHCl_3$, Hexane, ACN, Diethyl Ether, $CHCl_3$:ACN:MeOH (1:1:1) and $CHCl_3$:Hexane (1:1, and 1:2)

Isocratic mobile phase

Isocratic methods using MeOH:ACN in the following ratios were observed: [0:100], [9:91], [13:87], [15:85], [88:12], [100:0].

Gradient mobile phase

The following gradient methods were evaluated:

- 100% MeOH and 0% ACN was held for a period of time, followed by a timed ramp to 10% MeOH and 90% ACN, then held for a period of time. Solutions were ramped back to 100% MeOH and 0% ACN for baseline stabilization before the next run.
- 100% MeOH and 0% ACN was held for a period of time, followed by a timed ramp to 13% MeOH and 87% ACN, then held for a period of time. Solutions were ramped back to 100% MeOH and 0% ACN for baseline stabilization before the next run.
- 20% MeOH and 80% ACN was held for a period of time, followed by a timed ramp to 10% MeOH and 90% ACN, then held for a period of time. Solutions were ramped back to 20% MeOH and 80% ACN for baseline stabilization before the next run.

Mobile phase

A final mobile phase of 13% MeOH and 87% ACN was selected. Using the binary system the HPLC used one line for MeOH and one for ACN, and the solvents were mixed at 13:87 MeOH:ACN and degassed before introduction to the column.

Flow rate

Flow rates of 0.100 mL/min, 0.200 mL/min, 0.500 mL/min, 1.000 mL/min, and 2.000 mL/min were examined.

Sample preparation

Two methods of extraction were evaluated.

1. Diethyl Ether Method: The following method was used on plasma samples. Maintaining a 3:1 ratio of diethyl ether: sample for each extraction. 600 μL diethyl ether was added to 200 μL of plasma and vortexed for 1 min. Samples were held at room temperature for 30 minutes to allow phase separation, and the upper diethyl ether fraction containing lipid soluble components is isolated with the aqueous phase remaining. The diethyl ether phase containing extractants is transferred into a second test tube. An additional 600 μL diethyl ether is added to the aqueous layer, vortexed for 1 minute, then allowed to separate at room temperature for 30 minutes. The diethyl ether phase is combined with the first extractant. The aqueous phase is then disposed of, and the extract is stored in the diethyl ether at -20°C.
2. Saponification + Diethyl Ether Method: Plasma vitamins were recovered through the diethyl ether method above and were saponified in toluene and -.5 mol KOH l^{-1} in methanol for 8 min at 86°C.

Final methodology

The method used for sample analysis utilized a flow rate of 0.5 ml min^{-1} and a mobile phase of 87:33 ACN:MeOH. The diethyl ether method of extraction was used.

Validation

Reproducibility

Reproducibility was assessed through a repetition of 10 runs of a 1mg/mL standard solution for retinol and PGB_1 and cholecalciferol. Concentration in micrograms of each vitamin was calculated using the following equation:

$$[Vitamin\ in\ sample] = \left(\frac{AUC\ of\ Vitamin}{AUC\ of\ PGB_1}\right)([\mu g\ of\ PGB_1])\left(\frac{\varepsilon\ PGB_1}{\varepsilon\ Vitamin}\right)$$

The coefficient of variation of micrograms of vitamin was used to test reproducibility.

CHAPTER IV

PRESENTATION OF FINDINGS

Resuspension solvent

MeOH:ACN (13:87), $CHCl_3$, Hexane, ACN, Diethyl Ether, $CHCl_3$:ACN:MeOH (1:1:1) and $CHCl_3$:Hexane (1:1, and 1:2) were each evaluated for reconstitution of the standards prior to analysis. If one vitamin was less or not identifiable than 13:87, the remaining vitamins were not tested.

Mobile phase

The isocratic methods [13:87] was the selected method based on best average percent of vitamin identification and recovery. Data not shown.

A gradient method was not selected due to significant baseline drift that complicated integration of low vitamin quantity. A representative chromatogram and 3D graph is below with the baseline shown in Figure 9 and Figure 10 respectively.

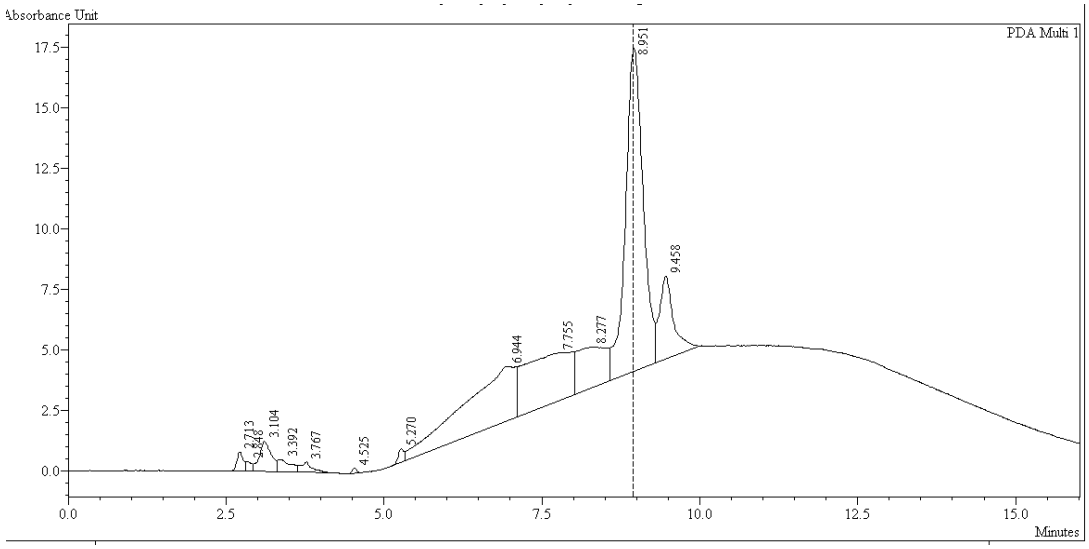

Fig. 9. Vitamin D_3 Chromatogram at 264nm of a [100:0] transition to [13:87] [MeOH:ACN] ramp.

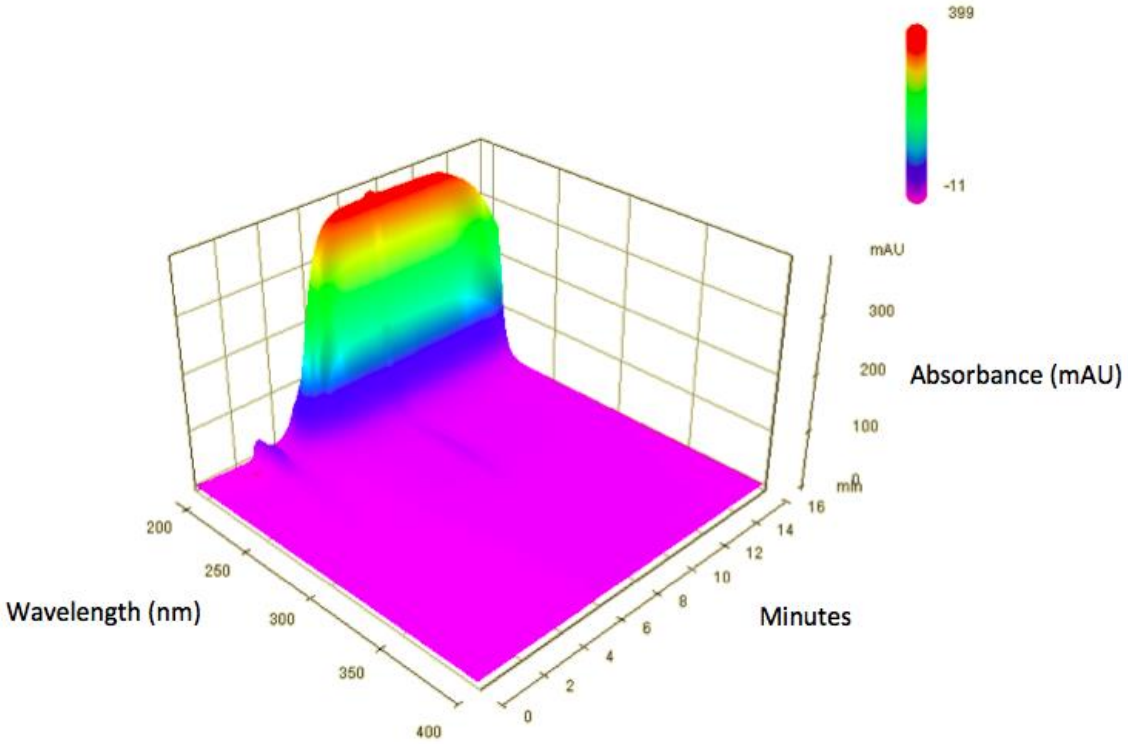

Fig. 10. Graph of vitamin D_3 [100:0] transition to [13:87] [MeOH:ACN] ramp at 264 nm.

