QUANTITATIVE ANALYSIS FOR THE PRESENCE OF VITAMIN A,D&E IN BUTTER USING UFLC OR UV- VISIBLE TECHNIQUE

BY
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(03/AS/012)

This thesis is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in physical sciences & technology of the faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka

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Declaration

This analysis described in this thesis was carried out by my self at the chemical laboratory of SGS Lanka (Pvt) Ltd under the supervision of Dr.Nirmali Wickramaratne, Senior Lecturer, Department of Physical Sciences & technology of Faculty of Applied Sciences of Sabaragamuwa University of Sri Lanka and Mrs. Kolitha amarasinghe of SGS Lanka (Pvt) Ltd during the industrial training from 2nd November 2008 to 19th March 2009.

A report on this has not been submitted to any other university for another degree.

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Date

Affectionately dedicated to

My parents

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Abstract

Vitamin A, D, E are fortified to dairy products such as butter, margarine, milk, cheese, and many other foods in the form of vitamin A as Retinol or Retinyl Ester, vitamin D as ergocalciferol or cholecalciferol and vitamin E as α- tocopherol. It is important to quantify the amount of vitamin A, D, E content in food products because both high and low dietary intake of vitamin A, D, E causes certain malfunctions in human body.

Quantification of vitamin A, D, E in butter or margarine is difficult as it is in minute amount in sample; also vitamins losses occur in the process of analysis during saponification due to isomerisation (2.4% loss) and 1.5% loss during separation during column chromatography (Burnius vitamin A commission report). Therefore precautions must be taken to minimize exposure to air, heat especially during chromatography and evaporation.

The vitamin A, D, E absolute amounts in marketed butter or margarine was analyzed thus to ensure the consumer safety. Initially the most suitable method was identified through trials for the analysis among several standard methods reported. The vitamins content was qualitatively identified using the standard chromatograms. The percentage recovery was also measured. Ultra Fast Liquid Chromatography (UFLC) procedure was used in determining vitamin A and vitamin D. Determination of Vitamin E was done using UV- visible spectroscopy.

Results indicated that, qualitatively and quantitatively satisfied for vitamins levels. Recovery percentage was 89.5% for vitamin and 89.8% for vitamin A2. The recovery percentage was 88.2% for vitamin D and 87.5% for vitamin E

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Chapter 01

1.1. Introduction

The fat soluble vitamin (A, D, E and K) are absorbed by complex processes that parallel the absorption of fat. Thus, any condition that causes malabsorption of fat (e.g. celiac disease, tropical sprue, regional enteritis) may result in deficiency of one or all of these vitamins. Fat-soluble vitamins affect permeability or transport in various cell membranes act as oxidation-reduction agents, coenzymes, or enzyme inhibitors. They are stored principally in the liver and excreted in the feces. Because these vitamins are metabolized very slowly, over dosage may produce toxic effect. So these are added to food products in trace amounts. (David and Michael, 2005). Therefore quantitatively identification of fat soluble vitamin is very essential to customer safety.

Vitamin A and D are added to fluid milk products and semisolid milk product. Manufactures of milk products with added vitamins A and D must have the fortification levels of their products checked and labeled properly. (Lloyd et al). Labeling of food regulations required that vitamin A is calculated as micrograms of retinol equivalent on the basis that 6-μg of β-carotene or 12-μg of other biological active caratenoids equal 1-μg of retinol equivalent. (Ronald et al, 1991).

SGS (Societe Generale de Surveillance) is one of the world's leading inspection, verification, testing and certification company. SGS is recognized as the global benchmark for quality and integrity. The core services offered by SGS can be divided into three categories. There are inspection, testing and certification services. SGS certifies that products, systems or services meet the requirements of standards set by governments (e.g. GOST R), standardization bodies (e.g. ISO 9000) or by SGS customers. SGS also develops and certifies its own standards at the certification services. SGS Lanka (pvt) Ltd always develop their method of testing to obtain correct and accurate result. Therefore SGS Lanka (pvt) Ltd is essential to develop correct and accurate method to determine fat soluble vitamin in Butter or margarine sample.

Vitamin A and D found are found in animal and fish liver oil and dairy product but little in animal and vegetable oils and fats, meat and cereals. The richest source of vitamin E is oils and fats of vegetable origin, cereals and eggs. Little comes from animal fats, meat, fruit and vegetables

In the past methods of analysis for the fat soluble vitamins have not been very successful because each of the vitamins consists of several closely related compounds. However, in recent years improved extraction, clean –up column materials and detection system have permitted the development of HPLC techniques that can provide full data on vitamin activity in oils, fat and lipid extracts foods. For example, Mulholland (1986) reported very sensitive HPLC determination of vitamin A, D and E using narrow bore column and diode array detection.

Papers published on the applications of HPLC to the determination of fat – soluble vitamins in foods and feeds over the period 1977- 88 have been thoroughly reviewed and listed (1988) and van niekerk (1988). General techniques such as separation on sephadex resin have been described by bell (1971) and gas chromatography by Sheppard el al. (1972). However in the 2-3 years new micro particulate reverse phase packing have permitted the development of HPLC techniques which will provide full data on vitamin activity in oils, fats and lipids extracts of food (Barnett et al, 1980)

The fat soluble vitamins are generally associated, albeit with exceptions, as (1) occurring in fatty oils or "invisible" lipids in cells, (2) being absorbed with the aid of bile and chylomicrons (lipoproteins), (3) being transported to target tissues bound to carrier proteins or lipoproteins, (4) being stored in the body and (5) being a threat by hypervitaminosis (Adverse relations due to excess vitamins)

1.2 Objectives

- Determine suitable methods to extract vitamin A,D,E form various types of method
- Develop the accuracy of that suitable methods
- Develop the suitable UFLC method to determine vitamin A and D
- Preparation of secondary working standard of vitamin A,D,E using vitamins capsules
- To analyze whether the samples contains the labeled amount of vitamin A, D, E using butter sample.
- Develop the suitable UV – visible spectrophotometer method to determine vitamin E by using standard curve
- Determine the recovery of vitamin A, D, E procedures.

Chapter 02

2. Literature review

2.1 Vitamins

When animals are maintained on a chemically defined diet containing only purified proteins, carbohydrates, fats and necessary minerals, will not sustain the life. An additional factors present in food is required; although only mininute amount necessary. These additional factors are called Vitamins. Now it is generally accepted that vitamin is an organic compound which can be distinct from Carbohydrates, fats, proteins, and water and present in normal food in minute amounts and essential for development of normal tissue and for normal health, growth and maintenance. When vitamins are absent from the diet or not properly absorbed or utilized it will causes a specific deficiency case or syndrome. These cannot be synthesized by the host and there for must be available from the diet or from the micro-organisms of intestinal tract.

The vitamins generally divided in to two groups; fat-soluble vitamins and water-soluble vitamins. The fat-soluble vitamins which are usually found associated with the lipids of natural foods; include vitamin A, D, E, and K. The vitamin B complex and vitamin c complex are in water-soluble group. Vitamin A was the first accessory food factors to be identified as a component of specific foods.

2.2 Vitamin A and β -Carotene

Vitamin A exists in animal products largely as the alcohol-Retinol, and it is stored in the animal body in combination with fatty acids. During the participation of vitamin A in metabolic functions the esterified molecules becomes free. Because of the alcohol group, retinol readily forms esters. Vitamin A is a fat soluble vitamin and most abundantly occurs as esters of long chain fatty acids Palmitate. Extraction of vitamin A from butter sample is difficult as they contain trace amount of vitamin A and also for its high sensitivity to light, air and high temperatures.

Vitamin A activity in food is mainly due to the all-trans isomer of retinol, which is the most abounded biological active member of vitamin A group. In animal sources vitamin A occurs as mixed esters of long chain fatty acids, mainly palmitate, except in egg it is principally unspecified. In the diet β-carotene and other carotenoids provides most of

vitamin A.Retinyl acetate or palmitate is used to supplement a variety of food products. Vitamin A prevents retardation of growth and preserves the integrity of the epithelial cells.(Ronald et al ,1991)

Vitamin A is sensitive to ultra violet (UV) light, air (proxidants for that matter), High temperatures and moisture. There for steps need to be taken to avoid any adverse changes in this vitamin due to such effects, using low actinic Glassware, Nitrogen and/or vacuum, as well as avoiding excessively high Temperatures. Addition of antioxidant reagents onset is highly recommended. (Augustin, 2002).

As vitamin A is a fat soluble vitamin, the over dosage of vitamin A should be avoided as it will cause toxic effects, such as in Osteoporosis. It is expected to carryout sensory evaluation on vitamin A concentration in butter sample

The precursor molecule of vitamin A is Carotenoids. These provitamin carotenoids can be converted to vitamin A in animal bodies. The most active and most widely distributed form of carotenoid is β-Carotene. It is a symmetrical molecule.

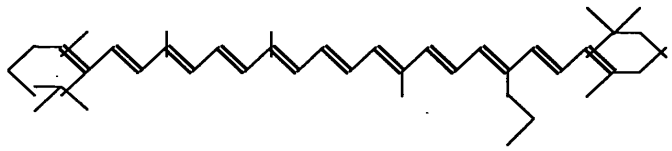


Figure 2.1 the molecule of β-Carotene

The β-carotene molecule consists of two β-ionone rings connected by a chain of four isoprene units. (Budavari et al, 1989)

2.2 Conversion of β-Carotene to Retinol

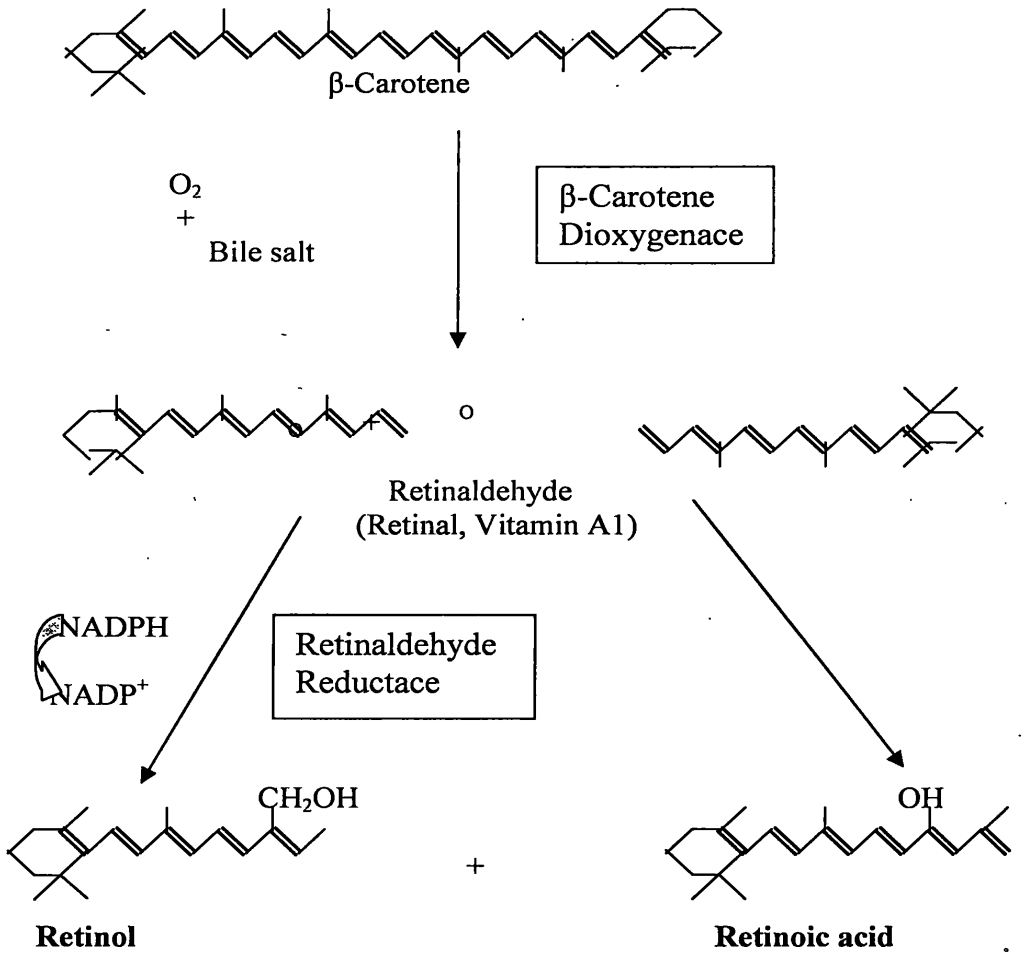


Figure 2.2 : β-carotene and its cleavage to retinaldehyde and reduction of Retinaldehyde to retinol and oxidation of retinaldehyde to retinoic acid

The β-Carotene molecule will converted into two retinaldehyde molecules in the presence of β-Carotene Dioxygenace enzyme, bile salt and O_2 and retinaldehyde molecule will reduce to produce retinol molecule in the presence of Retinaldehyde reductace enzyme and NADPH.

The vitamin a is a pale yellow crystalline solid, insoluble in water, but soluble in fat and various fat solvents. It exists in different isomers and the most biological active isomer of vitamin A is; all-Trans retinol. (Banergee, 1981)

In an attempt to bring order to the problem of nomenclature and relative values, various factors used to describe vitamin A activity are presented in fallowing table. (Phillips R.W., 1982)

Vitamin A should be quantified in terms of **Retinol Equivalent (RE)** or **β-Carotene Equivalent (CE).**

Table 2.1 Conversion of Vitamin A activity

International Unit (IU) of vitamin A	= 1 USP unit = RE(Vitamin A activity)of 0.300 μg Crystalline Vitamin A or 0.550 μg Vitamin A Palmitate.
1-μg RE	3.3 IU
1-β-CE	Standard Provitamin A
β-CE	0.5 μg RE = 1.66 IU

2.4 Importance of Vitamin A

Vitamin A its analogues and its metabolites functions in vision, cell differentiation, embryogenesis, the immune response, reproduction and growth. The best defined function of Vitamin A is in vision. Vitamin A has been implicated in many physiological process including spermatogenesis, taste, hearing appetite, and growth and development of bone.

Vitamin A is necessary for the spermatogenesis in males and maintenance of pregnancy in females and without sufficient vitamin A, fetus resorption occurs. And also it support for the Osteobalastic activity and bone growth.

In the cell nucleus; the vitamin A derivative retinoic acid regulates gene expression in the development of epithelial tissue including skin. Retinoic acid is the active ingredient in the drug tretinoin used in the treatment of serve acne and wrinkled skin. (Phillips R.W., 1982)

Vitamin A in its various forms functions as a hormone and as the visual pigment of vertebrate eye. The vitamin A derivative retinal is the pigment that initiates the response of rod and cone cells of the retina to light producing a neuron signal to brain. (Nelson and Cox, 2005).

In the visual process vitamin A is converted to an aldehyde and combines in the retina with the protein Opsin. The resultant compound Rhidopsin or visual purple reacts with light and in the process initiates the activation of the visual neural pathways.

Retinoic acid is an oxidized metabolite that is rapidly absorbed from the portal system and not stored in the liver. It has partial vitamin A activity in that it will permit normal body growth and epithelial surface differentiation. However, it is not effective in the retina, nor will support reproduction. (Phillips R.W., 1982)

Vitamin A is a fat soluble vitamin and most abundantly occurs as esters of long chain fatty acids Palmitate. Extraction of vitamin A from infant formulas is difficult as they contain trace amount of vitamin A and also for its high sensitivity to light, air and high temperatures. Vitamin A activity in food is mainly due to the all-trans isomer of retinol, which is the most abounded biological active member of vitamin A group. In animal sources vitamin A occurs as mixed esters of long chain fatty acids, mainly palmitate, except in egg it is principally unspecified. In the diet β-carotene and other carotenoids provides most of vitamin A.Retinyl acetate or palmitate is used to supplement a variety of food products. Vitamin A prevents retardation of growth and preserves the integrity of the epithelial cells.(Ronald et al ,1991)

2.5 Dietary sources and intakes of vitamin A

Common dietary source of preformed vitamin A are liver, dairy products, such as milk, cheese, and ice cream and oily fish. The richest source of performed vitamin A is liver oils of Shark, of Marie fish, of Cod and Halibut, and Marine mammals such as polar bears.

Common dietary source provitamin A caratenoids are Carrots, yellow Squash, dark green leafy vegetables, corn, tomatoes, and oranges in southern Asia and Africa caratenoids provide major source of Vitamin A.

Commercial supplement of vitamin A as pills are available in most countries in most instances multivitamin pill contain, 5000 IU. A supplement containing 10000IU or even larger amount per pill is also available. Thus some individuals are almost certainly ingesting large unneeded and possibly toxic supplement of vitamin A. (Olson et al, 2001).

2.6 Toxicity of vitamin A

Vitamin A is one of the few vitamins that have toxic manifestations; this has caused sporadic problems. The toxic effects of vitamin A are categorized in to three categories. These are Acute, Chronic, and Tetratogenic.

However β-Carotene from dietary will not causes these problems and are not to be toxic when ingested in large amounts.

Acute toxicity is produced by one or several closely spaced; very large doses of vitamin A, usually >100 times the recommended intake in adults and >20 times in children. Early signs of acute toxicity are usually transient and disappear within few days.

Chronic toxicity , which is much more common than acute toxicity , is induced by the recurrent ingestion over a period of weeks to years of excessive doses of Vitamin A that are usually >10 times of recommended intake. Permanent damage to liver bone, vision and skeletal pain may result. Excessive amount of vitamin A may increase the breakdown of our bones and interfere with vitamin D, which is essential to absorb Calcium to human body.

Most Tetratogenic effects of vitamin A include fetal resorption; abortion, birth defects and permanent learning disabilities in the pregnancy. But recovery usually occurs slowly when intake ceases.

Recommended dietary allowances are 3000 IU for men and 2300 IU for women per day. Daily intake over 10000 IU of retinol in form of vitamin A are not recommended. The recommended amount of Vitamin A in infant formulas is between 750-1500 IU (Mcgowan et al, 2004)

2.7 Analysis of vitamin A

There are biological, colorimetric, and spectrographic and chromatographic methods to qualitatively analyze Vitamin A in foods and food formulas. The most preferred technique for the accurate determination of vitamin A is reversed phase HPLC (High Performance Liquid Chromatography) methods. There are several HPLC methods are found and few of these methods were tried by using UFLC instrument instead of HPLC instrument.

2.8 Ultra fast liquid chromatography (UFLC)

2.8.1 Introduction

There is a growing trend toward the use of smaller (< 3 um) particle size columns to reduce analysis time and increase sample throughput. The use of sub-2um particle size columns has required specialized instrumentation for the high backpressures generated by these columns. There is concern that high backpressure is associated with increased system maintenance and also leads to heat generation within the column, which can adversely affect reproducibility results. The use of a steep gradient over a short run time may also provide poor retention time reproducibility if the pump resolution is not high enough. Recently, a novel 2.2 um particle size column was introduced that allows efficiencies similar to sub-2um columns, but at greatly reduced system backpressures. The use of this column with a conventional LC system that features high resolution pumps (3nL/step) and a high-speed auto sampler (10 second cycle time) now allows higher throughput to be obtained with backpressures similar to traditional HPLC conditions. Retention time and peak area reproducibility results for an ultra-fast LC separation will be presented.

Ultra fast reversed-phase high-performance liquid chromatographic (HPLC) separations are often needed for analyses related to combinatorial chemistry, studies in liquid chromatography–mass spectrometry, and other applications in which very rapid sample turnaround is paramount. Unfortunately, no consensus exists regarding the best column technology for optimally performing the desired rapid separations.

This overview compares the advantages and limitations for columns of ultramicroporous, ultramicrononporous, and superficially porous particles and monolith structures for the very fast separation of solutes by reversed-phase HPLC. Data from literature and the author's laboratory are used to illustrate the strengths and limitations of the various approaches that can be used for ultra fast separations.

Prominence UFLC responds to increased customer requests for ultra-fast analysis. Based on the existing Prominence components, improvements in flow path pressure (auto sampler), high-speed gradient (controller and solvent delivery units), and reduction of peak broadening in flow paths (auto sampler and detector cell) have been implemented High-throughput analysis requires shortening of the total cycle time, which is defined as the separation, the injection interval and the column equilibration auto samplers boast the world's fastest injection (10 sec, 10 µL injection time), as well as suppressed carryover achieved by special surface coating of the needle and the improved needle seal.

2.8.2 Advantages of UFLC system

- Maximizing reliability

The UFLC modules have many design improvements to consumable as check valves, pistons, and the auto sampler rotor seal. These improvements allow UFLC components to operate reliably as a conventional or UFLC system, whereas with other systems currently on the market, the extremely high backpressure may contribute to more frequent replacement of consumable part

Steep gradients for performing short cycle time analyses and heat generated by high pressure in the column may produce poor reproducibility of retention time and peak area. Responsive tracking of a quickly changing flow rate is essential in high speed gradient analysis. The Prominence UFLC features excellent solvent delivery flow performance with its pumping resolution of 3.7 nL using micro stroke technology (10 µL/stroke) and superior solvent delivery flow control (response of 0.1 second) to provide excellent retention time repeatability at higher flow rates, columns such as the XR-ODS, with an internal diameter of 2 mm to 3 mm, are most appropriate as they can better handle the increased mobile phase flow rate and minimize wear on instrument components, such as pump pistons and seals, auto sampler rotor, etc

As the column's internal diameter becomes smaller, it is necessary to decrease the sample injection volume in proportion to the column cross-sectional area (about 2 μL to 5 μL injection volume with 3 mm internal diameter column). With the Prominence UFLC, uses a high-performance measuring pump and enhanced air tightness of flow lines to achieve peak area repeatability (RSD) of 0.3% or less, even with injections of 2 μL to 5 μL.

Prominence UFLC provided excellent reproducibility on all compounds with a 2μl injection. When using instruments designed for high-pressure operation, it is conceivable that there may be problems with the durability of maintenance parts, ease of maintenance and quality of analysis.

- **Ultra-fast analysis times, accurate and complete data, easy to use and flexible**

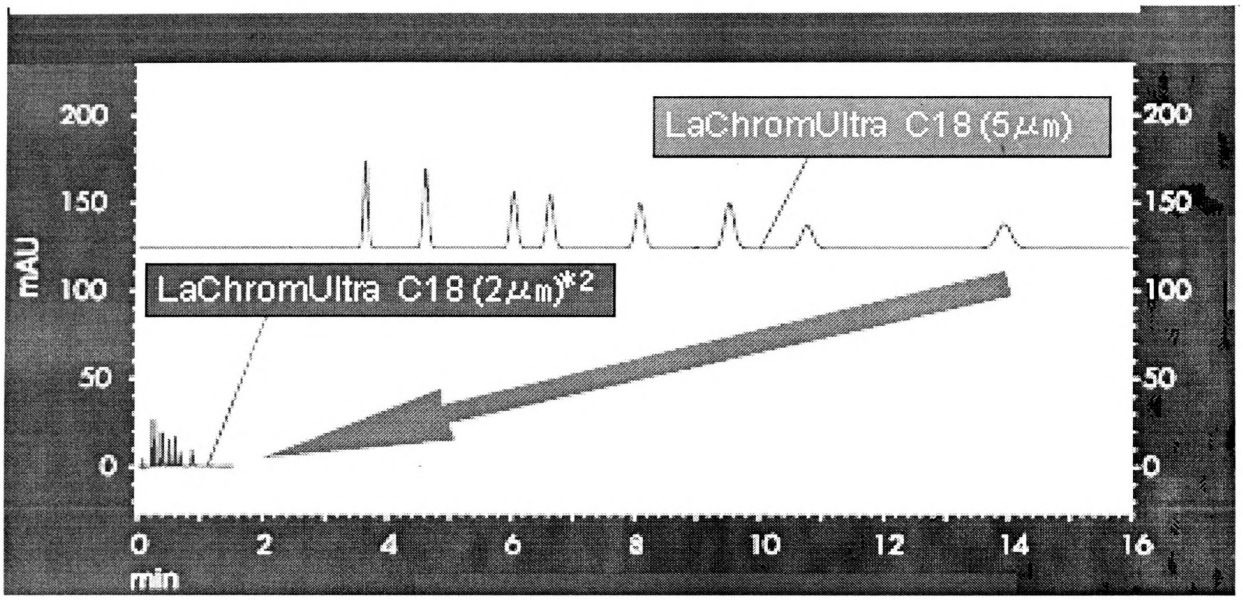
LaChromUltra delivers ultra-fast, high-resolution analyses through a balanced optimization of a 2μm particle column and a maximum 60MPa system pressure

1) Ultra-fast analysis times: Reduced to about 1/10, as compared with our conventional HPLC.

2) Accurate and complete data: detector response times (0.01sec) and sampling periods (10msec) designed for high speed analysis.

3) Easy to use: finger-tight column switching system allows you to easily change between ultra and conventional LC columns.

Flexible: Choose Hitachi's 3 or 5μm particle columns for easy method transfer between ultra and conventional LC applications on one system.



Example of Analysis of DNPH-Aldehyde

Figure 2.3 Analysis time reduce to 1/10

- **High Speed Analysis and Superior Resolution at Significantly Lower pressures.**

High speed liquid chromatography performance is a balance between many factors including particle size, system pressure and column chemistry. 2μm particle size columns offer the permeability and performance to achieve ultra fast analyses with excellent chromatographic resolution but at much lower pressures than other sub-2μm particle size columns. In addition, the stable end capping makes columns the ideal choice for a broad range of applications

Features

1) lowered pressure loss and high-speed high-separation analysis using Sub- Uses a 2-μm particle size column developed specially for ultra high-speed liquid chromatographs
2) Excellent processing of the end capping make columns and well-balanced separation
3) Achieve 2μm columns

The Shim-pack XR-ODS, which is packed for use in reverse-phase chromatography, has 2.2μm diameter particles of totally porous high-purity silica gel with chemically bonded octadecylsilane (ODS) as the base material. Generally, columns using smaller particles provide good separation when using shorter length columns and help to minimize the reduction in column efficiency even if the mobile phase flow rate is high. This makes them well suited to high-speed analysis.

However, since column pressure increases inversely proportional to the square of the diameter of the packing material particle, special HPLC instruments that have resistance to high pressure are required

The specialized instruments that allow use of sub- 2μm columns often sacrifice operability, reliability and application flexibility. The design of the new Shim-pack XR-ODS is based on an in-depth evaluation of a variety of factors such as separation performance, durability and column pressure. Use of 2.2 μm silica gel-based material reduces column flow resistance, allowing high-speed analysis, even with systems comprised of conventional HPLC hardware. Since the XR-ODS achieves a good balance between separation efficiency and pressure, resolution performance is maintained as in a general purpose column (4.6mmi.d. x 150mm, 5μm) while greatly shortening analysis time. Moreover, column pressure is maintained at 30 MPa or less in most analytical conditions without using a specialized system for extremely high pressure. We reported the development concept and some features and applications of the Shim-pack XR-ODS columns

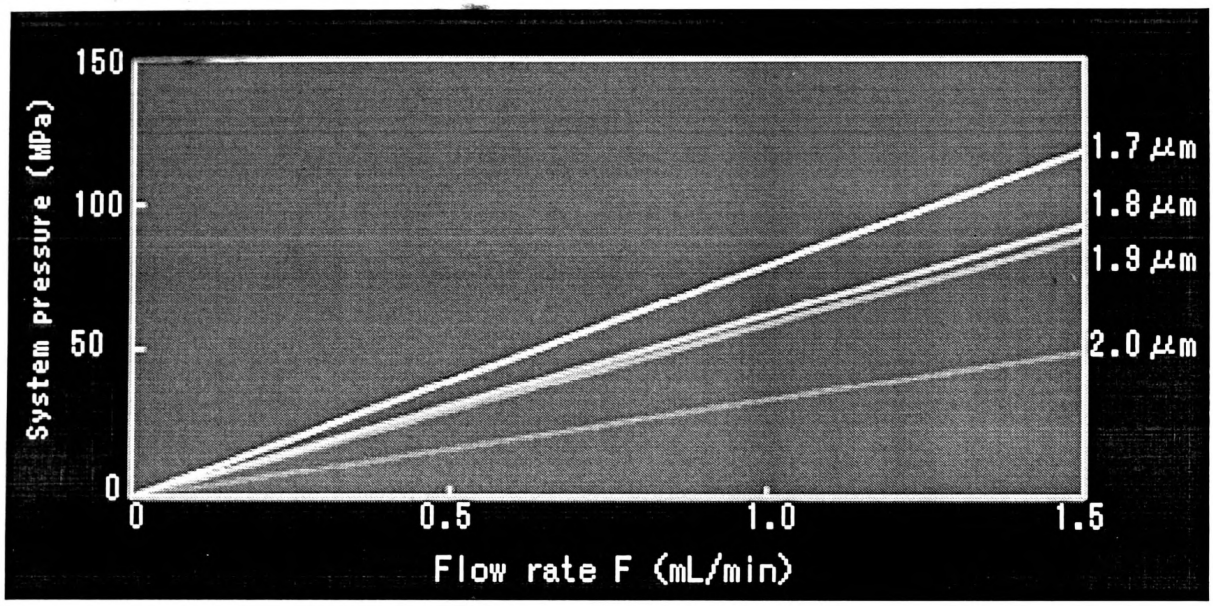


Figure 2.3 **Example of Ultra High Speed Analysis Using Methanol as the Mobile Phase**

Today's demand for increased efficiency in the analytical industry has highlighted the important technical challenge of providing faster separation in HPLC.

The direct approach is to increase the mobile phase flow rate (linear velocity); however, increasing the mobile phase flow rate in the widely used 5mm particle diameter packed column diminishes column efficiency. For enhancing performance, there are multiple possible approaches but the following two seem the most promising:

1) Use of smaller particle packing material
2) Separation under elevated temperature

The common goal of the two approaches is to reduce the Height Equivalent to Theoretical Plate (HETP) and, consequently, to increase the column performance per a given column length in a high flow-rate region. There are several factors to consider in both approaches.

The technological approach of decreasing particle size in the packing material is one way to enable more rapid separation, as the HETP remains small even under a higher flow-rate range if smaller particle size packing material is used.

However, this approach is problematic because hardware will be subjected to a greater load due to increased column pressure (which is inversely proportional to the square of the particle diameter), requiring limited column length. Additionally, the back pressure drastically increases as the particle size decreases. This makes it difficult to use the smaller particle columns under an optimum (higher) flow-rate range or a long column to get high resolution.

In response, specialized instruments with improved pressure resistance for high-speed separation have been developed. However, these specialized instruments have certain limitations regarding pressure tolerance and sacrifice injection accuracy and detection sensitivity, precluding their use in normal analysis. The question arises: is it really necessary or beneficial to use sub-2μm packing material for increasing the speed when the back pressure increases so much?

Shimadzu has proposed a solution that makes it possible for any user to enjoy high speed and high resolution simultaneously with their existing instrumentation. The solution involves the use a column packed with very uniform 2.2μm packing material. With this material, the differences between a 2.2μm column and a 1.8μm column are minimal but the expected back pressure is less than 2/3 with the 2.2μm column, meaning the highest performance level is achieved without sacrificing operability.

Additionally, in some cases, smaller particle columns are not suitable for separating closely eluted peak pairs. If resolution is critical, the natural strategy is to use a longer column. With a small particle column, it may be impossible to use a longer column because the back pressure exceeds the allowed operating pressure. As a result, it is necessary to choose a lower flow rate or a smaller particle size to reduce the back pressure since, as mentioned above, column back pressure is inversely proportional to the square of the particle diameter.

The selection of the appropriate particle size is critical to obtaining the best results and smaller particles do not necessarily offer these results. Too-high pressure is just a consequence of too-small particles unless you want high pressure. Shimadzu believes it would be more useful if the same or better separation with a shorter run time can be achieved with lower back pressure.

➢ **Separation under elevated temperature**

There is increasing interest in elevated temperature LC since better and faster separation can be simultaneously obtained by using higher temperature, which has the effect of accelerating diffusion of the species while decreasing resistance to flow in the column.

It is a characteristic of columns that efficiency is highest at a particular mobile phase flow rate, but at flow rates less than and greater than that flow rate, efficiency decreases (HETP increases). This relationship is typically expressed using the following van Deemter equation:

$$\mathbf{H = AdP + B/n + CdP2n}$$

H is the HETP (column length required to obtain 1 theoretical plate - the smaller this value, the greater the efficiency), dP is the packing particle diameter, and n is the mobile phase linear velocity. The applicability of smaller packing material with a micro-sphere packed column for performing higher-speed separation is attributed to the lack of deterioration in efficiency at high flow rates through two additional terms in the equation. Both factor A (eddy diffusion) and factor C (coefficient of mass transfer) decrease as temperature increases.

Additionally, separation at high temperature has an effect similar to that of decreasing particle diameter. Increasing temperature improves the diffusion aspect of the C term in the van Deemter equation, allowing for a flatter curve at higher flow rates. The resistance to column flow is inversely proportional to temperature, such that the column pressure at 80°C is about 40% lower than that at 40°C.