Flow rate

Flow rates of 0.100 mL/min, 0.200 mL/min, 0.500 mL/min, 1.000 mL/min, and 2.000 mL/min were examined.

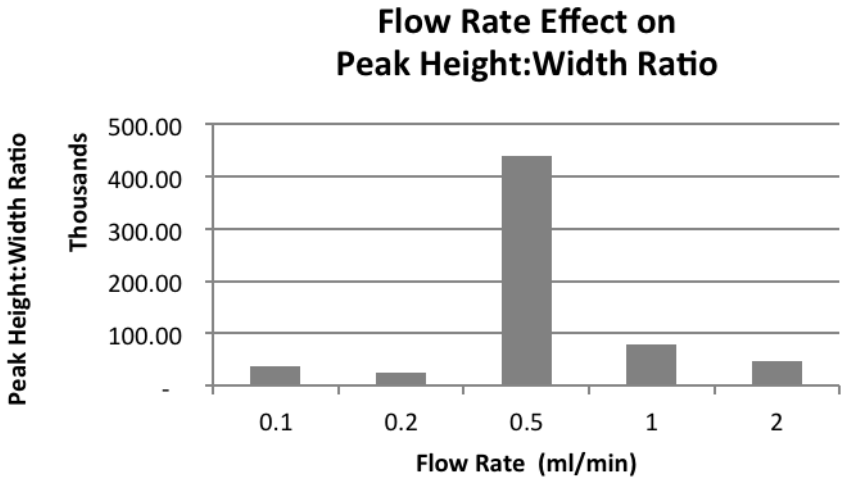

Fig. 11. Flow Rate Effect on Peak Height: Width Ratio

Extraction:

Saponification

Results of Saponification are found in Table 3

Table 3:
Results of Saponification Shown as AUC Maximum Percent. NT indicates Not Tested

Solute	Saponified	Non Saponified
Retinol	*NT*	*NT*
D3	*NT*	*NT*
25-OH-D3	12.294%	100%
Alpha Tocopherol	*NT*	*NT*
Gamma Tocopherol	*NT*	*NT*
Phylloquinone	0.509%	100%
PGB1	*NT*	*NT*

Validation

Reproducibility

10 repetitions of 1 mg mL^{-1} known standard of retinol and PGB_1 showed a calculated average of 1,628.0 ± 27.4 mg mL^{-1}. Based on these data, the coefficient of variation was 1.7%, demonstrating significant reproducibility.

10 repetitions of 1 mg mL^{-1} known standard of D_3 showed a AUC average of 551334.65 ±13726.07. Based on this data, the coefficient of variation was 2.5%, demonstrating significant reproducibility.

CHAPTER V

DISCUSSION AND SUMMARY

While there have been many methods for HPLC detection of fat-soluble vitamins in the literature [40–49], there is an absence of a method for simultaneous detection of retinol, cholecalciferol, calcidiol, α-tocopherol, γ-tocopherol, and phylloquinone in human plasma. The purpose of this study was to establish a method for simultaneous evaluation and quantification of fat-soluble vitamins, including retinol, cholecalciferol, calcidiol, α-tocopherol, γ-tocopherol, and phylloquinone in human blood samples by HPLC coupled PDA detection. Additionally, this method provides the novel use of prostaglandin B_1 as an internal standard for quantification and utilizes a simple method of extraction without derivitization or saponification.

Analytes

The vitamins chosen specifically for plasma isolation as they are the most abundant forms found in circulation. Ultimately, these isomers were chosen because they remain the most measured and therefore comparable in their respective literature.

PGB_1 as an internal standard

The use of PGB_1 as an internal standard reduces the workload and cost of analysis in the assessment of fat-soluble vitamins. A typical method of quantification is through the use of a calibration curve and a series of external standard dilutions to approximate

the composition of the sample. While still the most utilized method for fat-soluble vitamin analysis by HPLC, the use of this method is particularly labor intensive for retinol due to light-induced isomeration. The use of an external standard requires frequent standardization [5]. Because retinol is susceptible to isomeration and is labor intensive to maintain, the use of a calibration curve requires constant repurchasing of small quantities of standard. PGB_1 as an internal standard alleviates the requirement for constant repurchasing since it is more stable in light and doesn't degrade as quickly. Additionally, PGB_1 unlike retinol, does not require spectrophotometry to assess degradation prior to analysis, reducing sample preparation workload. Secondly, The use of PGB_1 alleviates the need for a calibration curve for each vitamin analyzed and provides the ability to assess multiple vitamins using a single standard. Finally, internal standards control for sample evaporation in the auto-sampler as well as day-to-day variations in slope, migration, and baseline movements due to temperature changes.

Recent methodologies using internal standards incorporate spiking the samples prior to extraction [40,42–44,50] while other still spike the samples after extraction [48] or are not reporting the timing of internal standard addition [45,47]. When samples are spiked prior to extraction it controls for extraction efficiency, neutralizing the considerable limitation of using external standardization or spiking the samples with internal standards after extraction.

Isocratic vs. gradient mobile phase

It was noticeable for vitamins D_3, 25-OH-D_3, alpha-tocopherol, and gamma tocopherol that a gradient mobile phase seemingly produced better peak identification and integration, particularly a gradient of [100:0] to [10:90] MeOH:ACN. The limitations of this method, however, outweighed the benefits. Beyond the visible limitation of its low detection of D_3, gradient methods also have significant baseline shift and band broadening. This most often requires manual integration for the calculation of AUC, and is therefore variable based on technician and its accuracy is limited. The [13:87] [MeOH:ACN] Isocratic method showed on average the most reproducible with accurate program integration.

Flow rate

If the flow rate was rapid, peak separation became an issue, whereas if the flow rate was too slow, run time and band broadening became the issue. The data shown in Figure 11 demonstrate the effectiveness of the 0.5ml min^{-1} flow rate in increasing peak height: width ratio and is therefore most effective prevention of band broadening observed

Saponification

The significant reduction of AUC in the saponification of 25OHD_3 and Phylloquinone shown in Table 3 demonstrates a degradation of these vitamins during saponification. This is likely due to the vitamin exposure to increased temperature.

Because such significant reductions were seen in both $25OHD_3$ and Phylloquinone, it was unnecessary to examine saponification in the remaining vitamins.

Reproducibility

In the standardized 13:87 MeOH:ACN method, the standard deviation of retinal was 1.7% of the mean, and the standard deviation of D_3 was 2.5% of the mean, demonstrating high reproducibility. It is important to note, however, that the wavelength of detection of vitamin D_3 increased from 266nm seen in earlier data to 298nm shown in later data used for the reproducibility calculations. It has been shown that degradation is negligible after storage in amber tubes at refrigerator temperatures after 1 year in a plasma matrix, however free D_3 can be oxidized and inactivated by moist air within a few days, and is susceptible to photo-degradation [12]. The photoisomerization of vitamin D into $Lumisterol_3$ and $Tachysterol_3$, shown in Figure 12, induces a shift wavelength absorbance maximum, supported by the vitamin D_3 spectra observed in Figure 13. The change observed in the spectrum from 266nm to 298nm highlights the importance of managing standard and sample preparation, collection, and storage. Care should be taken to store standards and samples in amber vials in the dark, and preparation should be done in a dim or yellow light setting.