This highlights the importance of temperature as a factor in accelerating separation by demonstrating that analysis time can be effectively shortened by raising the column temperature while increasing the mobile phase flow rate.

As long as the packing material is stable under the elevated temperature, this technique is quite powerful to get higher resolution and higher speed without making many changes to the system configuration.

➢ **Both Repeatability and speed**

The high-precision solvent delivery system provides excellent durability and precision due to its micro displacement pump design and resolution of delivery steps. This pump resolution is necessary to perform steep gradient profiles required for high-speed HPLC. After extended-use endurance tests, the auto sampler demonstrates excellent repeatability for small-volume injections due to its proprietary valves and design. The new XR columns were developed in consideration of separation, durability and applicable pressure. The 2.2um particle size with controlled particle size distribution skillfully balances separation efficiency, high-speed flow and pressure.

➢ **Upgradeability**

Liquid chromatography separation speed becomes faster by using analytical columns with ultra-fine particle packing and increasing the mobile phase flow rate. When the actual analysis time is reduced, other factors required for the analysis cycle time become important, such as the time required for auto sampler injection movement, gradient delay time and system conditioning time.

High Performance Liquid Chromatography is a technique that a solvent (Mobile phase) is passed through a sorbant (Stationary phase) to effect the separation of vitamin A from compound mixture. It differs from normal column chromatography that the high efficiency sorbant is packed in to a column and the solvent is pushed through with a high pressure pump. In normal phase HPLC columns are packed with Silica gel and composed primarily of low polarity solvents.

In reversed phase HPLC columns are packed with silica to which has been bound to a particular functionality (C8, C18, Cyano, Phenyl) which produces relatively non-polar stationary phase. Mobile phase will be quite polar, normally consisting of combination of water, Methanol, Acetonitrile, etc. After vitamin A elute from column these immediately passes through detector (usually UV detector for vitamin A). For the quantization, the absorbance of vitamin A standard solution is compared with unknown concentration.

The separation of compounds is controlled by the polarity of the mobile phase that uses or the polarity of solvent use to dissolve the compounds. Usually the polarity of mobile phase will determine the retention time of the elute.

The state of the column also very important. It should be thoroughly washed with relevant solvents before uses and also the extracted mixtures to be analyzed should be filter through micro filter to avoid turbidity. As with any HPLC methods evidence not only for peak identity but also of peak purity is essential to provide good quantitative result.

2.8.3 Major part of UfLC instrument

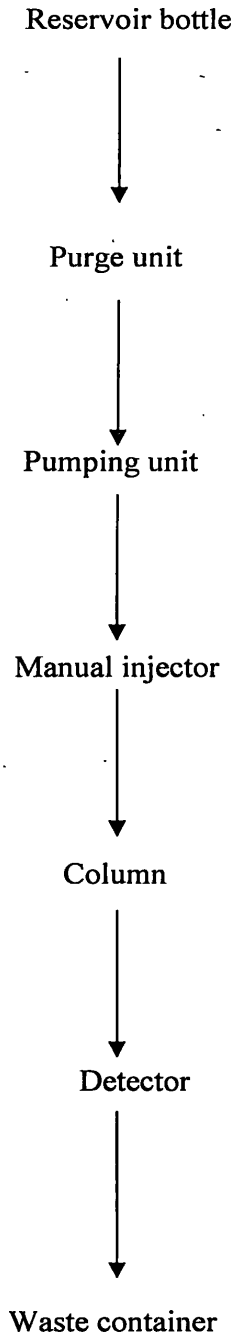


Figure 2.5 Parts of UFLC and solvent system

Reservoir bottle : mobile phase is drawn out of the reservoir bottle and pumped through the tubing by the pumping unit.

Purge unit : Removes dissolved air from the mobile phase preventing air bubbles and consequent rise drift or other baseline irregularity caused by dissolved air.

Pumping unit : pumps the mobile phase through the manual injector, column and detector, in that order and finally to the waste container.

Automatic injector: samples are injected to the system via automatic injector, with micro syringe (20µl)

Column : reversed phase column is used. In the column component are separated by means of the mutual interactions of the mobile phase and the stationary phase of the column

Detector : As vitamin A consists of unsaturated double bonds, the UV detector is being used here. The detector will detect the elute from column and the corresponding UV absorbance.

Waste container: the mobile phase and samples injected will drains in to waste container after passing the detector

2.9 Vitamin D

2.9.1 Chemistry of vitamin D

There are two main recognized forms of the vitamin. Vitamin D3 (cholecalciferol) and Vitamin D2 (Ergocalciferol) which are steroid derivatives.

The structures of these vitamins are given below

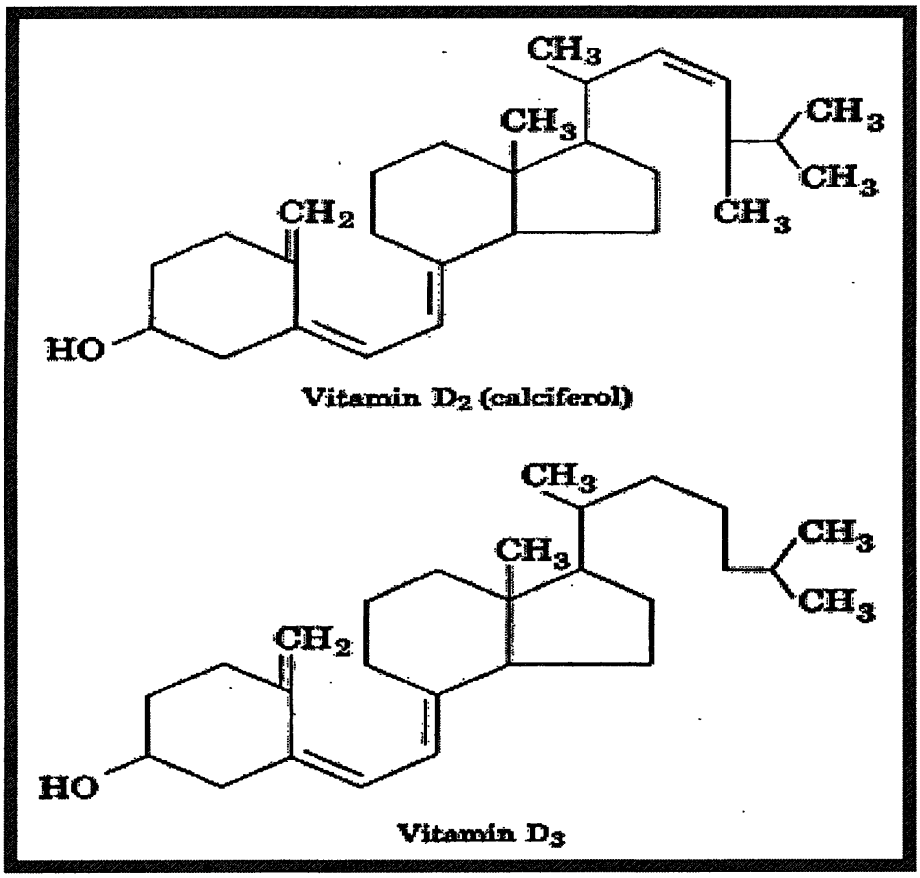


Figure 2.6 Types of vitamin D

The precursors of vitamin D3 and D2 are cholesterol (animal) and ergosterol (plants) respectively. Although ergosterol is widely distributed in plant it is not considered a significant source of vitamin D activity as it is poorly absorbed. However direct administration of vitamin D2 as a supplement produces the desired effects.

2.9.2 Synthesis of vitamin D

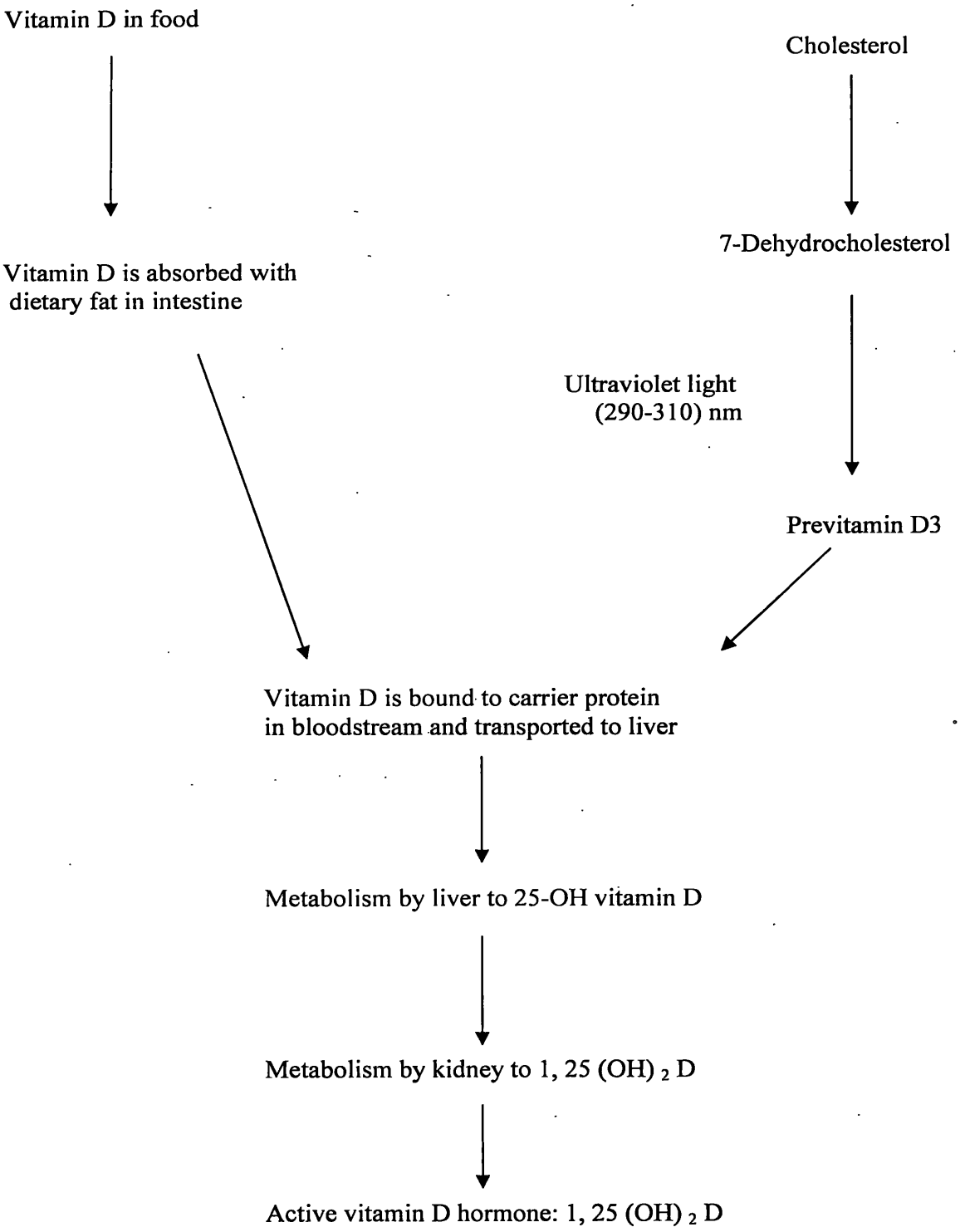


Figure 2.7 Synthesis of the vitamin D

The precursor of vitamin D, cholesterol is synthesized in human body. A Point significance is that the provitamin of vitamin D3 is 7 dehydrocholesterol and is formed from cholesterol in the intestinal mucosae. So that both exogenous and endogenous cholesterol are converted to D3.The last two steps of the sequence occur in the skin, one in a photochemical reaction.

The reaction in the skin is intercellular and occurs while the molecule is membrane proteins and lipids appear to play a catalytic role in the kinetics and thermodynamics of the reaction. The reversibility of conversion of pre-vitamin D3 to lumisterol and tachysterol enables the vitamin to be stored in separate reservoirs for a short period of time. This together with slow diffusion from the skin has the nett effect of vitamin D3 formed by skin having a better effect, molecule for molecule, than vitamin D3 arising from the diet.

Vitamin D3 provided directly by the diet comes from animal food such as fish oil, egg yolk, margarine, liver, lard and to a lesser extent butter and cheese. Vitamin D3 is absorbed in the duodenum and jejunum aided by bile and carried by chylomicrons to the plasma by way of the lymph. Plasma contains a vitamin D binding globulin. Vitamin D is not stored in human liver.

2.9.3 Functions and Mechanism of Action of Vitamin D3 (Calcitriol)

The primary function of calcitriol or 1,25 (OH)2D3 in the intestine is increased absorption of calcium and phosphorous. Vitamin D is carried into the nucleus of the enterocyte, where it binds to receptor proteins and acts as a steroid hormone, resulting in the stimulation and synthesis of new messenger RNA molecules these messenger RNA molecules are then translated to produce a protein, calbindin. Calbindin, a calcium binding protein in the intestinal mucosa is synthesized in response to the action of calcitriol (active vitamin D). This calcium binding protein is needed for Ca transport across the cell membranes Action of Vitamin D on Intestinal Absorption of Calcium A Model for Action of Calcitriol in Gene Transcription

Calcitriol is involved in parathyroid hormone (PTH) stimulation of Ca and P "reabsorption" in the distal renal tubule.

Vitamin D seems to have more effect on Increasing P reabsorption than Ca reabsorption in the kidney. PTH, alone or with calcitriol, directs the mobilization of Ca and P from the bones to help achieve a normal blood Ca concentration

The main biologically active form of vitamin D3 is calcitrol. It is synthesized by successive hydroxylation in the liver and kidney. Calcitriol (1,2dihydroxicholecalciferol) acts like a steroid hormone by affecting protein synthesis in its target tissues. Its main effects are in the intestine, bone and kidney where specific proteins are synthesized to meditate its effects which mainly involve Ca2+ and PO43-. Its major effects are Intake of Ca2+ from the gut by induction of calcium binding protein calbindin and an ATP ase for a Ca2+/ATP pump. Intake of PO43- through gene expression of an alkaline phosphate. Mobilization of bone through induction of a Ca2+ binding protein, osteocalcin. Mobilization of Ca2+ in bone by increasing alkaline phosphates activity to raise Ca 2+, PO43- levels in the blood when necessary. In association with the parathyroid hormone promoting renal reabsorbtion of Ca2+ and PO43-(via binding proteins) inducing an intestinal phytase to promote free ca2+ formation from complexed Ca2+.

Calcitriol acts having bound vitamin D receptor (VDR) synthesized by a VDR gene as Apo VDR. A number of newer functions have been elicited for vitamin D including a change in amino acid composition of proteins of connective tissue, and an increase in wound healing strength and re-epithelialisation. Vitamin D3 enhances the expression of transforming growth regulator of (1) breast cancer cells, and (2) of the expression of proto-oncogenes of regenerating skin.

It also plays a role in inhabiting proliferation and diffreciation of keratinocytes and thereby has use in the treatment of psoriasis. The anti – hypertensive effect of high Ca2+ intake is suspected to be connected with serum vitamin D3 levels.

The clinical applications of many of the effects of vitamin D are restricted due to its high calcaemic effect and therefore the need arose for vitamin D analogs with a reduced calaemic activity. Such analogs have been studied for the treatment of a number of clinical conditions. For examples : (1) The inhibition of breast and prostate cancer cells, (2) increase in cellular immunity (3) protection against neuron loss (4) treatment of osteoporosis (5) treatment of leukemia (6) treatment of asthma and (7) prophylaxis against renal bone disease.

2.9.4 **Vitamin D Deficiency**

The traditional manifestations of vitamin D deficiency are rickets in (in children) and osteomalacia (soft bone, in adults).While the latter is manifested due to loss of Ca 2+ from bone in the elderly, in pregnancy and lactation, rickets is a disease of the young mainly due to the lack of sunlight. It is called the disease of poverty and darkness. This is brought about by environmental and social condition such as cold, smog, urban high-rise tenements, and customs (e.g. Purdah), where sunlight is blocked off and addition these social strata have no money for vitamin D supplements.

Outside these circumstances the disease is rare as only 15 minutes exposure to sunlight in white skinned individuals, suffices to prevent rickets and osteomalacia .Both form have same chemical and ultraviolet spectrometric properties and there separation has provide extremely difficult. The difficulty is emphasized by the low content of most natural materials.

The major problem of analysis is separation from vitamin A which usually present at comparatively high level. It is, therefore, not surprising that the method of choice for analysis of vitamin D has been biological assay.

Vitamin D3 occurs naturally in animal products. It may arise either from the animal's diet or by the action of sunlight on the animal skin. Vitamin D2 appears to be as effective as in man and is manufactured from plant materials

Chemically vitamin D2 and D3 are sterols and may be found along with the other sterols such as phytosterols and cholesterol and but at much lower concentrations in the unsaponifiable matter of oil, fats and food lipid extracts. Vitamin D3 and vitamin D2 in solution are in equilibrium with their previtamins. It follows therefore that previtamins provide potential vitamin D activity.

2.9.5 Sources of Vitamin D

Vitamin D is found naturally in very few foods. Dietary source: fatty fish (mackerel, salmon, sardines), cod-liver oil, eggs yolks, Margarine Fortified milk (vitamin D2/D3),butter, cereals and bread products, orange juice Infant formula is fortified with vitamin D (400 IU per quart)

2.9.6 Causes of Vitamin D Deficiency

Impaired availability of vitamin D: Inadequate intake of vitamin D Fat malabsorption, Lack of photoisomerization. Impaired hydroxylation to 25-hydroxy-vitamin D: liver disease. Impaired production of 1, 25(OH) 2-vitamin D: kidney disease .

There are several Risk Factors is occurred by Vitamin D Deficiency. Exclusively breast fed infants - human milk 25 IU/L of vitamin D , Dark skin – lower the vitamin D synthesis with exposure to sunlight , Fat malabsorption - lower the absorption of vitamin D , Elderly - lower the synthesis of vitamin D in the skin + stay indoors , Institutionalized adults , Obesity - vitamin D deposited in body fat stores, lower the intestinal absorption of calcium and phosphorus: Hypocalcaemia ,Hypophosphatemia , Phosphaturia , Secondary hyperparathyroidism (bone resorption), Demineralization of bones: Osteoporosis/ostoemalacia in adults ,Rickets in children ,Muscle pain/weakness, Vitamin D Deficiency Children: Rickets Failure of bone mineralization in infants and children: Delayed closure of the fontanels (soft spots) in the skull, Deformed rib cage in infants ,Seizures from hypocalcaemia

2.9.7 Vitamin D Toxicity

Excessive exposure to sun does not cause toxicity by Overproduction of endogenous cholecalciferol. Excessive ingestion of vitamin D causes a much greater increase in 25-OH D3 levels because exogenous vitamin is absorbed and delivered to the liver. In the liver, exogenous vitamin D (vitamin D3) is hydroxylated in position 25, and converted to 25-OH D3 and released into the blood. Although the efficiency of 25 hydroxylase decreases when the intake of vitamin D is high, an excessive amount of metabolite can still be produced with over supplementation.

Toxicity results in the increased oxidation of membranes and deposition of "calcium in soft tissue'. The symptoms of vitamin D toxicity include anorexia, vomiting, hypertension, renal insufficiency and failure to thrive (in infants).

2.9.8 Vitamin D – Doses

- ✓ Adequate intake (AI)
- ✓ 19-50 years: 5 microgram / day
- ✓ 51-70 Years: 10 microgram/ day
- ✓ More than 70 years: 15 microgram/day
- ✓ Upper level for adults: 50 microgram/ day.

2.9.9 Assessing of Vitamin D Status

- Serum 25(OH)D3 level
  - Normal level ⇒ 75-125 nmol/L
  - Severe deficiency ⇒ < 20-25 nmol/L
- Serum 1,25(OH)2 D level
  - Undetectable in severe deficiency

2.10 Vitamin E

These vitamins comprise the Tocols and Tocotrienols alpha – tocopherol being the major vitamer found naturally as a RRR isomer. The tocopherols are master antioxidants which scavenge electrons from free radicals. Free radicals are a highly reactive molecular species with an unpaired electron.

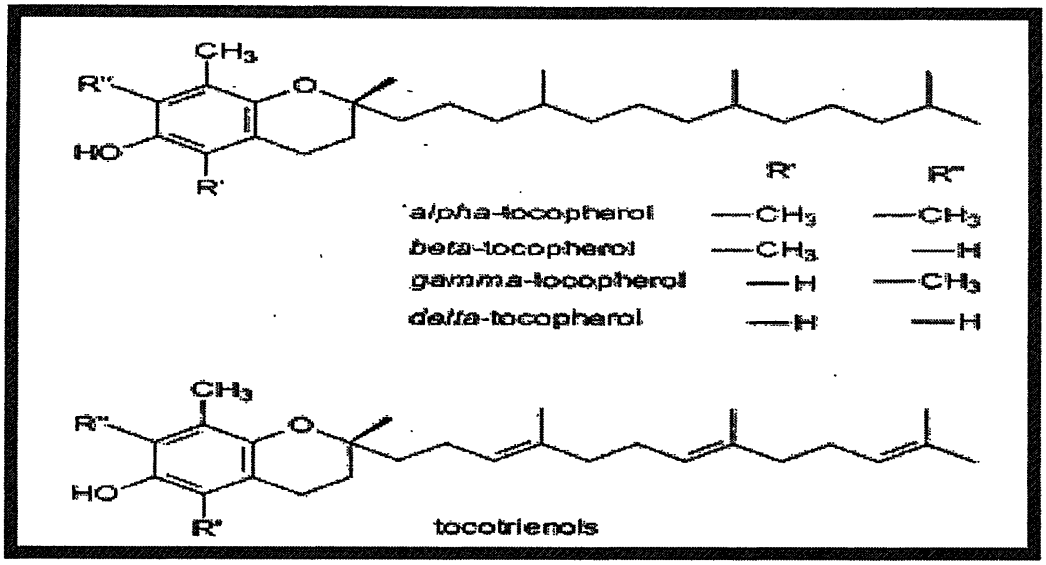


Figure 2.8 T he structure of tocopherol and tocotrienols

The aerobic environment of our planet produces a degree of oxidative stress on biological species which by means of enzyme reactions produce free radicals. Such oxidative stress is increased in high O^2 environments (oxygen tent) and during vigorous exercise. The result is the production of various types of oxygen based free radicals such as the singlet oxygen ('0^2), the oxygen super oxide (O^2)-, the hydroxyl free radical (.OH), and various alkyl peroxides (ROO.). Vitamin E together with other natural antioxidants such as Vitamin C, bioflavonoid, Se (in glutathione peroxidase) vitamin A and riboflavin can neutralize the effects of these free radicals.

Although vitamin E is the master anti- oxidant, on reaction with a free radical it forms the tocopherol free radical (a pro-oxidant) and has to be regenerated by another antioxidant. The antioxidants therefore act synergistically.

2.10.1 Mechanism of vitamin E action

The major function of vitamin E appears to be to protect membrane systems. Particularly by protecting the oxidation of polyunsaturated fatty acids (PUFA'S) from peroxidase action i.e. it is a peroxyl trapping antioxidant. This is important as oxidation causes profound changes in membrane architecture and physiological function e.g. the inability to respond to hormonal stimuli.

Vitamin E by its hydrophobic nature can penetrate the cell membrane lipids. It is unevenly distributed in membranes and is suspected to alter the physical state and modulate membrane functions. In addition to being an antioxidant it can otherwise act as a membrane stabilizer together with Zn^{2+}.

Since it protects PUFA'S from oxidation, increased PUFA in diet requires increased intake of dietary vitamin E increased oxidative stress also increases requirement for vitamin E has been shown to reduce thiobarbituric acid reducing substances (which is an index of lipid peroxidation). Vitamin E however, is supposed to increase O2 utilization by affecting mitochondrial membrane systems.

Vitamin E binds to all membranes and lipoproteins, significantly low density lipoprotein (LDL) and also protects red blood cells from hemolysis. Its antioxidant property protects DNA from damage by free radicals.

2.10.2 Vitamin E and disease

Vitamin E has been prophylactically and therapeutically used to reduce atherosclerosis, the pathogenesis of which is lipid peroxidation and fibrinolysis. Vitamin E has been shown to protect endothelial cells and platelet membranes involved in this disease. Further atherosclerosis which is partly due to oxidatively modified LDL (by free radicals) is decreased by vitamin E

Vitamin E in combination with Se has been used to prevent cancer. It has been recently shown that administration of vitamin E decreases DNA and RNA content and inhibits human breast cancer cells. The general protective role of vitamin E to DNA may also be a contributory factor.

Vitamin E is reputed to be beneficial in the treatment of ulcers, maintaining defence mechanisms and treatment of myocardial infarction and retardation of aging although the exact mechanisms are not clear

The brain and spinal cord are high in lipid and any damage to them causes extensive peroxidation. Further, it has been suspected that scavengers of free radicals, like vitamin E could steam brain cell loss e.g. In Parkinson's disease. However clear results have still not emerged in the therapeutic use of vitamin E in these cases.

2.10.3 Sources and Requirements of vitamin E

It is mainly found in vegetable oil but not in fish oil. Fruit contains smaller amount of vitamin E. vitamin E is lost in commercial food processing and even on storage in a deep freezer. Ingested vitamin E is transferred via chylomicrons and LDL. Requirement of vitamin E depend on a number of factors such as age, PUFA in diet, oxidative stress, etc. Deficiency of the vitamin after depletion of stores causes increased hemolysis and creatinuria. Supplementary doses up to 600mg/day are not reported to cause hypervitaminosis. Present indications are that natural (RRR) and synthetic alpha-Tocopherrol (Racemic) is equally potent.

2.10.4 Vitamin E dose

- ✓ The daily requirement of vitamin E: about 3 to 15 mg daily.
- ✓ Tolerance upper intake level: adults 1000 mg/ day.

2.10.5 Toxicity of Vitamin E

- ✓ Supplements up to 800 IU is harmless probably
- ✓ Upper Level is 1,000 mg/day of any form of supplementary alpha-tocopherol
- ✓ Upper Level is 1500 IU (natural sources) or 1100 IU (synthetic forms)
- ✓ Inhibit vitamin K metabolism and a anticoagulants.

2.10.6 UV- visible spectrophotometer

Vitamin E analysis can be done using UV- visible spectrophotometer. An absorbance spectrophotometer is an instrument that measures the fraction of the incident light transmitted through a solution. In other words, it is used to measure the amount of light that passes through a sample material and, by comparison to the initial intensity of light reaching the sample, they indirectly measure the amount of light absorbed by that sample. Spectrophotometers are designed to transmit light of narrow wavelength ranges (visible light, near-ultraviolet, and near-infrared). A given compound will not absorb all wavelengths equally–that's why things are different colors (some compounds absorb only wavelengths outside of the visible light spectrum, and that's why there are colorless solutions like water). Because different compounds absorb light at different wavelengths, a spectrophotometer can be used to distinguish compounds by analyzing the pattern of wavelengths absorbed by a given sample. Additionally, the amount of light absorbed is directly proportional to the concentration of absorbing compounds in that sample, so a spectrophotometer can also be used to determine concentrations of compounds in solution. Finally, because particles in suspension will scatter light (thus preventing it from reaching the light detector), spectrophotometers may also be used to estimate the number of cells in suspension. We will be using a spectrophotometer several times this semester to quantify the concentration of chemicals present in a solution.

When studying a compound in solution by spectrophotometer, you put it in a sample holder called a cuvette and place it in the spectrophotometer. Light of a particular wavelength passes through the solution inside the cuvette and the amount of light transmitted (passed through the solution—Transmittance) or absorbed (Absorbance) by the solution is measured by a light meter.

While a spectrophotometer can display measurements as either transmittance or absorbance, in biological applications we are usually interested in the absorbance of a given sample. Because other compounds in a solution (or the solvent itself) may absorb the same wavelengths as the compound being analyzed, we compare the absorbance of our test solution to a reference blank. Ideally, the reference blank should contain everything found in the sample solution except the substance you are trying to analyze or measure. Absorbance is related logarithmically to transmission as

A = -log T and There is a relationship between concentration and absorbance. This relationship is expressed by the Lambert-Beer law, which is more commonly known as Beer's law. This law states that the absorbance of a light absorbing material is proportional to its concentration in solution.

$$A = \varepsilon l c$$

ε = the *extinction coefficient* of the substance, (unique for each substance)

l = the sample path length measured in centimeters (i.e. the width of the cuvette—almost always 1 cm)

c = the molar concentration

Many spectrophotometers must be calibrated by a procedure known as "zeroing." The absorbency of some standard substance is set as a baseline value, so the absorbencies of all other substances are recorded relative to the initial "zeroed" substance. The spectrophotometer then displays % absorbency (the amount of light absorbed relative to the initial substance)

Butter and margarine

Butter is a dairy product made by churning fresh or fermented cream or milk. It is generally used as a spread and a condiment, as well as in cooking applications such as baking, sauce making, and frying. Butter consists of butterfat, water and milk proteins.

Most frequently made from cows' milk, butter can also be manufactured from that of other mammals, including sheep, goats, buffalo, and yaks. Salt, flavorings and preservatives are sometimes added to butter. Rendering butter produces clarified butter or ghee, which is almost entirely butter fat.

Butter is an emulsion which remains a solid when refrigerated, but softens to a spreadable consistency at room temperature, and melts to a thin liquid consistency at 32–35 °C (90–The density of butter is 911 kg/m^3 (1535.5 lb/yd^3).

It generally has a pale yellow color, but varies from deep yellow to nearly white. Its color is dependent on the animal's feed and is commonly manipulated with food colorings in the commercial manufacturing process, most commonly annatto or carotene Butter contains 81 percent fat, of which 54 percent is saturated fat, 20 percent is monounsaturated fat, 3 is percent polyunsaturated fat and between 4-8 percent is trans fats.

Almost all margarine begins as chemically-extracted, refined vegetable oil. This is a poor quality product to begin with. The oil is extracted at high temperature, which damages the oil. It also destroys the vitamin E in the oil, an important nutrient.

To make margarine, the oil must be hardened. This is done by by bubbling hydrogen through the vegetable oil at high temperature. The hydrogen saturates some of the carbon-carbon bonds of the oil. The product then becomes hard or solid at room temperature

There are several types of butter can be founded in sir lankan market. Such as Anchor Butter 100g, Astra Margarine 500g, Highland Butter -200g ... etc. Astra yellow pack butter is contained only Vitamin A and Vitamin D but blue pack one is contained vitamin A,D,E. other butter and margarine are contained one or more those vitamins. But Highland butter is not contained any of those vitamins. That only contained vitamin C (ascorbic acid)

2.11.1 Butter and Nutrition

Butter contains about 74 calories per 10 grams. Hard margarine has a similar energy content while low fat soft margarines may contain as little as 27 calories per 10 grams. Butter contains Vitamin A, a little Vitamin D and Vitamin E. Margarine typically contains more Vitamin A, Vitamin E and Vitamin D.

Butter and margarine are high in fat, contain no carbohydrate but contain very small amounts of protein Abnormalities of the heart and larger blood vessels occur in babies born to vitamin A deficient mothers. Butter is America's best and most easily absorbed source of vitamin A. Butter contains lecithin, a substance that assists in the proper assimilation and metabolism of cholesterol and other fat constituents. Butter also contains a number of anti-oxidants that protect against the kind of free radical damage that weakens the arteries.

Vitamin A and vitamin E found in butter both play a strong anti-oxidant role. Vitamin A and the anti-oxidants in butter--vitamin E, selenium and cholesterol--protect against cancer as well as heart disease.

Vitamin A found in butter is essential to a healthy immune system; short and medium chain fatty acids also have immune system strengthening properties. Vitamins A and D in butter are essential to the proper absorption of calcium and hence necessary for strong bones and teeth. Butter also has anti-cariogenic effects, that is, it protects against tooth decay. Butter is a good source of iodine, in highly absorbable form. Vitamin A in butter is essential for proper functioning of the thyroid gland.

Vitamin A is a group of compounds that play an important role in vision, bone growth, reproduction, cell division, and cell differentiation. Vitamin A helps regulate the immune system, which helps prevent or fight off infections by making white blood cells that destroy harmful bacteria and viruses. Vitamin A also may help lymphocytes (a type of white blood cell) fight infections more effectively. Vitamin A promotes healthy surface linings of the eyes and the respiratory, urinary, and intestinal tracts

Vitamin D is found only in small and highly variable amounts in butter, cream, egg yolk and liver. Most dried whole milk and evaporated milk are fortified as well as some margarine, butter, selected cereals and some infant products. The active form of Vitamin D is essential for normal growth and development and is important for the formation of normal bones and teeth. Like other steroid hormones, the active form of Vitamin D is localized in the nuclei through a receptor mechanism. Vitamin D has an important role in the maintenance of the appropriate serum levels of calcium and phosphorus to support normal mineralization of bone. Active form of Vitamin D has the following functions within the body:

- It stimulates the active, energy-requiring intestinal absorption of calcium. This is through stimulation of the synthesis of calcium-binding protein in the brush border of the intestinal mucosa. Alkaline phosphates, whose synthesis is also induced by the active form of Vitamin D, may also be involved.
- It stimulates the active phosphate-transport system in the intestine.
- In conjunction with parathyroid hormone, it acts to mobilize calcium from bone in order to maintain proper serum calcium levels.