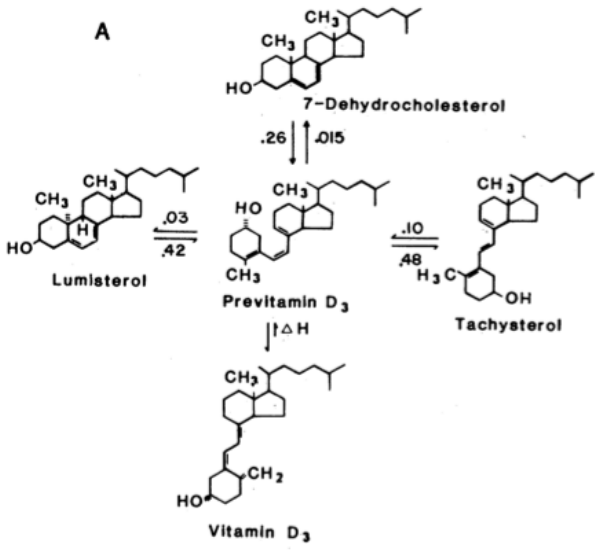

Fig. 12. The photochemical reaction in human epidermis of 7-DHC to $preD_3$. The $preD_3$ either thermally converts to vitamin D_3 or photoisomerizes to $lumisterol_3$ and $tachysterol_3$ [51].

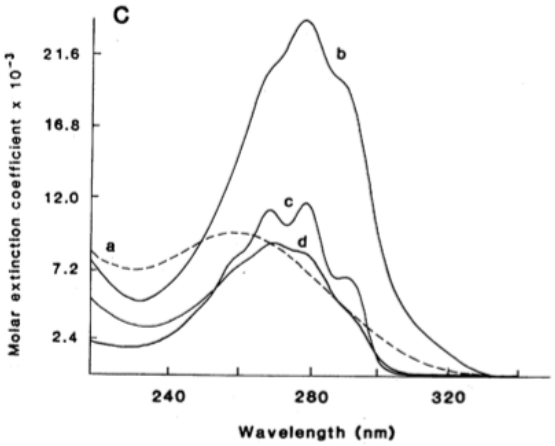

Fig. 13. Ultraviolet absorption spectrums of (a) $preD_3$, (b) $tachysterol_3$, (c) 7-DHC, and (d) $lumisterol_3$, isolated from human epidermis [51].

That degradation products can be used for evaluation with this method is based on the high reproducibility of D_3 standard injected, without migration of retention time.This

allows the program to identify at 298nm and quantitate the AUC without manual adjustment for degradation. While limited in the ability to detect for coelution, the ratio of photoisomers to cholecalciferol can thus be accounted for. The wavelength maximums used for identification must therefore be reported in the findings.

Use of photodiode array detection compared to mass spectroscopy

Kopec et al. (2013) published a comparison of HPLC methods using PDA and mass spectrometry (MS) for the quantification of carotenoids, retinyl esters, α-tocopherol, and phylloquinone in chylomicron-rich fractions of human plasma [46]. This is a worthwhile comparison as HPLC-MS is quickly becoming more available and seemingly advantageous in chromatographic analysis. They concluded that, particularly for α-tocopherol and some carotenoids, HPLC-PDA provided slightly higher sensitivity but noted that co-elution can be a problem with PDA detectors. This induced them to decide that while the absence of ion suppression/enhancement made HPLC-PDA a better choice for carotenoids and α–tocopherol, MS provides compound identification confirmation and should therefore be preferred for low-level detection. The confirmation of chemical identification is undoubtedly an advantage for MS, however the cost for an MS detector can be an unreasonable expense for many labs, especially with appropriate methodology utilizing the more commonly owned PDA detector. Furthermore, increased sensitivity demonstrated in PDA detectors allow for more accurate quantification in biologic samples, and the ability to scan different wavelengths while detecting small volumes of analytes minimizes any significant limitation of co-elution.

Limitations

Limitations in this study include the inability to identify cis-/trans-isomerization, therefore potentially causing peak isomer separation and reducing the overall AUC of the peak. This is particularly a concern for retinol. This was minimized by keeping the samples in the stabilizing blood matrix for as long as possible before extraction, and by monitoring vitamin standard degradation. Another limitation is this method is only designed to detect for free fat-soluble vitamins. The diethyl ether extraction method is efficient enough to isolate both free and esterified vitamins. It is unknown if esterified is being evaluated by this HPLC method. Saponification was tested in attempt to free retinol from RBP, and cholecalciferol and calcidiol from DBP. Vitamin D proved unstable in the increased temperature required to hydrolyze the esterification between retinol and its carrier protein. Therefore any vitamin still bound to its carrier protein was left in the protein layer in extraction, or was undetectable in the methodology. An additional limitation is the requirement for a skilled HPLC technician required for analysis and integration.

Future research

A common limitation in chromatography is the requirement for sample preparation and large space requirements for instrumentation, extending the time between data collection and analysis. This specifically limits research in many hard to reach populations. The desire for analysis at the location of sampling has driven improvements in portable liquid chromatography instrumentation [52], however portable PDA detectors do not yet exist.

Sharma et al. have identified advancements in UV-absorption detectors made, particularly in LED based detection systems ideal for portable LC units [53], however the technology is not yet able to transition into the field.

Summary

The purpose of this study was to establish a method for simultaneous evaluation and quantification of fat-soluble vitamins, including retinol, retinal, retinoic acid, cholecalciferol, calcidiol, α-tocopherol, γ-tocopherol, and phylloquinone, in human blood samples using RP-HPLC coupled with photodiode array detection. This method is novel in the use of prostaglandin B_1 as an internal standard for quantification and utilizes a simple method of extraction without derivatization or saponification. The method had a high degree of reproducibility as evidenced by standard deviations 1.7% and 2.5% of the mean for retinal and D_3 respectively.

REFERENCES

[1] IOM. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. National Academies Press; 2001. doi:10.17226/10026.

[2] IOM. Dietary Reference Intakes for Calcium and Vitamin D. vol. 32. Washington DC: National Academies Press; 2011. doi:10.1542/peds.2012-2590.

[3] Ross AC, Caballero B, Cousins RJ, Tucker KL, Ziegler TR, editors. Modern Nutrition in Health and Disease. 11th ed. Baltimore: Lippincott Williams & Wilkins; 2014.

[4] Antioxidants D, Compounds R, Reference U, Dris U, Committee S, Evaluation S, et al. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. National Academies Press; 2000. doi:10.17226/9810.

[5] Skoog DA, Holler FJ, Crouch SR. Principles of Instrumental Analysis. 6th ed. Belmont: Thomson Brooks/Cole; 2007.

[6] Gropper SS, Smith JL. Advanced Nutrition and Human Metabolism. Sixth. Belmont: Wadsworth Cengage Learning; 2013.

[7] National Center for Biotechnology Information. SID=24899395. PubChem Subst Database n.d. https://pubchem.ncbi.nlm.nih.gov/substance/24899395 (accessed June 19, 2017).

[8] Cediel G, Olivares M, Brito A, Lopez de Romana D, Cori H, La Frano MR. Interpretation of Serum Retinol Data From Latin America and the Caribbean. Food Nutr Bull 2015;36:S98-108. doi:10.1177/0379572115585743.

[9] Pee S De, Dary O, de Pee S, Dary O. Biochemical Indicators of Vitamin A Deficiency: Serum Retinol and Serum Retinol Binding Protein. J Nutr 2002;132:2895S–2901.

[10] Borges MC, Martini LA, Rogero MM. Current perspectives on vitamin D, immune system, and chronic diseases. Nutrition 2011;27:399–404. doi:10.1016/j.nut.2010.07.022.

[11] Holick M. Vitamin D deficiency. N Engl J Med 2007;357:266–81. doi:10.1056/NEJMra070553.

[12] National Center for Biotechnology Information. SID=24893192. PubChem Subst Database n.d.

[13] National Center for Biotechnology. SID=57647690. PubChem Subst Database n.d.

[14] Herrmann M, Farrell C-JL, Pusceddu I, Fabregat-Cabello N, Cavalier E. Assessment of vitamin D status – a changing landscape. Clin Chem Lab Med 2017;55:3–26. doi:10.1515/cclm-2016-0264.