- It mobilizes phosphate from the bone in order to maintain serum phosphate levels.
- In a minor way, it acts to increase the reabsorption of calcium by the kidney.
- It increases renal tubular reabsorption of phosphate.

Usually vitamin E is added to the butter and margarine higher amount than vitamin And Vitamin D. because of the vitamin E is very essential for the human body. Vitamin E is lost in commercial food processing and even on storage in a deep freezer. Ingested vitamin E is transferred via chylomicrons and LDL. Requirement of vitamin E depend on a number of factors such as age, PUFA in diet, oxidative stress, etc. Deficiency of the vitamin after depletion of stores causes increased hemolysis and creatinuria. Supplementary doses up to 600mg/day are not reported to cause hypervitaminosis. Present indications are that natural (RRR) and synthetic alpha-Tocopherrol (Racemic) are equally potent.

The major function of vitamin E appears to be to protect membrane systems. Particularly by protecting the oxidation of polyunsaturated fatty acids (PUFA'S) from peroxidase action i.e. it is a peroxyl trapping antioxidant

Chapter 03

3 .Materials and Methodology

3.1 Sample collection

Samples to be analyzed were collected randomly from the market and they were selected if only they are being provided the vitamin A,D,E concentration of the manufacturer.

3.2 Materials

3.2.1 Equipment

(a) Beaker or conical flask, of capacity 250 ml

(b) Saponification flask, of capacity approximately 200ml, fitted with a reflux condenser

(c) One mark volumetric flasks, capacities 100 ml and 200ml

(d) One mark pipettes, of capacities 100ml and 200ml

(e) Steam bath, boiling water bath electrical heating mantle

(f) Water bath, capable of operating at a temperature of up to 40 ^{0}C

Separating funnel, of capacity 500ml, preferably with a polytetrafluorethylene (Pttfe) stopper

(g) Unitrasonic bath

(h) Filter paper, of diameter 9cm

(i) Spatula

(j) Analytical balance

(k) Separating funnels

(l) Hot plate

(m) Nitrogen blanket apparatus (liquid Nitrogen tank)

(n) Reflux condenser

(o) Side armed Saponification flask

(p) UV Spectrophotometer (UV-1601 (SHIMADZU)

3.2.2 Instruments

(a) Ultra fast liquid chromatography (UFLC)

SCL-10A VP System controller

SPD-10A VP UV-Visible detector (325nm)

HPLC Column: (XR-ODS Column with initial diameter 2μm or 3μm)

(b) Instrument software

Class VP SCL-10A

VP version 5.30

(c) Micro filter

Nylon 66 membrane

0.45μm× .47mm (SUPELCO 5-8063)

(d) Vacuum pump

Model 13156

Pressure vacuum pump

(Gelman Little Glant-Gelman science)

(e) Rotary evaporator (SHIMADZU)

(f) UV Spectrophotometer

UV-1601 (SHIMADZU)

(g) Sonicator

Branson 1510, 70W, 42 kHz

3.3 Reagents

3.3.1 Solvents

(a) All trans-Retinol pure standard (Sigma Chemical cor.)

(b) Sodium ascorbate solution, 200 g/l

Prepare this by dissolving 3, 5 g of ascorbic acid in 20mg of 1mol/l sodium hydroxide (NaOH) solution and mix. Prepare this solution fresh daily.

(c) Methanol - HPLC grade

(d) Ethanol 95% (by volume), free from aldehyde

(e) Potassium hydroxide aqueous alcoholic solution, 30 g/l

Dissolve 3g of KOH in water and add 10 ml of ethanol in a 100ml Volumetric flask. Dilute with water to the 100ml mark and mix

(f) KOH solution - 50%

Dissolve 50g of KOH in 50 ml of water.

(g) Butylated Hydroxy Toluene (BHT) Solution – 1mg/ml

(h) Petroleum Ether solution – boiling point 40^0C-60^0C

(i) Distilled water

(j) Cod liver oil as standard

(k) diethyl ether.

(l) 20 ml M alcoholic sulphuric acid

Prepare by mixing 1.1ml sulphuric acid with 18.9 ml alcohol.

3.3.2 Mobile phase

(a) Methanol : Water (95:10) (for vitamin A)

(b) Acetonitrile : Methanol (95:5) (for vitamin D)

3.3.3 Analysis of vitamin A using reversed phase UFLC

There are four basic steps for analyzing of vitamin A in infant formulas which are saponification, extraction, concentrating and analyses.

Saponification can be done by using excess amount of KOH solution and reflux the sample: KOH mixture for sufficient time and the nitrogen flow must bubble through the mixture. Because the vitamin A is sensitive to Oxygen and also it's better if it can be carried out in dark place. Also antioxidant (pyrogallol, sodium ascorbate) can be used to avoid oxidation of vitamin A. samples are saponified to convert retinol esters to retinol and to destroy interfering substances. So this treatment permits to separate saponifiable components (glycerol, fatty acid salt) which could interfere with vitamin A. To eliminate interference determined by proteins and carbohydrate ethanol is used to saponification process. Because protein and carbohydrate are soluble in organic phase and ethanol are completely miscible with water.

Extraction can be done using Petroleum ether fraction of boiling point 40^0C-60^0C. It is essential to use the low boiling fraction to extract vitamin A due to its heat sensitivity. It will decompose above 65^0C. All steps in extraction procedure must be done to minimize exposure to air, heat especially during chromatographic & evaporative step. Those vitamins are extremely light sensitive, to prevent destruction all sampling and analysis step was done under subdued light condition and also to prevent the losses of vitamins minimum amount of glass ware was used. In the AOAC method the extraction is done directly to Ethanol and Tetra Hydro Furan (THF) – (50:50) solution. But THF is an expensive and highly toxic solution. It is better to avoid these types of solutions regularly and precautions must be taken when using.

As butter contain residue amount of vitamin A it is better if it can be dissolved in minimum amount of solvent before injecting to the UFLC. After extracting to Pet ether solution, using rotary evaporator it can be concentrated. But using Nitrogen flow also it can be concentrated. We can do this using a water bath of 40^0C and Nitrogen flow and can dissolve the residue in minimum amount of solvent to obtain a high ppm value.

Vitamin A in oils may be determined by the rapid Carr-Price method, in which the blue color formed with Antimony tri Chloride solution is measured by the measurement of ultra violet (UV) absorbance in an organic solvent.

When the expected concentration in the sample is high, the blue value or the absorption spectrum can be determine directly on a solution of the sample; but with most samples the determination should be made on solution of the unsaponifiable matter. (Ronald et al, 1991)

3.3 Methodology

As determination of Vitamin A is difficult two standard methods were tried and achieve accurate results.

3.4.1 Method 01

As a first method ISO 12080 specifies method was done. At the beginning of the project the vitamin A standard solution was not available. So vitamin A tablet was used as a secondary standard.

3.4.1.1 Saponification of butter sample

A 5g of butter sample was measured to a saponification flask .25 ml of distilled water added to in 20ml of potassium hydroxide and 10ml of sodium ascorbate solution. Add 50ml of ethanol and mix well.

Reflux for 30 min on a steam bath and swirl from time to time. Cool immediately

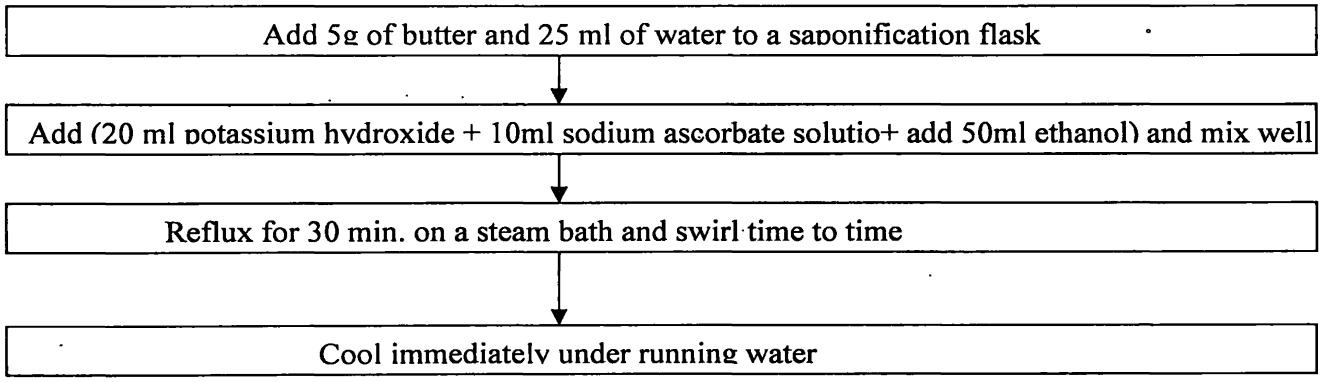


Figure 3.1 Flow chart for method 01 butter sample

3.4.1.2 Saponification of vitamin A standard solution

Cut the capsule containing the vitamin A standard solution and express the oil into a saponification flask. Weight, to the nearest 0, 1 mg, approximately 2 mg of the standard solution. Add 40ml of ethanol, 10ml of sodium ascorbate solution and 10 ml of potassium hydroxide and solution and mix well.

Reflux for 30 min on a steam bath and swirl from time to time. Cool immediately under running water.

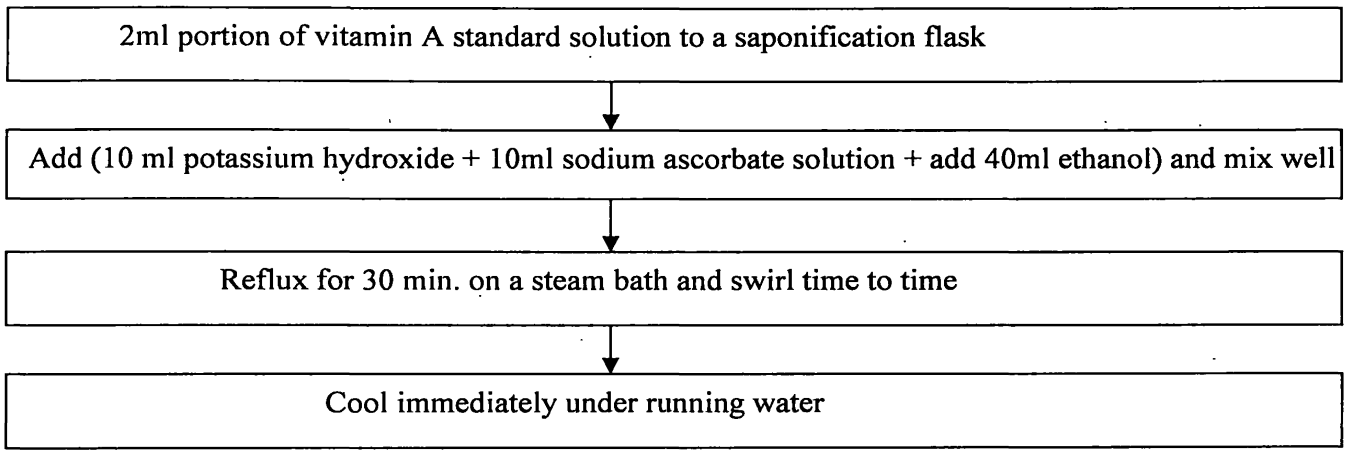


Figure 3.2 Flow chart for method 01 standard solution

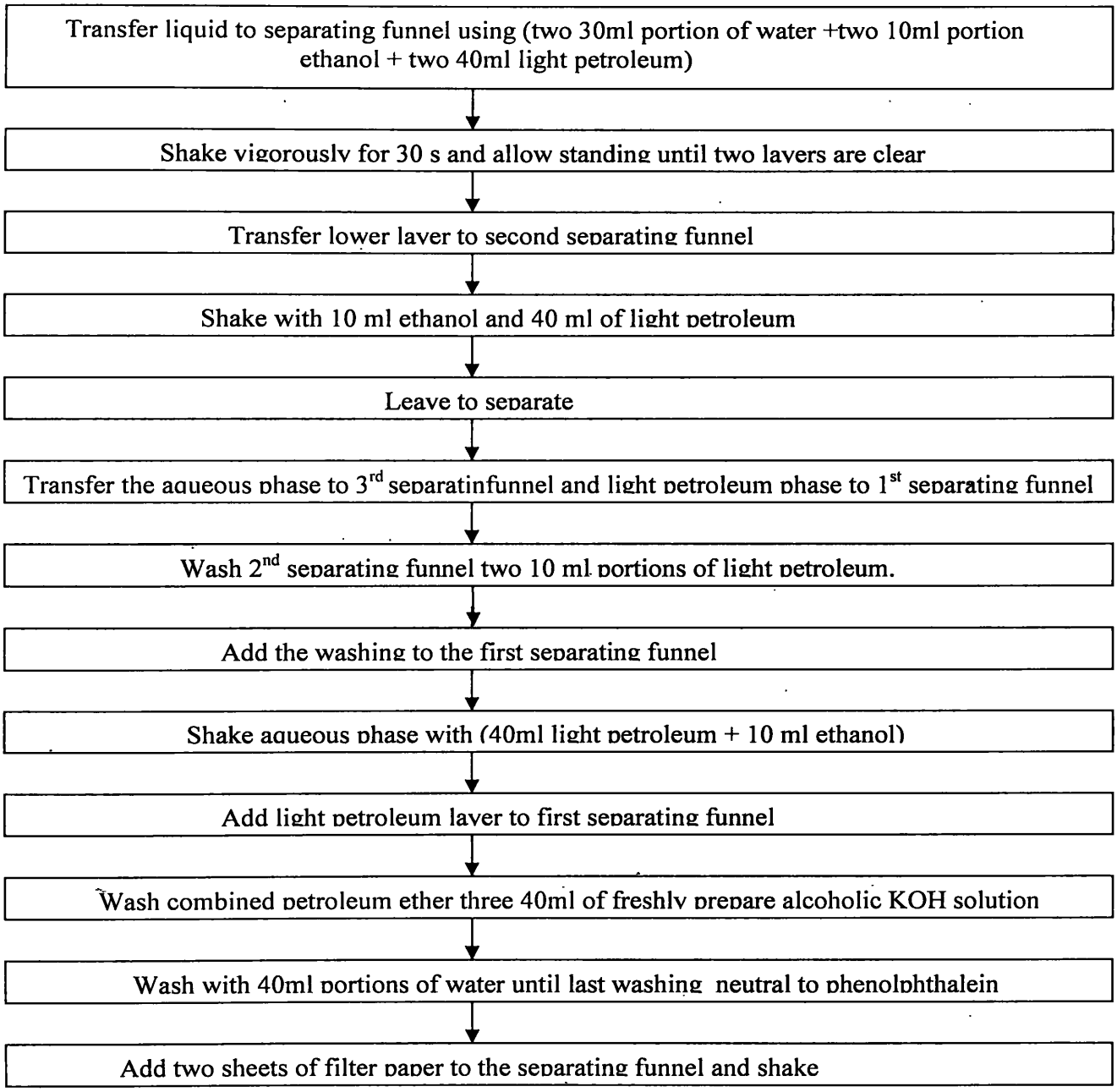
3.4.1.3 Extraction of vitamin A standard solution and butter solution

Transfer the liquid to a separating funnel using two ml portion of water, two 10ml portion of ethanol and two 40ml portion of light petroleum. Shake vigorously for 30s and allow standing until the two layers are clear. Transfer the aqueous (lower) phase to a second separating funnel and shake with mixture of 10ml of ethanol and 40ml of light petroleum. Layers are leaved to separate.

Transfer the aqueous phase to a third separating funnel and the light petroleum phase to the first separating funnel. Wash second separating funnel with two 10ml portion of light petroleum. Add the washing to the first separating funnel.

Shake the aqueous phase with 40ml of light petroleum and 10ml of ethanol. Add the light petroleum phase to the first separating funnel. Wash combined light petroleum extracts with three 40ml portions of freshly prepared potassium hydroxide alcoholic solution, shaking vigorously. Then wash with 40ml portions of water until the last washing is neutral to phenolphthalein. Drain the last few drops of water, add two sheets of filter paper, cut into strips, to the separating funnel and shake.

Transfer the light petroleum extract, dried as described above, to a 200ml one mark volumetric flask and add 10mg to 20mg of BHT. Dilute with light petroleum to the 200ml mark.



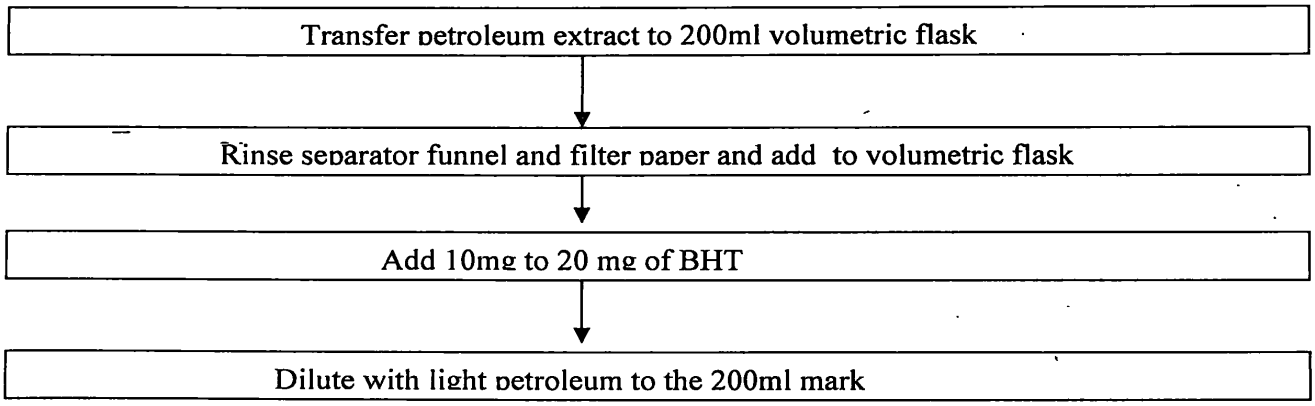


Figure 3.3 Flow chart for method 01 extraction

3.4.1.3 Preparation of test and reference solutions

Pipette dilute extracts obtained from both the test solution and the vitamin A standard solution in to separate round bottom flasks. Evaporate to dryness under vacuum by swirling in a water bath at a temperature not exceeding 40^0C. Cool under running water and restore atmospheric pressure, preferably with nitrogen. Dissolve the residue immediately in 10, 0 ml methanol

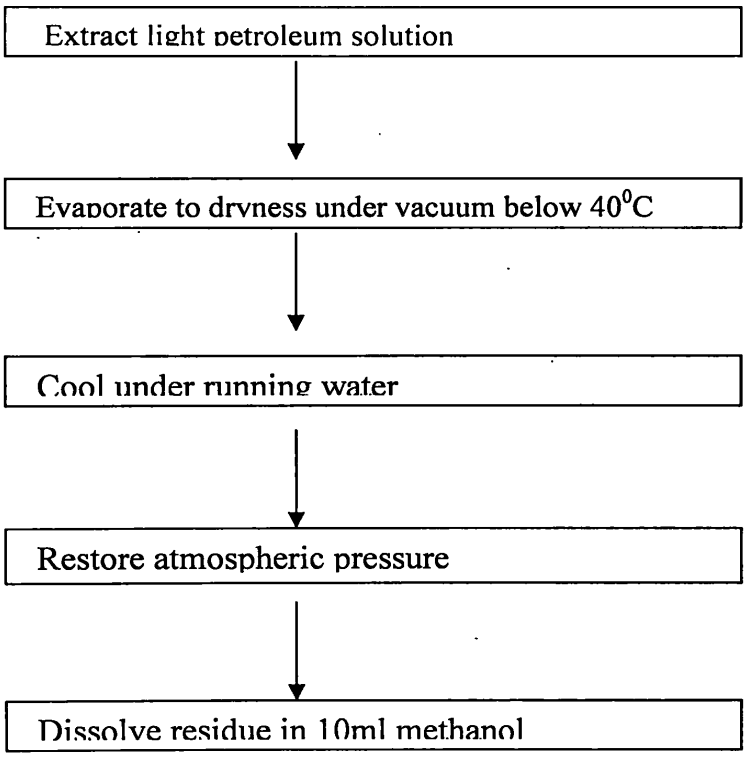


Figure 3.4 Flow chart for preparation test and standard solution

3.4.3.5 Recovery test

About 3.8 mg of standard was weighed accurately in to saponification flask which contained 5g of butter and procedure was done according to above test and standard method.

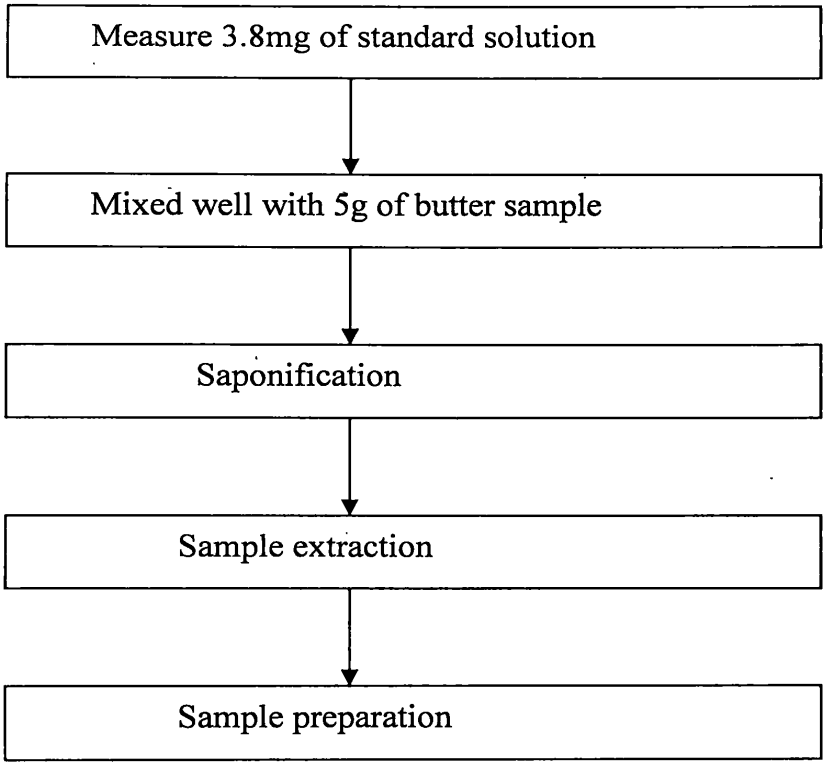


Figure 3.5 Flow chart for recovery samples with method 1 procedure

3.4.1.4 Analysis

Mobile phase was run through column of UFLC. Extract solution was automatically injected at the volume of 20 µl to UFLC through injection port. The mobile phase was run through column at a flow rate of 2.0 ml/min. Vitamin A was monitored at the wave length of 325nm by UV detector. Standard solution was also automatically injected through injection port.

3.4.2 Method 02

As a Second method published HPLC procedure was done. That is reviewed by Ball (1998) and Niekerk (1998). At the beginning of the project the vitamin A standard solution was not available. So vitamin A tablet was used as a secondary standard.

3.4.2.1 Preparation of sample and extraction

About 5g of sample was weighted in to saponification flask fitted with a side –arm and delivery tube.0.25g (approximate) of pyrogalloll as antioxidant, and 37.5 ml ethanolic KOH solution (28% m/v). The mixture was saponified for 30 min under reflux on a boiling water bath. A steam of nitrogen was bubbled through the solution via the side-arm of the flask the solution was quantitatively transferred, whilst still warm, to a separating funnel using 37.5 ml of water and cool under ruining water. 125ml of pet ether was added, using a potion to rinse the saponification flask. Separating funnel plus contents was shaked vigorously for 3 min. the mixture was allowed to stand until the layers separate and the bottom layer was run off into a second 500ml separating funnel. The solution was extracted with a further 125ml portion of mixed ether, shaking vigorously for 3 min. The mixture was allowed to stand until the layers separate, and bottom layer was run off to the waste.

The separate extract was washed with 37.5ml water, inverting the separating funnel gently several times to avoid the formation of emulsions. The mixture was allowed to stand until the layers separate and bottom layer was run off to waste. The mixed ether extracts was washed with further 37.5ml portions of water using progressively more vigorous shaking until the wash solutions are neutral to phenolphthalein.

The wash extracted was transferred to a 500ml amber rotary evaporation flask using few ml of ethanol to rinse the separating funnels. 2ml of BHT solution 1mg/ml as antioxidant was added into it and evaporated to dryness on a rotary evaporator at 40 ^{0}C. Any remaining trace of water was removed by adding more ethanol and evaporating. The flask was immediately stoppered to preserve the inert atmosphere. Immediately cooled to room temperature. The residue was dissolved in 10ml methanol. The sample solution was transferred to a specimen tube, for chromatography and protected immediately from light by enclosing in aluminum foil.

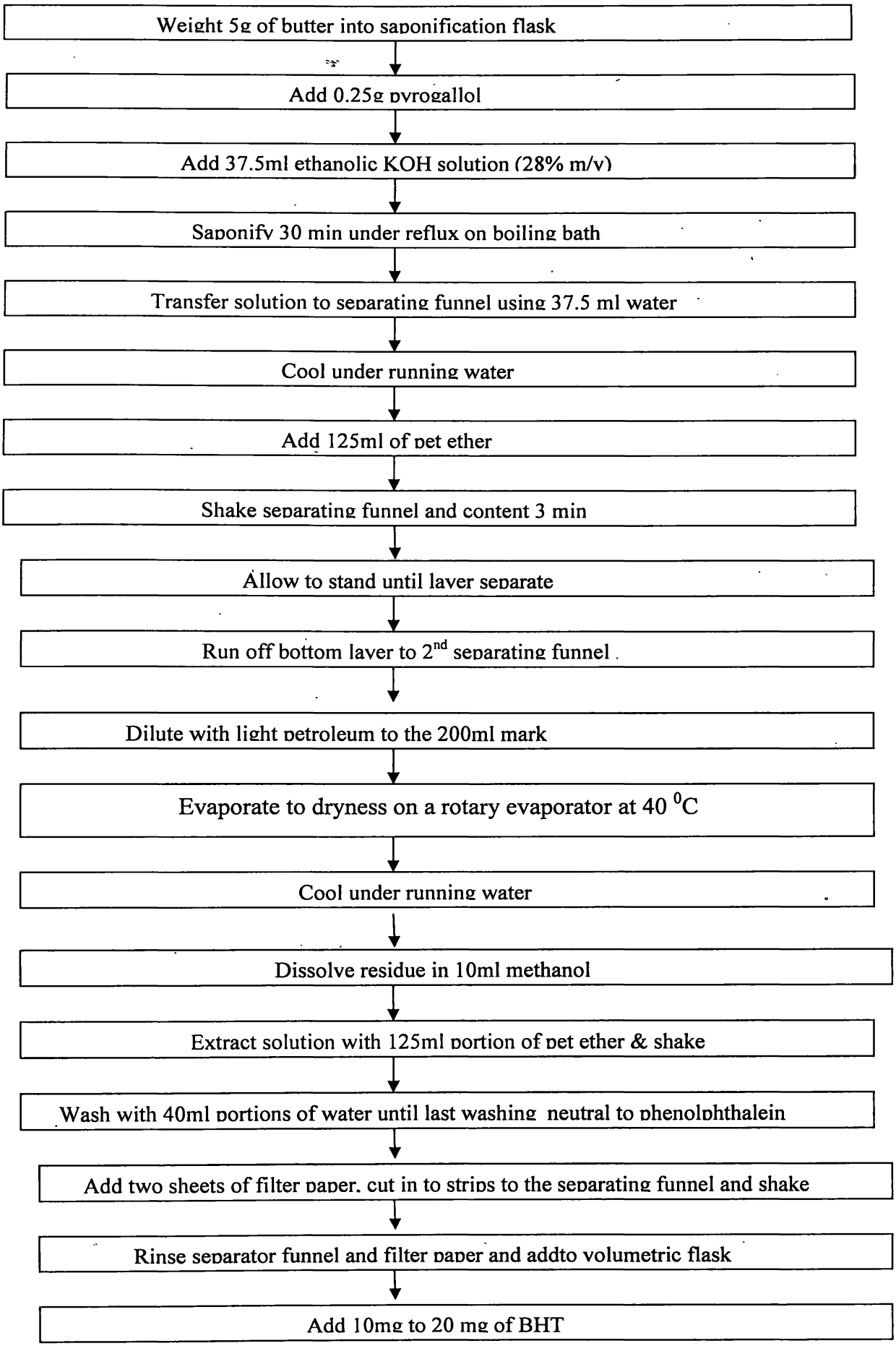


Figure 3.6 Flow chart for method 02

3.4.2.2 Preparation of mobile phase

The mobile phase was prepared using the ISO 12080 method for vitamin A analysis using UFLC for LC-8 column the mobile phase was a mixture of Methanol: Water (95:5). The prepared mobile phase filtered through micro filter.

3.4.2.3Preparation of standard solution

2 mg of vitamin A standard solution was saponified and extracted according to the 3.4.2.1 sample extraction & preparation procedure.

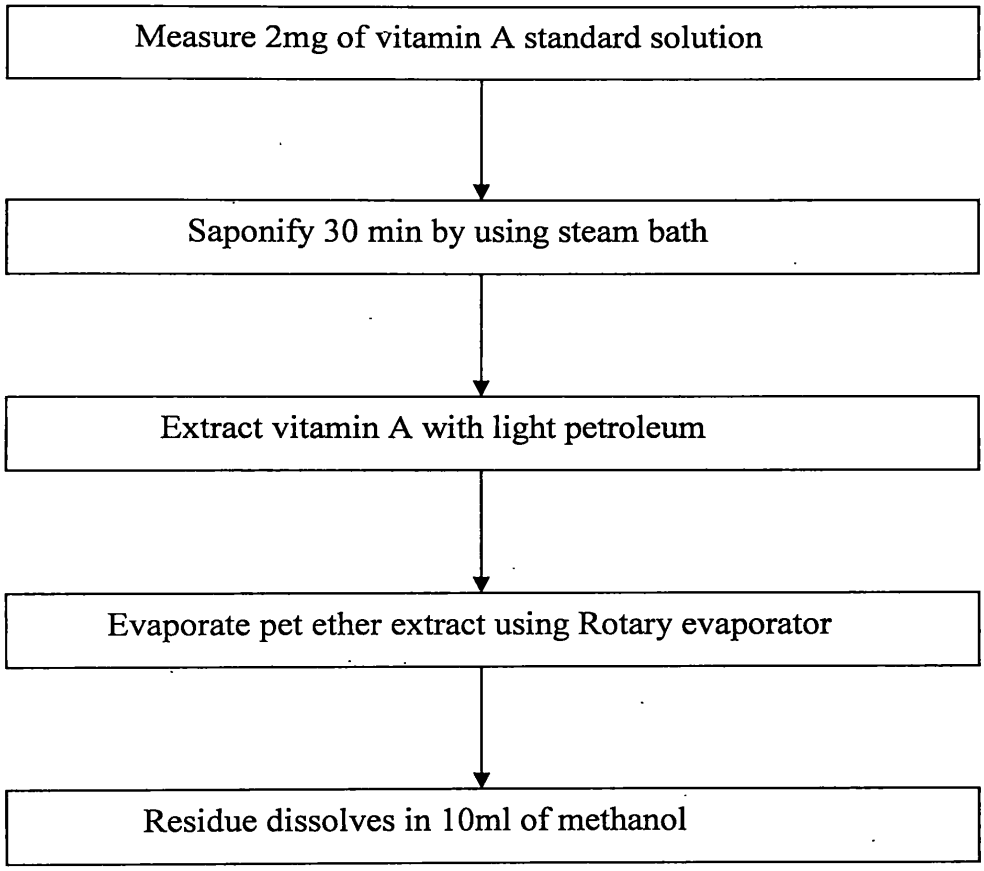


Figure 3.7 Flow chart for vitamin A standard solution with method 02 procedure

3.4.2.4 Recovery test

About 2.3 mg of standard was weighed accurately in to saponification flask which contained 5g of butter and procedure was done according to above method 2 test and standard method.