[15] Leung PS. The potential protective action of vitamin D in hepatic insulin resistance and pancreatic islet dysfunction in type 2 diabetes mellitus. Nutrients 2016;8. doi:10.3390/nu8030147.

[16] Hu S, Liu Q, Huang X, Tan H. Serum level and polymorphisms of retinol-binding protein-4 and risk for gestational diabetes mellitus: a meta-analysis. BMC Pregnancy Childbirth 2016;16:52. doi:10.1186/s12884-016-0838-7.

[17] Skowrońska P, Pastuszek E, Kuczyński W, Jaszczoł M, Kuć P, Jakiel G, et al. The role of vitamin D in reproductive dysfunction in women – a systematic review. Ann Agric Environ Med 2016;23:671–6. doi:10.5604/12321966.1226865.

[18] Bischoff-Ferrari HA, Dawson-Hughes B, Orav EJ, Staehelin HB, Meyer OW, Theiler R, et al. Monthly High-Dose Vitamin D Treatment for the Prevention of Functional Decline. JAMA Intern Med 2016;176:175. doi:10.1001/jamainternmed.2015.7148.

[19] Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: An endocrine society clinical practice guideline. J Clin Endocrinol Metab 2011;96:1911–30. doi:10.1210/jc.2011-0385.

[20] Torquato P, Ripa O, Giusepponi D, Galarini R, Bartolini D, Wallert M, et al. Analytical strategies to assess the functional metabolome of vitamin E. J Pharm

Biomed Anal 2016;124:399–412. doi:10.1016/j.jpba.2016.01.056.

[21] National Center for Biotechnology Information. SID=24900118. PubChem Subst Database n.d. https://pubchem.ncbi.nlm.nih.gov/substance/24900118 (accessed June 19, 2017).

[22] National Center for Biotechnology Information. SID=24899992. PubChem Subst Database n.d. https://pubchem.ncbi.nlm.nih.gov/substance/24899992 (accessed June 19, 2017).

[23] Evans H, Bishop KS. On the Existence of a Hitherto Unrecognized Dietary Factor Essential for Reproduction: On the Existence of a Hitherto Unrecognized Dietary Factor Essential for Reproduction Author (s): Herbert M . Evans and K . Scott Bishop Published by : Science (80-) 1922;56:650–1.

[24] Farina N, Mgekn I, Ar C, Rusted J, Tabet N. Vitamin E for Alzheimer ' s dementia and mild cognitive impairment (Review). Cochrane Collab Cochrane Database Syst Rev 2012. doi:10.1002/14651858.CD009524.pub2.www.cochranelibrary.com.

[25] Grimm M, Mett J, Hartmann T. The Impact of Vitamin E and Other Fat-Soluble Vitamins on Alzheimer´s Disease. Int J Mol Sci 2016;17:1785. doi:10.3390/ijms17111785.

[26] Galli F, Azzi A, Birringer M, Cook-Mills JM, Eggersdorfer M, Frank J, et al. Vitamin E: Emerging aspects and new directions. Free Radic Biol Med 2017;102:16–36. doi:10.1016/j.freeradbiomed.2016.09.017.

[27] Kyla Shea M, Booth SL. Concepts and controversies in evaluating vitamin K status in population-based studies. Nutrients 2016;8:1–25. doi:10.3390/nu8010008.

[28] National Center for Biotechnology Information. SID=24871410. PubChem Subst Database n.d. https://pubchem.ncbi.nlm.nih.gov/substance/24871410 (accessed June 19, 2017).

[29] Hao G, Zhang B, Gu M, Chen C, Zhang Q, Zhang G, et al. Vitamin K intake and the risk of fractures 2017.

[30] Cheung C-L, Sahni S, Cheung BMY, Sing C-W, Wong ICK. Vitamin K intake and mortality in people with chronic kidney disease from NHANES III. Clin Nutr

2015;34:235–40. doi:10.1016/j.clnu.2014.03.011.

[31] Araujo MC, Yokoo EM, Pereira RA. Validation and Calibration of a Semiquantitative Food Frequency Questionnaire Designed for Adolescents. J Am Diet Assoc 2010;110:1170–7. doi:10.1016/j.jada.2010.05.008.

[32] Novotny J a, Kurilich AC, Britz SJ, Baer DJ, Clevidence B a. Vitamin K absorption and kinetics in human subjects after consumption of 13C-labelled phylloquinone from kale. Br J Nutr 2010;104:858–62. doi:10.1017/S0007114510001182.

[33] Jones KS, Bluck LJC, Wang LY, Stephen AM, Prynne CJ, Coward WA. The effect of different meals on the absorption of stable isotope-labelled phylloquinone. Br J Nutr 2009;102:1195–202. doi:10.1017/S0007114509371688.

[34] Martin a. JP, Synge RLM. A new form of chromatogram employing two liquid phases. Biochem J 1941;35:1358–68. doi:10.1042/bj0351358.

[35] De Leenheer AP, Lambert WE, Van Bocxlaer JF, editors. Modern Chromatographic Analysis of Vitamins. 3rd ed. New York: Marcel Dekker, Inc; 2000.

[36] National Center for Biotechnology Information. CID=5280388. PubChem Compd Database n.d. https://pubchem.ncbi.nlm.nih.gov/compound/5280388 (accessed June 19, 2017).

[37] Bligh EG, Dyer WJ. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION1. Can J Biochem Physiol 1959;37.

[38] Suttie JW. Vitamin K in Health and Disease. Boca Raton: CRC Press; 2009.

[39] Lianos EA, editor. Eicosanoid Protocols. Humana Press Inc.; 1999.

[40] Sau Man Po E, W. Ho J. J, Yi Gong B. Simultaneous chromatographic analysis of eight fat-soluble vitamins in plasma. J Biochem Biophys Methods 1997;34:99–106. doi:10.1016/S0165-022X(97)01210-4.

[41] Moreno P, Salvadó V. Determination of eight water- and fat-soluble vitamins in multi-vitamin pharmaceutical formulations by high-performance liquid chromatography. J Chromatogr A 2000;870:207–15. doi:10.1016/S0021-

9673(99)01021-3.

[42] Lee BL, New AL, Ong CN. Simultaneous Determination of Tocotrienols, Tocopherols, Retinol, and Major Carotenoids in Human Plasma. Clin Chem 2003;49:2056–66. doi:10.1373/clinchem.2003.022681.

[43] Quesada JM, Mata-Granados JM, Luque De Castro MD. Automated method for the determination of fat-soluble vitamins in serum. J Steroid Biochem Mol Biol 2004;89–90:473–7. doi:10.1016/j.jsbmb.2004.03.056.

[44] Chatzimichalakis PF, Samanidou VF, Papadoyannis IN. Development of a validated liquid chromatography method for the simultaneous determination of eight fat-soluble vitamins in biological fluids after solid-phase extraction. J Chromatogr B Anal Technol Biomed Life Sci 2004;805:289–96. doi:10.1016/j.jchromb.2004.03.009.

[45] Hermans N, Cos P, Berghe D Vanden, Vlietinck AJ, De Bruyne T. Method development and validation for monitoring in vivo oxidative stress: Evaluation of lipid peroxidation and fat-soluble vitamin status by HPLC in rat plasma. J Chromatogr B Anal Technol Biomed Life Sci 2005;822:33–9. doi:10.1016/j.jchromb.2005.05.040.

[46] Kopec RE, Schweiggert RM, Riedl KM, Carle R, Schwartz SJ. Comparison of high-perfomance liquid chromatography/tandem mass spectrometry and high-performance liquid chromatography/photo-diode array detection for the quntitation of carotenoids, retinyl esters, α-tocopherol and phylloquinone in chylomicron-rich frac. Rapid Commun Mass Spectrom 2013. doi:10.1002/rcm.6576.

[47] Gleize B, Steib M, André M, Reboul E. Simple and fast HPLC method for simultaneous determination of retinol, tocopherols, coenzyme Q10 and carotenoids in complex samples. Food Chem 2012;134:2560–4. doi:10.1016/j.foodchem.2012.04.043.

[48] Hrvolová B, Martínez-Huélamo M, Colmán-Martínez M, Hurtado-Barroso S, Lamuela-Raventós RM, Kalina J. Development of an advanced HPLC-MS/MS method for the determination of carotenoids and fat-soluble vitamins in human plasma. Int J Mol Sci 2016;17. doi:10.3390/ijms17101719.

[49] Colmán-Martínez M, Martínez-Huélamo M, Miralles E, Estruch R, Lamuela-

Raventós RM. A new method to simultaneously quantify the antioxidants: Carotenes, xanthophylls, and Vitamin A in human plasma. Oxid Med Cell Longev 2016;2016. doi:10.1155/2016/9268531.

[50] Billupsa J, Jonesa C, Jacksonb TL, Ablordeppeyc SY, Spencer SD. Simultaneous RP-HPLC-DAD quantification of bromocriptine, haloperidol and its diazepane structural analog in rat plasma with droperidol as internal standard for application to drug-interaction pharmacokinetics. Biomed Chromatogr 2010. doi:10.1146/annurev-cellbio-092910-154240.Sensory.

[51] MacLaughlin JA, Anderson R., Holick MF. Spectral Character of Sunlight Modulates Photosynthesis of Previtamin D3 adn its Photoisomers in Human Skin. Science (80-) 1982;216:1001–3.

[52] Sharma S, Tolley LT, Tolley HD, Plistil A, Stearns SD, Lee ML. Hand-portable liquid chromatographic instrumentation. J Chromatogr A 2015;1421:38–47. doi:10.1016/j.chroma.2015.07.119.

[53] Sharma S, Tolley HD, Farnsworth PB, Lee ML. LED-based UV absorption detector with low detection limits for capillary liquid chromatography. Anal Chem 2015;87:1381–6. doi:10.1021/ac504275m.

Appendix A

Texas Woman's University Institutional Review Board Human Subjects Review Committee Approval

Texas Woman's University Institutional Review Board

Application for Expedited and Full Review

For office use only:

Protocol #: _____

Name of Principal Investigator (PI): Sarah Tucker Phone: 970-691-3777

Status: ☐ faculty ☒ student ☐ staff ☐ other : _____ E-mail: stucker5@twu.edu

Department: Nutrition and Food Sciences

Colleague ID# (this is the 7-digit # on your ID): 1103854

Title of Study: HPLC-DA Method for the Determination of Fat-Soluble Vitamins.

If the PI is a student, provide the following information for the faculty advisor:

Name of advisor: K Shane Broughton, PhD kbroughton@twu.edu
Nancy Dimarco, PhD, RDN E-mail: ndimarco@twu.edu

TWU Department: Nutrition and Food Sciences

Estimated beginning date of study: 05/09/2016 Estimated duration of study 3 months

Campus (Denton, Dallas, or Houston) Denton Level of review: ☐ expedited ☒ full

Type of Project : (check all that apply)
☒ thesis ☐ professional paper ☐ dissertation ☐ class project
☐ faculty research ☐ pilot ☐ other ____

Has project has been submitted for funding (internal or external)? ☐ yes ☒ no
If yes, funding source:

Signatures:

Principal Investigator (PI): Signature certifies that the investigator has primary responsibility for all aspects of the research project.

Sarah Tucker
Principal Investigator

5/2/16
Date

Faculty Research Advisor (for student research only): Signature certifies that the faculty member has read, reviewed, and approved the content of the application and is responsible for the supervision of this research study.

Nancy M. DiMarco
Faculty Research Advisor

5/3/16
Date

Academic Administrator: Signature certifies that the administrator has read, reviewed, and approved the content of the application.

K Shane Broughton
Academic Administrator (Department Chair, Program Director, or Associate Dean)

5/2/16
Date

Appendix B

Consent Form

TEXAS WOMAN'S UNIVERSITY
CONSENT TO PARTICIPATE IN RESEARCH

Project Title:	HPLC-DA Method for the Determination of Fat-Soluble Vitamins
Principal Investigator:	Sarah Tucker, BS
Investigator's Email:	stucker5@twu.edu
Investigator's Phone:	[redacted]
Faculty Advisors:	K Shane Broughton, PhD Kbroughton@twu.edu [redacted] Nancy DiMarco, PhD, RDN, CSSD ndimarco@twu.edu [redacted]
Research Assistants:	Desiree Patterson Dpatterson1@twu.edu [redacted] Titus Sombuor, MS tsombuor@twu.edu [redacted]

Purpose of the Research Study:

The primary purpose of this study is to develop a time- and cost-efficient method to evaluate fat-soluble vitamin status in human blood. This data will be used to test reproducibility of the method.

You are being asked to participate in this study because you are an adult above the age of 18, willing to donate blood, and willing to give consent.

Procedures:

If you agree to participate in this investigation, you will be asked to provide a blood sample. A maximum of 25 mLs, or ~5 tsp of blood, will be collected for the measurement of vitamins A, D, E, and K. This will be drawn by research staff and will take a maximum of 30 minutes to complete.

After data collection, A TWU lab technician will analyze the blood sample. In the case of irregular lab findings, you will be advised to contact your physician for treatment. Adverse outcomes may be completely incidental to the study. If there is a problem, we ask that you let the study staff know at once and they will help you. However, TWU does not provide medical services or financial assistance for injuries that might happen because you are taking part in this research study.

Length of Participation:

Total participation time will last approximately 30 minutes. During this time you will be asked to provide a blood sample.

Participant Initials: _____

Potential Risks: There is a possibility of certain risk occurrence during data collection. They include: risk of coercion, loss of confidentiality, loss of anonymity, embarrassment, physical discomfort of blood draw, bruising at the site of blood draw, infection at the site of blood draw, fatigue, and latex allergy.

- *Coercion:* The possibility of coercion exists in research studies. To minimize this risk, the PI will explain why the study is to be done and give you the opportunity to ask questions. Every effort will be made to ensure proper treatment of participants by research staff. At any time, you may ask questions or withdraw from the study. You may take breaks at any time or withdraw from the study without question or penalty.
- *Loss of anonymity:* Identification will be protected as much as possible through personal identification numbers and privacy during data collection, but anonymity cannot be assured.
- *Loss of confidentiality:* Confidentiality will be kept to the extent that is allowed by law. All data will be coded and names of those in the study will not be used. The PI will keep hard copies of all data and personal information in a folder locked in a filing cabinet. Only the PI will have access to the data. Research personnel will only use the coded data. A master list with names related to codes will be kept in a separate locked filing cabinet until the study completion when it will be destroyed. After study completion, all private data will be shredded within 5 years. Results of the study may be in peer-reviewed journals; however private data will not be used. There is a potential loss of confidentiality during any and all email or Internet transactions
- *Physical discomfort of blood draw:* You may experience physical discomfort or pain during the blood draw. Every effort will be made to take blood samples quickly by using trained phlebotomists. In addition, research staff will ensure you are well hydrated before the blood draw by having you drink at least 8 oz. of fluid prior to blood draw. If you express a desire to stop at any time, you will be allowed to do so without any penalty.
- *Bruising at site of blood draw:* The risk of bruising resulting from drawing blood is minimalized due to procedures being performed by trained personnel. Universal precautions and techniques will be used during blood draw procedures. To minimize bruising, pressure will be applied for approximately five minutes after the blood draw.
- *Infection at site of blood draw:* The risk of infection resulting from drawing blood is small due to procedures being performed by trained personnel. Universal precautions and techniques will be used during blood draw procedures. Sites for blood draws will be cleaned with alcohol immediately before each venipuncture. New needles will be used for each blood draw and placed in biohazard box immediately after use.
- *Fatigue:* You may experience fatigue during the blood draw. Every effort will be made to take blood samples quickly by using trained research staff. If you express a desire to stop at any time, you will be allowed to do so without any penalty.
- *Latex allergy:* The phlebotomist will wear gloves during the blood draw. Before the blood draw, you will be asked if you are allergic to latex. If you are allergic, non-latex gloves and tourniquet will be used.