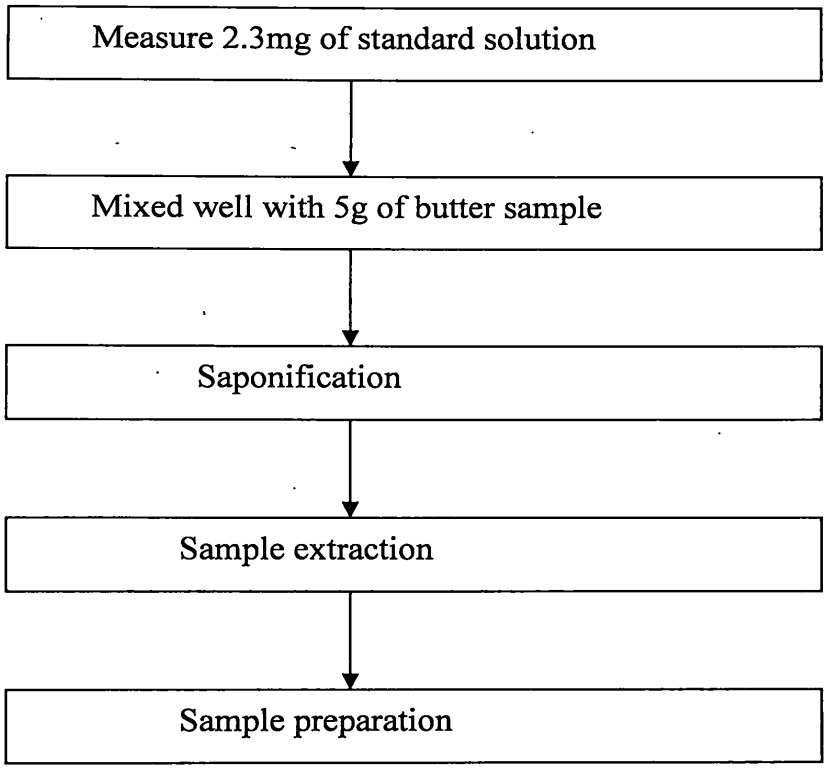


Figure 3.8 Flow chart for recovery samples with method 2 procedure

3.4.2.5 Analysis

Mobile phase was run through column of UFLC. Extract solution was automatically injected at the volume of 20 µl to UFLC through injection port. The mobile phase was run through column at a flow rate of 2.0 ml/min. Vitamin A was monitored at the wave length of 325nm by UV detector. Standard solution was also automatically injected through injection port.

3.5 Analysis of vitamin D

3.5.1 Background

Because of the very lower levels of vitamin D in food, even in vitamin fortified foods, chromatographic interference from sterols, vitamin E, carotenoids and other substances necessitates extensive clean- up and extraction procedure for UFLC. Saponification of the samples necessary to release the vitamin, followed by ethers extraction. Published HPLC procedures have been reviewed by Ball (1988) and van niekerk (1988). In the vitamin D analysis AOAC official method 979.24 was followed.

3.5.2 Method of analysis vitamin D

At the beginning of the project the vitamin D standard solution was not available. So vitamin D tablet was used as a secondary standard.

3.5.2.1 Preparation of sample and extraction

4.5g of butter sample was weighted into saponification flask. 25ml alcohol, 5ml sodium ascorbate solution and 3ml KOH solution was added in small portions, with gentle swirling. Solution was refluxed on H2O bath 30 min and cooled rapidly under running water. Test solution was transferred to separator with aid of two 15 ml portions H_2O, 10ml alcohol, and two 50ml portions of ether.

The mixture was shaked vigorously 30 s and let stand until clear. Aqueous phase was Transferred to second separator and shaked with 10ml alcohol and 50ml n-pentane. The solutions was let to separate and transferred lower phase to third separator and upper pentane phase to first separator. Combined extracts was washed with three 50ml portions 3% KOH solution, shaking vigorously. Then solution was washed with successive 50ml portions H2O until last washing is neutral to phenolphthalein. Last few drops of H2O was drained by adding 2 sheets of 9cm filter paper in strip to funnel, and shaked. Washed pentane extract was transferred to round-bottom flask, rinsing separator and filter paper with pentane. Extract was evaporated to dryness under vacuum by swirling in H2O bath at about 400C. Dry extract was cooled under running water and restored atmospheric pressure with nitrogen. Residue is immediately dissolved in 10ml of mobile phase

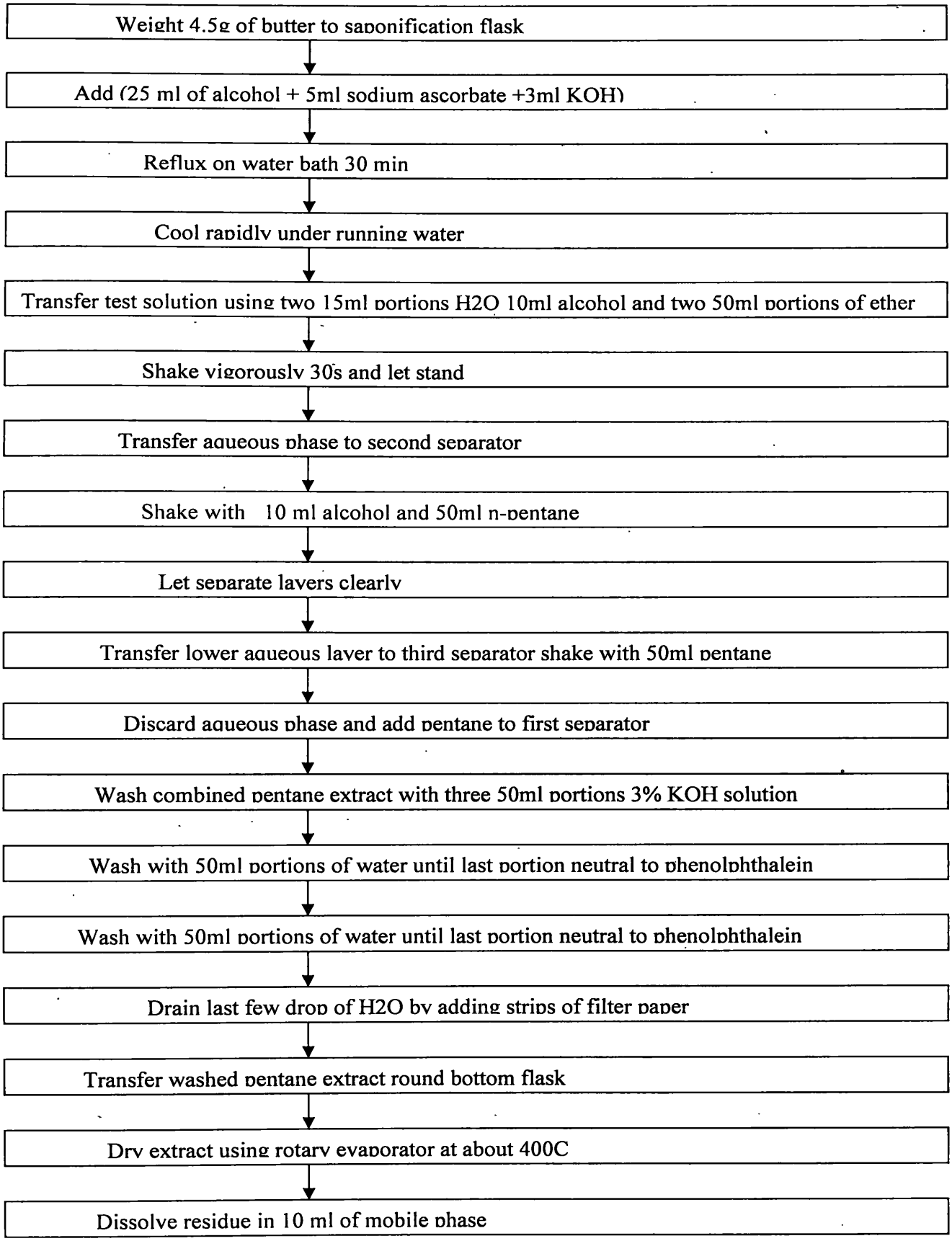


Figure 3.9 flow chart for vitamin D extraction

3.5.2.2 Recovery test

About 5.6mg of standard (cod-liver oil) was weighed accurately in to saponification flask which contained 5g of butter and procedure was done according to above method D test and standard method.

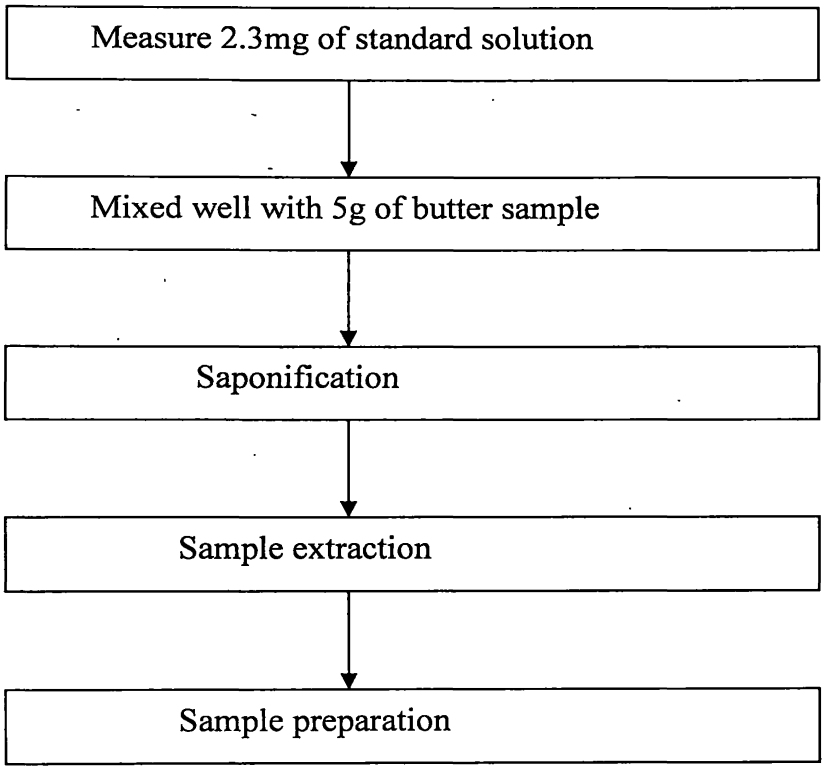


Figure 3.10 Flow chart for recovery samples with Vitamin D procedure

3.5.2.3 Analysis

Mobile phase was run through column of UFLC. Extract solution was automatically injected at the volume of 20 µl to UFLC through injection port. The mobile phase was run through column at a flow rate of 2.0 ml/min. Vitamin D was monitored at the wave length of 265nm by UV detector. Standard solution was also automatically injected through injection port.

3.6 Analysis of vitamin E

3.6.1 Background

To determine vitamin E different analytical procedure (TLC, GC, HPLC, UV-visible) are utilized. Because of their easier applicability, the Furter- Meyer method makes use of the tocopherols by nitric acid to the red tocoquinone. It is more specific than other method. This method was published the association of vitamin Chemists in 1966.

3.6.2 Analysis of vitamin E using UV- visible method

There are five basic steps for analyzing of vitamin A in butter which are saponification, extraction, concentrating, derivatization and analyses.

To determine vitamin E concentration several methods are available, depending on the goal of the analysis and on the matrix. In some cases, it is necessary to quantify α – tocopherol, while in other cases it is necessary to quantify both tocopherol and tocotriens. Anyway , sample has to be pre-treated to break structures into which vitamin E bind (membranes, lipoproteins,liposomes), to eliminate interferences determined by proteins and carbohydrates which are soluble in organic phase, and to create a medium in which vitamin E should be soluble and could freely elute.

For this purpose, usually, ethanol or methanol is used. In food with a low lipid concentration method provides directly for the extraction with diethyl ether. In more fatty food it is necessary to apply a saponification.

This treatment permits to separate saponifiable components (glycerol, fatty acid salts) which could interfere with vitamin E determination. As these compounds are soluble in the aqueous status, they can be removed. Saponification is carry out utilizing potassium hydrate associated to antioxidants to avoid oxidation

After saponification, not saponifiable components, like vitamin E, are extracted adding an apolar organic solvent (hexane, acetone, diethyl ether) and the solution mixed: Fatty acids, glycerol and other undesired substances stay in the alkaline aqueous phase and do not interfere with vitamin E determination.

Comparison between methods with or without saponification shows that saponification permit to quantify α forms, while δ- forms are degraded. Method without saponification permits to quantify Υ- and δ- forms too.

Derivatization is a technique used transforms a chemical compound into a product of similar chemical structure, called derivative. Derivative is a compound that is formed from a similar compound or a compound that can be imagined to arise from another compound, if one atom is replaced with another atom or group of atoms. Because of that the reaction is reliable and proceeds to completion. Less unreacted starting material will simplify analysis. Also, this allows a small amount of analyte to be used. Also the reaction is general, allowing a wide range of substrates, yet specific to a single functional group, reducing complicating interference and the products are relatively stable, and do form degradation products within a reasonable period, frustrating analysis. The tocopherols are oxidized rapidly I the presence of alkali but are stable in acid so the initial saponification is carried out with alcoholic sulphuric acid.

After extracting to Petroleum ether solution, using rotary evaporator it can be concentrated. But using Nitrogen flow also it can be concentrated. We can do this using a water bath of 40 ^{0}C and Nitrogen flow and can dissolve the residue in minimum amount of solvent to obtain a high ppm value. By using vitamin E standard solution 1ppm solution was prepared. Using that calibration curve is prepared and according to curve the vitamin E concentration of sample was calculated.

3.6.3.3 Saponification and extraction of butter sample

5g of butter sample was weighted into a 100ml flask fitted with a reflux condenser. Then 10ml of absolute alcohol and 20mlM alcoholic sulphuric acid were added into it. The condenser and flask was wrapped in aluminum foil. The solution was refluxed for 45 min and cooled immediately. 50ml of water was added .The solution transferred to a separating funnel with the aid of a further 50ml of water. The unsaponify matter was Extract with 5×30 ml diethyl ether. The combined ether extracts was washed free from acid and dried over anhydrous sodium sulphate. The extract was evaporated at a low temperature whilst protecting it from light, the final traces of solvent was removed in a steam of nitrogen. Then the residue was immediately dissolved in 10ml absolute alcohol.

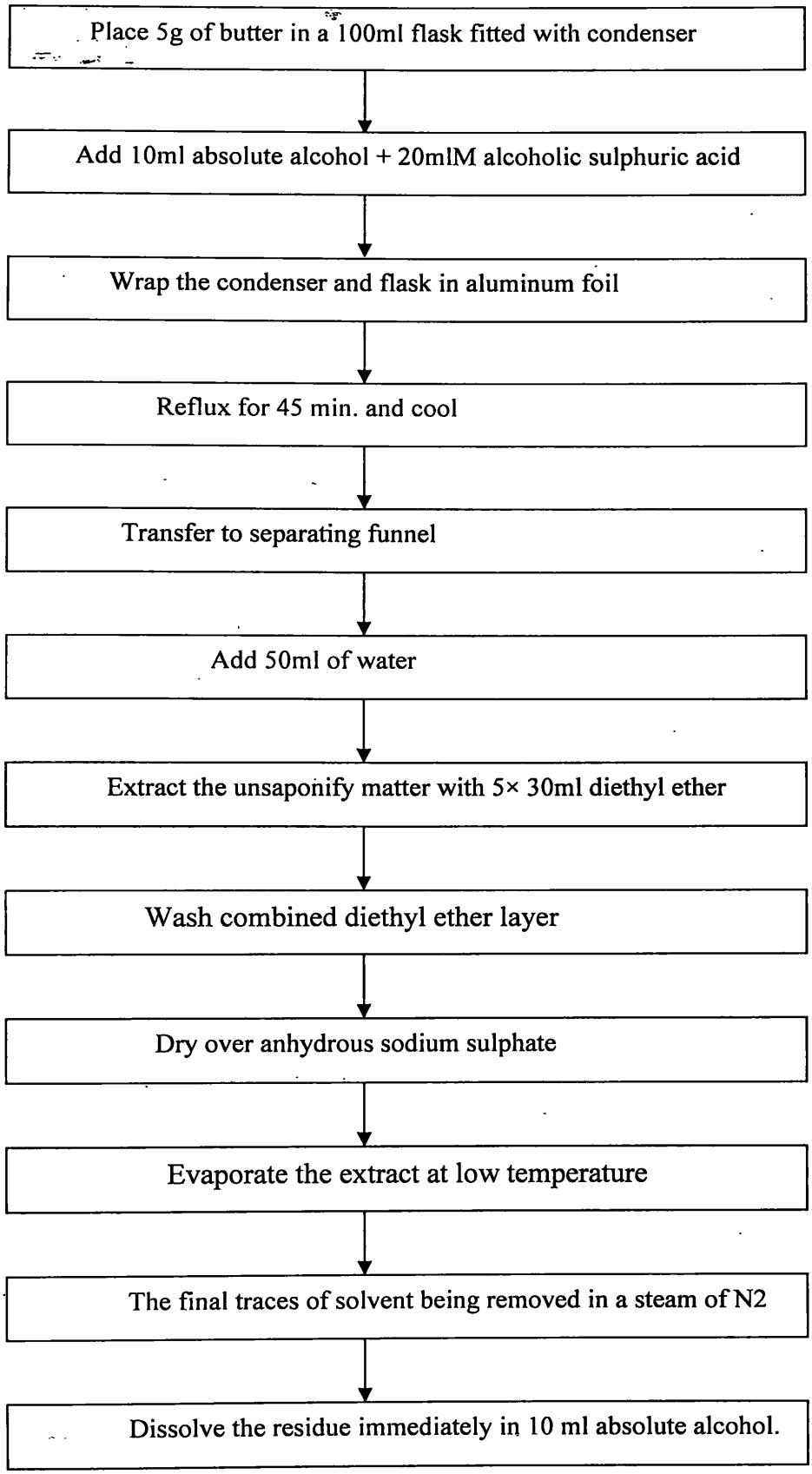


Figure 3.11 Flow chart for vitamin E extraction

3.6.3.4 Preparation 1 ppm vitamin E solution

0.1 g of vitamin e was put into a 100ml volumetric flask and 25 ml of ethanol and 5 ml of conc. Nitric acid were added into it.