Voluntary Nature of the Study:

Your participation in the study is voluntary and can be withdrawn at any point without penalty. Results of this study may be obtained by request.

Participant Initials: _____

Disclaimer: The researchers will try to prevent any problem that could happen because of this study. You should let the researchers know at once if there is a problem and we will help you to the best of our abilities. TWU does not provide medical services or financial assistance for injuries that might happen because you are taking part in this research.

Contacts and Questions:

If you have any questions, concerns, or complaints about the procedures, the researchers conducting this study can be contacted at:

Sarah Tucker
[redacted]
stucker5@twu.edu

Authorization:

You will be given a copy of this signed and dated consent form to keep. If you are not given a hard copy of this consent form or lose it and would like a replacement, please request one. If you have any questions about the research study please contact the primary investigators, listed at the top of this form. If you have questions about your rights as a participant in this research or the way this study has been conducted, you may contact the Texas Woman's University Office of Research and Sponsored Programs at 940-898-3378 or via email at IRB@twu.edu.

Signature

Printed Name Date

If you would like to receive a summary of the results of this investigation, please provide an address or email address to which this summary should be sent:

Appendix C

Recruitment Flier

TWU Research Opportunity

Volunteers needed for Fat Soluble Vitamin Research

The goal of this research is to develop a method for identification of fat-soluble vitamins (A, D, E, and K) in blood to determine subclinical and clinical deficiencies and potential opportunities for future research.

[You may be able to participate if you are:]

- **Female**
- **Between the ages of 18 to 45**

[For more information please contact:]

Sarah Tucker
stucker5@twu.edu

Confidentiality Statement: There is a potential risk of loss of confidentiality in all email, downloading, and Internet transactions. This study is voluntary and you may discontinue at any time.

Appendix D

Questionnaire

Fat Soluble Vitamin Questionnaire.

Contains:

Health and Demographic Questionnaire
Modified NHANES Food Frequency Questionnaire
TWU Sunlight Questionnaire

These questionnaires are designed to assess your health, the sources of fat soluble vitamins A, D, E, and K in your diet, and your general knowledge of vitamin D.

Questions vary from how frequently you consume specific foods to your weekly habits. The questions are asked in different answer formats. Some of them may ask your frequency of an activity and others may ask you to agree or disagree on a scale with the statement presented. In all cases answer the question by checking the corresponding answer.

Your honesty with the questions will help to validate research methods and support values in your provided blood samples. This is not a diagnostic tool. Please choose the closest match, and only choose one answer unless stated otherwise.

GENERAL INSTRUCTIONS

- Answer each question as best you can. Estimate if you are not sure. A guess is better than leaving a blank.
- If you answer NO for a question, please follow any instructions that direct you to the next question.

Health and Demographic Questionnaire

1. What is your Date of Birth: _____/____
   month/year
2. Ethnicity: (Check all that apply)
   - African American
   - American Indian
   - Asian/Pacific Islander
   - Caucasian (non-Hispanic)
   - Hispanic
   - Scandinavian
   - Other: _____

3. Have you ever been, or are you currently a smoker?
   - Yes
   - No
4. Are you currently taking androgen suppressants or non-steroidal anti-inflammatory drugs (ibuprofen, acetaminophen, aspirin, etc.)?
   - Yes
   - No
5. Are you taking any birth control medication?
   - Yes
   - No
6. Are you currently taking any kind of hormonal therapy (excluding oral contraceptives)
   - Yes
   - No
7. Is there a chance you might be pregnant?
   - Yes
   - No
8. Are you currently breastfeeding?
   - Yes
   - No
9. Have you been diagnosed with Polycystic Ovarian Syndrome (PCOS)?
   - Yes
   - No
   - Unknown
10. Have you been diagnosed with infertility due to endometriosis or some other condition (excluding polycystic ovarian syndrome)?
    - Yes
    - No
11. Have you been diagnosed with cardiovascular disease
    - Yes
    - No
12. Have you been diagnosed with metabolic syndrome?
    - Yes
    - No
13. Have you been diagnosed with Type 1 Diabetes Mellitus?
    - Yes
    - No
    - Unknown
14. Have you been diagnosed with Type 2 Diabetes Mellitus?
    - Yes
    - No
    - Unknown
15. Have you been diagnosed with a clotting/bleeding disorder (i.e. hemophilia, sickle cell anemia, etc.)?
    - Yes
    - No

    15.1. Are you taking any drugs to control blood clotting?
    - Yes
    - No

Please list all prescription medications and/or dietary supplements that you are currently taking:

Medication/Dosage/Date Started/Reason _____
_____
Medication/Dosage/Date Started/Reason _____
_____
Medication/Dosage/Date Started/Reason _____
_____
Medication/Dosage/Date Started/Reason _____
_____
Medication/Dosage/Date Started/Reason _____
_____
Medication/Dosage/Date Started/Reason _____
_____
Medication/Dosage/Date Started/Reason _____
_____
Medication/Dosage/Date Started/Reason _____
_____
Medication/Dosage/Date Started/Reason _____
_____
Medication/Dosage/Date Started/Reason _____
_____

Modified NHANES Food Frequency Questionnaire

Over the <u>past 12 months</u>:

1. How often did you drink **tomato juice** or **vegetable juice**?
   - NEVER
   - 1 time per month or less
   - 2-3 times per month
   - 1-2 times per week
   - 3-4 times per week
   - 5-6 times per week
   - 1 time per day
   - 2-3 times per day
   - 4-5 times per day
   - 6 or more times per day
2. How often did you drink **orange juice** or **grapefruit juice**?
   - NEVER
   - 1 time per month or less
   - 2-3 times per month
   - 1-2 times per week
   - 3-4 times per week
   - 5-6 times per week
   - 1 time per day
   - 2-3 times per day
   - 4-5 times per day
   - 6 or more times per day
3. How often did you drink **apple juice**?
   - NEVER
   - 1 time per month or less
   - 2-3 times per month
   - 1-2 times per week
   - 3-4 times per week
   - 5-6 times per week
   - 1 time per day
   - 2-3 times per day
   - 4-5 times per day
   - 6 or more times per day
4. How often did you drink **grape juice**?
   - NEVER
   - 1 time per month or less
   - 2-3 times per month
   - 1-2 times per week
   - 3-4 times per week
   - 5-6 times per week
   - 1 time per day
   - 2-3 times per day
   - 4-5 times per day
   - 6 or more times per day
5. How often did you drink other **100% fruit juices** or **100% fruit juice mixtures** (such as pineapple, prune, or others)?
   - NEVER
   - 1 time per month or less
   - 2-3 times per month
   - 1-2 times per week
   - 3-4 times per week
   - 5-6 times per week
   - 1 time per day
   - 2-3 times per day
   - 4-5 times per day
   - 6 or more times per day
6. How often did you drink other **fruit drinks** (such as cranberry cocktail, Hi-C, lemonade, or Kool-Aide, diet or regular)?
   - NEVER (GO TO QUESTION 7)
   - 1 time per month or less
   - 2-3 times per month
   - 1-2 times per week
   - 3-4 times per week
   - 5-6 times per week
   - 1 time per day
   - 2-3 times per day
   - 4-5 times per day
   - 6 or more times per day

   6.1. How often were your fruit drinks **diet** and/or **sugar-free**?
   - Almost never or never
   - About ¼ of the time
   - About ½ of the time
   - About ¾ of the time
   - Almost always or always
7. How often did you drink **milk as a beverage** (NOT in coffee, NOT in cereal)? (Please include chocolate milk and hot chocolate.)
   - NEVER (GO TO QUESTION 8)
   - 1 time per month or less
   - 2-3 times per month
   - 1-2 times per week
   - 3-4 times per week
   - 5-6 times per week
   - 1 time per day
   - 2-3 times per day
   - 4-5 times per day
   - 6 or more times per day

   7.1. What kind of milk did you usually drink?
   - Whole milk
   - 2% fat milk
   - 1% fat milk
   - Skim, nonfat, or ½% fat milk
   - Soy milk
   - Rice milk

- Raw, unpasteurized milk
- Almond milk
- Other

8. How often did you drink **meal replacements**, **energy**, or **high-protein beverages** such as Instant Breakfast, Ensure, Slimfast, Sustacal or Others?
   - NEVER
   - 1 time per month or less
   - 2-3 times per month
   - 1-2 times per week
   - 3-4 times per week
   - 5-6 times per week
   - 1 time per day
   - 2-3 times per day
   - 4-5 times per day
   - 6 or more times per day

Over the <u>past 12 months</u>:

9. How often did you eat **cream of wheat** or **other wheat-based hot cereals**?
   - NEVER (GO TO QUESTION 10)
   - 1 time per month or less
   - 2-3 times per month
   - 1-2 times per week
   - 3-4 times per week
   - 5-6 times per week
   - 1 time per day
   - 2-3 times per day
   - 4-5 times per day
   - 6 or more times per day

   9.1. Was **milk** added to your hot cereal?
   - NO (GO TO QUESTION 10)
   - YES

   9.2. What kind of **milk** was usually added
   - Whole milk
   - 2% fat milk
   - 1% fat milk
   - Skim, nonfat, or ½% fat milk
   - Soy milk
   - Rice milk
   - Raw, unpasteurized milk
   - Almond milk
   - Other

10. How often did you eat **cold cereal**?
    - NEVER (GO TO QUESTION 11)
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day

    10.1. Was **milk** added to your cold cereal?
    - NO (GO TO QUESTION 11)
    - YES

    10.2. What kind of **milk** was usually added
    - Whole milk
    - 2% fat milk
    - 1% fat milk
    - Skim, nonfat, or ½% fat milk
    - Soy milk
    - Rice milk
    - Raw, unpasteurized milk
    - Almond milk
    - Other

11. How often did you eat tropical fruits (such as mango, papaya, etc.)? (fresh or dried)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day

12. How often did you eat **COOKED greens** (such as spinach, turnip, collard, mustard, chard, or kale)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day

13. How often did you eat RAW greens (such as spinach, turnip, collard, mustard, chard, or kale)? (*We will ask about lettuce later*.)
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week

- 1 time per day
- 2-3 times per day
- 4-5 times per day
- 6 or more times per day

14. How often did you eat **coleslaw**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
15. How often did you eat **sauerkraut** or **cabbage** (other than coleslaw)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day

Over the <u>past 12 months</u>:

16. How often did you eat **carrots** (fresh, canned, or frozen)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
17. How often did you eat **broccoli** (fresh or frozen)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
18. How often did you eat **mixed vegetables**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
19. How often did you eat **sweet** or **hot peppers**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
20. How often did you eat fresh **tomatoes** (including those in salads)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
21. How often did you eat **lettuce salads** (with or without other vegetables)?
    - NEVER (GO TO QUESTION 22)
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day

    21.1. How often were the lettuce salads you ate made with **dark green leaves**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week

- o 3-4 times per week
- o 5-6 times per week
- o 1 time per day
- o 2-3 times per day
- o 4-5 times per day
- o 6 or more times per day

22. How often did you eat **sweet potatoes** or **yams**?
    - o NEVER
    - o 1 time per month or less
    - o 2-3 times per month
    - o 1-2 times per week
    - o 3-4 times per week
    - o 5-6 times per week
    - o 1 time per day
    - o 2-3 times per day
    - o 4-5 times per day
    - o 6 or more times per day
23. How often did you eat **French fries**, **home fries**, **hash browned potatoes**, or **tater tots**?
    - o NEVER
    - o 1 time per month or less
    - o 2-3 times per month
    - o 1-2 times per week
    - o 3-4 times per week
    - o 5-6 times per week
    - o 1 time per day
    - o 2-3 times per day
    - o 4-5 times per day
    - o 6 or more times per day
24. How often did you eat **salsa**?
    - o NEVER
    - o 1 time per month or less
    - o 2-3 times per month
    - o 1-2 times per week
    - o 3-4 times per week
    - o 5-6 times per week
    - o 1 time per day
    - o 2-3 times per day
    - o 4-5 times per day
    - o 6 or more times per day

Over the <u>past 12 months</u>:

25. How often did you eat **catsup**?
    - o NEVER
    - o 1 time per month or less
    - o 2-3 times per month
    - o 1-2 times per week
    - o 3-4 times per week
    - o 5-6 times per week
    - o 1 time per day
    - o 2-3 times per day
    - o 4-5 times per day
    - o 6 or more times per day
26. How often did you eat **lasagna**, **stuffed shells**, **stuffed manicotti**, **ravioli**, or **tortellini**?
    - o NEVER (GO TO QUESTION 27)
    - o 1 time per month or less
    - o 2-3 times per month
    - o 1-2 times per week
    - o 3-4 times per week
    - o 5-6 times per week
    - o 1 time per day
    - o 2-3 times per day
    - o 4-5 times per day
    - o 6 or more times per day

    26.1. How often did the lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini contain cooked greens?
    - o Almost never or never
    - o About ¼ of the time
    - o About ½ of the time
    - o About ¾ of the time
    - o Almost always or always

    26.2. What kind (lasagna, stuffed shells, stuffed manicotti, ravioli, or tortellini) do you consume most often? Include brand name if applicable.
    - o _____

27. How often did you eat macaroni and cheese?
    - o NEVER
    - o 1 time per month or less
    - o 2-3 times per month
    - o 1-2 times per week
    - o 3-4 times per week
    - o 5-6 times per week
    - o 1 time per day
    - o 2-3 times per day
    - o 4-5 times per day
    - o 6 or more times per day
28. How often did you eat **bagels** or **English muffins**?
    - o NEVER
    - o 1 time per month or less
    - o 2-3 times per month
    - o 1-2 times per week
    - o 3-4 times per week
    - o 5-6 times per week
    - o 1 time per day
    - o 2-3 times per day
    - o 4-5 times per day
    - o 6 or more times per day
29. How often did you eat **peanut butter** or **other nut butters**?
    - o NEVER
    - o 1 time per month or less
    - o 2-3 times per month
    - o 1-2 times per week

- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2-3 times per day
- 4-5 times per day
- 6 or more times per day

30. How often did you eat **canned tuna** (including in salads, sandwiches, or casseroles)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
31. How often did you eat **beef hamburgers** or **cheeseburgers**?
    - NEVER (GO TO QUESTION 32)
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day

    31.1. How often were the beef hamburgers or cheeseburgers you ate made with **lean ground beef**?
    - Almost never or never
    - About ¼ of the time
    - About ½ of the time
    - About ¾ of the time
    - Almost always or always
32. How often did you eat **liver** (all kinds) or **liverwurst**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day

Over the <u>past 12 months</u>:

33. How often did you eat **smoked fish** or **seafood** (such as smoked salmon, lox, or others)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
34. How often did you eat **sushi**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
35. How often did you eat **all other fish** or **seafood** (including shellfish) that was **NOT FRIED, SMOKED,** or **RAW?**
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
36. Over the past 12 months, did you eat **soup**?
    - NO (go to question 37)
    - YES

    36.1. How often was the soup you ate **cream soup** (including chowder)?
    - Almost never or never
    - About ¼ of the time
    - About ½ of the time
    - About ¾ of the time
    - Almost always or always

    36.2. How often was the soup you ate **tomato** or **vegetable soup**?
    - Almost never or never
    - About ¼ of the time
    - About ½ of the time
    - About ¾ of the time

- Almost always or always

37. How often did you eat **pizza**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
38. How often did you eat **biscuits**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
39. How often did you eat **peanuts**, **walnuts**, **seeds**, or **other nuts**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
40. How often did you eat **granola bars**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
41. How often did you eat **yogurt** (NOT including frozen yogurt)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day

Over the <u>past 12 months</u>:

42. How often did you eat **cottage cheese** (including low-fat)?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
43. How often did you eat **cheese** (including low-fat; including on cheeseburgers or sandwiches or subs)?
    - NEVER (GO TO QUESTION 44)
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day

    43.1. How often was the cheese you ate **light** or **low-fat cheese**?
    - NEVER
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day
    - 4-5 times per day
    - 6 or more times per day
44. How often did you eat **ice cream, ice cream bars,** or **sherbet** (including low-fat or fat-free)?
    - NEVER (GO TO QUESTION 45)
    - 1 time per month or less
    - 2-3 times per month
    - 1-2 times per week
    - 3-4 times per week
    - 5-6 times per week
    - 1 time per day
    - 2-3 times per day

- 4-5 times per day
- 6 or more times per day

44.1. How often was the ice cream you ate **light**, **low fat**, or **fat-free ice cream** or **sherbet**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

45. How often did you eat **eggs**, **egg whites**, or **egg substitutes** (NOT counting eggs in baked goods and desserts)? (*Please include eggs in salads, quiche, and soufflés)*

- NEVER (GO TO QUESTION 46)
- 1 time per month or less
- 2-3 times per month
- 1-2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2-3 times per day
- 4-5 times per day
- 6 or more times per day

45.1. How often were the eggs you age **egg whites only**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

45.2. How often were the eggs you age **regular whole eggs**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

45.3. How often were the eggs you ate part of **egg salad**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

46. How many cups (8 oz) of **coffee or tea**, caffeinated or decaffeinated, did you drink?

- NEVER (GO TO QUESTION 46)
- 1 cup per month or less
- 2-3 cups per month
- 1-2 cups per week
- 3-4 cups per week
- 5-6 cups per week
- 1 cup per day
- 2-3 cups per day
- 4-5 cups per day
- 6 or more cups per day

46.1. How often was **cream** or **half and half** added to your coffee or tea?

- NEVER
- 1 time per month or less
- 2-3 times per month
- 1-2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2-3 times per day
- 4-5 times per day
- 6 or more times per day

46.2. How often was **milk** added to your coffee or tea?

- NEVER (GO TO QUESTION 47)
- 1 time per month or less
- 2-3 times per month
- 1-2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2-3 times per day
- 4-5 times per day
- 6 or more times per day

46.3. What kind of milk was usually added to your coffee or tea

- Whole milk
- 2% fat milk
- 1% fat milk
- Skim, nonfat, or ½% fat milk
- Soy milk
- Rice milk
- Raw, unpasteurized milk
- Almond milk
- Other

Over the <u>past 12 months</u>:

47. How often did you eat **margarine** on breads, bagels, English muffins, other muffins, pancakes, or waffles?

- NEVER (GO TO QUESTION 48)
- 1 time per month or less
- 2-3 times per month
- 1-2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2-3 times per day

- 4-5 times per day
- 6 or more times per day

47.1. How often was the margarine you ate on these breads **low fat** or **fat-free**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

48. How often did you eat **butter** on breads, bagels, English muffins, other muffins, pancakes, or waffles?

- NEVER (GO TO QUESTION 49)
- 1 time per month or less
- 2-3 times per month
- 1-2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2-3 times per day
- 4-5 times per day
- 6 or more times per day

48.1. How often was the butter you ate on these breads **low fat** or **fat-free**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

49. How often did you eat **margarine** on potatoes, cooked vegetables, rice, grains, or beans

49.1. How often was

- Almost never or never (GO TO QUESTION 50)
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

49.2. How often was the margarine you ate on these potatoes, cooked vegetables, rice, grains, or beans **low fat** or **fat-free**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

50. How often did you eat **butter** on potatoes, cooked vegetables, rice, grains, or beans?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

50.1. How often was the butter you ate on these potatoes, cooked vegetables, rice, grains, or beans **low fat** or **fat-free**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

51. How often did you eat **cream cheese**?

- NEVER (GO TO QUESTION 52)
- 1 time per month or less
- 2-3 times per month
- 1-2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2-3 times per day
- 4-5 times per day
- 6 or more times per day

51.1. How often was the cream cheese you ate **low fat** or **fat-free**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

Over the <u>past 12 months</u>:

52. How often did you eat **sour cream?**

- NEVER (GO TO QUESTION 53)
- 1 time per month or less
- 2-3 times per month
- 1-2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2-3 times per day
- 4-5 times per day
- 6 or more times per day

52.1. How often was the sour cream you ate **low fat** or **fat-free**?

- Almost never or never
- About ¼ of the time
- About ½ of the time
- About ¾ of the time
- Almost always or always

53. How often did you eat foods with **oils added** or with **oils used in cooking** (NOT including baked goods or salads)?

- NEVER
- 1 time per month or less

- 2-3 times per month
- 1-2 times per week
- 3-4 times per week
- 5-6 times per week
- 1 time per day
- 2-3 times per day
- 4-5 times per day
- 6 or more times per day

54. What kinds of oils did you usually eat (mark all that apply)?
    - Olive
    - Corn
    - Canola/rapeseed
    - Other

TWU Sunlight Questionnaire

1. How often do you seek sunshine?
    - Never
    - Seldom
    - Sometimes
    - Often
    - Always
2. How often do you avoid the sun?
    - Never
    - Seldom
    - Sometimes
    - Often
    - Always
3. How often do you wear a hat when it is sunny
    - Never
    - Seldom
    - Sometimes
    - Often
    - Always
4. How often do you cover your arms and legs when it is sunny?
    - Never
    - Seldom
    - Sometimes
    - Often
    - Always
5. How often do you use tanning facilities
    - Never (GO TO QUESTION 6)
    - Seldom
    - Sometimes
    - Often
    - Always

    5.1. How long do you tan per session?
    Minutes _____

    5.2. Does your tanning facility provide the correct UV to produce Vitamin D?
    - Yes
    - No
    - Unknown

    5.3. Do you purposefully use tanning facilities to make Vitamin D?
    - Yes
    - No

    5.4. Do you wear anything while tanning?
    - Tanning Accelerators
    - UV Protection
    - None
6. Do you wear makeup that contains UV protection
    - Yes
    - No (GO TO QUESTION 7)
    - Not Applicable (GO TO QUESTION 7)

    6.1. What SPF is it?
    SPF _____
7. Do you regularly take a vitamin D supplement?
    - Yes
    - No
8. How often do you use sunscreen in general, including makeup?
    - Never (GO TO QUESTION 11)
    - Seldom
    - Sometimes
    - Often
    - Always

8.1. What SPF do you usually use?

SPF _____

9. How often do you use sunscreen while working, including makeup?
   - o Never (GO TO QUESTION 10)
   - o Seldom
   - o Sometimes
   - o Often
   - o Always

9.1. What SPF do you use?

SPF _____

10. How often do you use sunscreen during recreational activities
   - o Never (GO TO QUESTION 11)
   - o Seldom
   - o Sometimes
   - o Often
   - o Always

10.1. What SPF do you use?

SPF _____

11. Are you likely to wear sunscreen during **Winter**?
   - o Yes
   - o No
12. Are you likely to wear sunscreen during **Spring**?
   - o Yes
   - o No
13. Are you likely to wear sunscreen during **Summer**?
   - o Yes
   - o No
14. Are you likely to wear sunscreen during **Autumn**?
   - o Yes
   - o No
15. Do you tend to Increase or Decrease your time spent **outdoors** during the **Winter**?
   - o Increase
   - o Decrease
16. When do you spend most of your time **outdoors** during the **Winter?**
   - o Midnight-7am
   - o 7am-10am
   - o 10am-3pm
   - o 3pm-6pm
   - o 6pm-Midnight
17. Do you tend to Increase or Decrease your time spent **outdoors** during the **Spring**?
   - o Increase
   - o Decrease
18. When do you spend most of your time **outdoors** during the **Spring?**
   - o Midnight-7am
   - o 7am-10am
   - o 10am-3pm
   - o 3pm-6pm
   - o 6pm-Midnight
19. Do you tend to Increase or Decrease your time spent **outdoors** during the **Summer**?
   - o Increase
   - o Decrease
20. When do you spend most of your time **outdoors** during the **Summer?**
   - o Midnight-7am
   - o 7am-10am
   - o 10am-3pm
   - o 3pm-6pm
   - o 6pm-Midnight
21. Do you tend to Increase or Decrease your time spent **outdoors** during the **Autumn**?
   - o Increase
   - o Decrease
22. When do you spend most of your time **outdoors** during the **Autumn?**
   - o Midnight-7am

- 7am-10am
- 10am-3pm
- 3pm-6pm
- 6pm-Midnight