3.6.3.5 Preparation vitamin E standard series

By using vitamin E 1ppm solution 0.5 ml, 1ml, 1.5ml, 2ml, 2.5ml samples was measured into labeled 25ml volumetric flask to get 0.02ppm, 0.04ppm, 0.06ppm, 0.08ppm and 0.1ppm solutions.

3.6.3.6 Preparation of test and reference solutions

The sample was transferred to a 25ml volumetric flask. 5ml absolute alcohol was added, followed by 1ml conc. Nitric acid(CARE: add drop wise with constant swirling). The sample volumetric flask and standard solution flasks were placed on a water bath at 90 ^{0}C for extractly 3min from the time the alcohol beings to boil. The solution was Cooled rapidly under running water and adjusted to volume with absolute alcohol. (cf. Association of vitamin chemists, 1966).

3.6.3.7 Recovery test

About 5.6mg of standard (cod-liver oil) was weighed accurately in to saponification flask which contained 5g of butter and procedure was done according to above method D test and standard method.

3.6.3.8 Analysis

The absorbance was Measure at 470nm against a blank containing 5ml absolute alcohol and 1ml conc. Nitric acid treated in a similar manner. The concentration value of standard series samples were measured by using spectrophotometer and standard curve was achieved. Then according to that curve the samples were measured and get concentration values of samples.

3.7 Calculations

3.7.1 Calculations for recovery test

The percentage loss of vitamin A during the method

$$\text{Loss percentage of the sample (L \%)} = \frac{\text{mass of loss} \times 100\%}{\text{Mass of add standard solution}}$$

The recovery percentage of sample = 100% - L%

3.7.1.1 Calculation for mass of vitamin A for method 1 and method 2 using UFLC Chromatograms

$$\text{Vitamin A mass of 100g of butter sample} = \frac{m \times A_{s \times 100}}{A_r \times m_s}$$

m - The mass of vitamin A standard solution used for analysis

A_s - the mean numerical values of the peak area of vitamin A in the test solutions

A_r - the numerical values of the peak area vitamin A in the test solution

m_s - The mass of butter sample used for analysis

$$As = \frac{S_1 + S_2 + S_3}{3}$$

S_1 - sample 1 peak area

S_2 - sample 2 peak area

S_3 - sample 3 peak areas

3.7.1.2 Calculation for mass of vitamin D using UFLC Chromatograms

$$\text{Mass of vitamin D from cod liver oil } (m_d) = \frac{2.5\ \mu g \times m_o}{525\ mg}$$

(Cod liver oil 525 mg is contained 2.5 μg of vitamin D)

m_o - mass of the cod liver oil sample

$$\text{Vitamin D mass of 100g of butter sample} = \frac{m_d \times D_s \times 100}{D_r \times m_s}$$

m - The mass of vitamin A standard solution used for analysis

D_s - The mean numerical values of the peak area of vitamin A in the test solutions

D_r - the numerical values of the peak area vitamin A in the test solution

m_s - The mass of butter sample used for analysis

$$D_s = \frac{S_1 + S_2 + S_3}{3}$$

S_1 - sample 1 peak area

S_2 - sample 2 peak area

S_3 - sample 3 peak area

3.7.1.3 Calculation for vitamin E concentration using standard curve

Calibration curve was obtained by using concentration values and absorbance values. The curve was plot by using spectrophotometer. Then the sample concentration was obtained by using that curve.

$$1\text{ppm} = \frac{1\text{g}}{1000\text{ml}}$$

$$\text{Vitamin E amount of the 100g of sample} = \frac{C_A\,(\text{ppm}) \times V_1 \times X \times 100}{M_s}$$

C_A - mean average concentration of samples

V_1 - The volume of ethanol which dissolve extract sample (25 ml)

X – Dilution factor of sample volume

M_s - The mass of butter which use for analysis

$$C_A = \frac{C_1 + C_2 + C_3}{3}$$

C1- average concentration of sample 1

C_2 - average concentration of sample 2

C_3 - average concentration of sample 3

Chapter 04

4.1 Results and Discussion

Quantification of vitamin A, D, E from butter or margarine is difficult as it is in minute amount in sample; also during saponification 2.4% due to isomerisation and 1.5% during column chromatography of vitamins losses can be happened. (Burnius vitamin A commission report). So all steps in this procedure must be done to minimize exposure to air, heat especially during chromatographic & evaporative step. Those vitamins are extremely light sensitive, to prevent destruction all sampling and analysis step was done under subdued light condition and also to prevent the losses of vitamins minimum amount of glass ware was used.

During my research two successive vitamin A procedures and vitamin D procedure was done to both butter sample and standard sample by using UFLC (ultra fast liquid chromatography). The results were obtained by compeering the area of standard solution chromatogram peak and the area of butter sample solution chromatogram peak.
The accurate peak for vitamin A and vitamin D can be obtained by using retention time of chromatogram. During my research always can be achieved same retention time for both butter sample and standard vitamin solution sample after doing several experiments.
By using that factor the results for vitamin A and vitamin D can be conformed. The standard curve for vitamin A and vitamin D were not prepared during my research. Because standard curve should be prepare by using correct vitamin standard solution sample. But those vitamins are fat soluble and not dissolve in many mobile phase solvent. That residue of the standard solution is affected to sensitive UFLC column. Also standard curve is prepared without saponification and extraction procedure steps. But sample is prepared with those two procedure steps. During saponification and extraction some vitamin losses can be happened. But according to vitamin A and vitamin D procedures both butter sample and standard solution was extracted according to same procedure. So the procedure error can be reduced by using those methods.

To get same retention time for both butter sample and vitamin standard sample both procedure have to be done within same time period. So the retention time is change day to

day conditions environment of lab. E.g. humidity, air flow rate. Also that is affected the mobile phase condition of at that time period.

At the beginning of research several procedures were tried to find suitable procedures for analysis vitamins samples. But most of the methods can't be done because of some chemical are very expensive, some chemicals are toxic, lack of some equipments.e.g. Also UFLC column is the reverse phase column, so the normal phase HPLC procedure can't be followed by using normal phase column.

The vitamins standard solution are very expensive. So that vitamins tablet are used as secondary standard. Vitamin A capsule is used for vitamin A standard, cod-liver oil is used as the vitamin D standard solution and vitamin E capsule is used as the vitamin E standard solution. But using UFLC instrument vitamins can be separately identified at their proper wave length. The standard solutions for vitamin analysis were some difficult. Because of vitamin standards were used in small amount for the experiment.

There is no any butter samples with single fat soluble vitamin is added. Because of vitamin A and D are added together with each other. Therefore the interference of other vitamins is affected to the vitamin analysis. The butter sample was obtained for recover test is difficult. The vitamins are automatically added in butter and margarine manufacturing process. The butter sample without any fat soluble vitamins is obtained for the recovery test for my research. But milk carotenoids very small amount is included in that sample.

According to the research vitamin D2 and vitamin D3 separately identification and α, β, γ, δ separate samples of vitamin E were not identified. Because of those vitamins standard couldn't be found. Carotenoids standard also can't be found. So identification of carotenoids in butter sample couldn't be done.

According to the vitamin A method 1 procedure successful chromatogram is obtained using three samples of butter (Appendix 01, Appendix 02, and Appendix 03,). The vitamin A standard chromatogram is obtained at the same retention time (Appendix 04). According to those chromatograms the peaks area values were obtained by using UFLC instrument.

According to the vitamin A method 2 procedure successful chromatogram was obtained using three samples of butter (Appendix 05, Appendix 06, and Appendix 07,). The vitamin A standard chromatogram is obtained at the same retention time (Appendix 08). According to those chromatograms the peaks area values were obtained by using UFLC instrument. During the vitamin A method 2 procedure huge emulsion is occurred. That emulsion was prevented by using ethanol sample. Because of the pyrogallol antioxidant the aqueous layer of separating mixture is separate clearly. So layers can be easily separated.

According to the vitamin D AOAC procedure successful chromatogram was obtained using three samples of butter (Appendix 09, Appendix10, and Appendix11). The vitamin A standard chromatogram is obtained at the same retention time (Appendix12). According to those chromatograms the peaks area values were obtained by using UFLC instrument.

Before analyze vitamin A and vitamin D using UFLC instrument should be purified very essential by using mobile phase which used to extract vitamin samples. Reverse phase UFLC describes methods that utilized a polar mobile phase in combination with nonpolar stationary phase. As state above, the non polar stationary phase structure more prefer to bind non polar structure. So polar mobile phase can be easily passed though that reverse phase column. Usually water, methanol, acetronitrile ...etc used for analysis of vitamins.

Table 4.1 Results of vitamin A & D in Butter Samples

Type of vitamin	sample type	Retention time	maximum Peak area
Vitamin A (Method 1)	1	2.855	213815
	2	2.858	212928
	3	2.863	218586
Vitamin A sandard		2.852	8100475
Vitamin A (Method 2)	1	2.880	230433
	2	2.844	235260
	3	2.847	233338
Vitamin A sandard		2.842	8519330
Vitamin D	1	1.012	2651984
	2	1.009	2921225
	3	1.010	3092879
Vitamin D standard		1.014	2165431

According to the above results the mass of vitamin A were calculated using calculation of vitamin A and D

Table 4.2 Results of sample testing

Type of vitamin	Mass of standard solution	Area of standard solution	mass of butter sample	mean Area of butter sample	Vitamin A in 100g of butter
Vitamin A Method 1	2 mg	8100475	5.590 g	215110	950 µg
Vitamin A Method 2	2 mg	8519330	5.698 g	233010	960 µg
Vitamin D Method 1	54.3 mg	2165431	4.5 mg	2888696	7.7 µg

Then the recover test for both vitamin A and vitamin D samples were done. By using UFLC instrument shape peak area chromatogram were obtained both vitamin A (Method 01) (Appendix13), (Method 02) (Appendix14) and vitamin A standard solutions. (Appendix15).

Table 4.3 Recovery Tests for Vitamin A& D in Butter Sample

Type of vitamin	Retention time	maximum Peak area
Vitamin A (Method 1)	2.726	644614
Vitamin A (Method 2)	2.718	396901
Vitamin A standard	2.701	758221
Vitamin D	1.007	1041508
Vitamin D standard	1.012	634097

The method for vitamin E analysis form butter sample by using reverse phase UFLC method was difficult. So that vitamin E was analyzed by using UV- visible spectrophotometer. In that experiment vitamin E standard series were prepared. According to procedure that should be prepared by using (3-0.3) g mass range of vitamin E samples are used. But by using analytical balance 0.3g of vitamin E can't be measured accurately. So that the standard series were prepared by using 1ppm solution of vitamin E. Then the absorbance of the those vitamin E concentrations were obtained

Table 4. 4 Absorbance of Vitamin E Standard Series

Sample number	Concentration of sample (ppm)	Absorbance of the sample
1	0.0200	0.006
2	0.0400	0.012
3	0.0600	0.015
4	0.0800	0.019
5	0.1000	0.025

Then standard curve were prepared by using spectrophotometer (Appendix16). By using that standard curve the vitamin E concentration of butter samples were measured. (Appendix17, Appendix18, Appendix19).

By using that concentration values according to procedure the mass of vitamin E in 100 g of butter were calculated.

Table 4. 5 Results of Vitamin E amount of Butter sample

Mean average concentration of samples (ppm)	The volume of ethanol dissolve sample extract	Dilution factor	Used butter mass (mg)	Mass of vitamin E 100g of butter(mg)
1.8400	25.00ml	20	5	18.4

Then the recover test for the vitamin E sample was done and got the concentration value for the sample. (Appendix 20) By using that the recovery mass of the sample was calculated.

Table 4. 6 Recovery results of Vitamin E

average concentration of samples (ppm)	The volume of ethanol dissolve sample extract	Dilution factor	Recovery mass of butter(mg)
1.217	25.00 ml	100	3.4771

By using the recovery mass of the samples the recovery percentage of the samples were calculated.

Table 4.7 Results of recovery tests for Vitamin A. D. E. sample

Sample	Vitamin A (Method 1)	Vitamin A (Method 2)	Vitamin D	Vitamin E
Amount of vitamin add to sample	3.8	2.3	0.0266	3.5
Amount of vitamin extract from sample	3.4	2.09	0.0235	3.04
Percentage loss	10.5%	10.2%	11.8%	13.2%
Recovery percentage	89.5%	89.8%	88.2%	86.8%

According to above procedure fat soluble vitamins of other samples were analyzed and get accurate results for those samples. By using fortify milk sample fat soluble vitamins were analyzed.

Chapter 05

5.1 Conclusion

Extraction method was developed according to the facilities that SGS laboratory does have. Results indicated that, qualitatively and quantitatively satisfied for vitamins levels. Recovery percentage was 89.5% for vitamin and 89.8% for vitamin A2. The recovery percentage was 88.2% for vitamin D and 87.5% for vitamin E. Therefore the recovery was around 85% to 90%. This lost may be occurred due to actinic light, heat and the Oxygen in the atmosphere. So the extraction should have been done more carefully.

In the research vitamin A, D, E are contained butter sample were used. 1000μg Of vitamin A was included 100g of that butter sample. According to method 1 procedure 950 μg of vitamin A can be extracted from that butter sample. Also by using vitamin a 2 nd method 960 µg of vitamin A can be extracted. To extracted this amount of vitamin method 1 and method 2 procedure had to be done several times. By that way the personal error and other errors can be reduced. The 2 method of vitamin D analysis was easier than the 1 method .because the extraction procedure of 2 method esier than 1st method.

According to the results of some samples contain exceeded amount of vitamin A and some contain lower amount of vitamin A, than the labeled amount. Vitamin A is usually fortified to the butter and it must be careful, as some formulas contained excess amount of vitamin A than the recommended intake. It will usually affect our health directly.

It is better if we can prepare samples freshly before injecting to the HPLC. Because according to the results obtained in the recovery test the percentage loss was around (10-12) %. That occurred mainly due to the sensitivity of vitamin A to actinic light, heat and Oxygen. As the extracted samples were kept in the refrigerator the vitamin A might have been degradated in to a more stable product.

When analysis of vitamin D, the chromatogram peak area for the vitamin A standard solution was obtained lower than the area of vitamin D test sample because of the obtained butter sample is contained higher amount of vitamin D amount than the obtained cod- liver oil sample.

According to the vitamin E standard curve, the 0.9883 of R^2 (The Pearson Coefficient of Determination) values can be obtained. So accuracy of the standard curve results is good and by using that curve can be achieved accurate results for the samples. By using that curve Recovery of the procedure can be obtained up to 86.8 %. The test butter sample was contained 8mg of vitamin E in 100g of butter. By using above standard curve 7.7 mg of vitamin E can be extracted.

During the testing time period protect of testing samples from light, heat and air is very essential. So that most of the equipments had to be used with covering aluminum foil. Also to reduce human errors all procedure had to be done many times.

5.2 Recommendation

- ✓ The results would be more accurate if the samples were prepared freshly before injecting to the HPLC.
- ✓ It's better if we can separately recognize the isomers of vitamin A and degradated products of vitamin A separately from the pure vitamin A.
- ✓ It's better to use a fresh standard vitamin A solution. Because the vitamin A will usually degraded in to more stable products easily.
- ✓ It's better if vitamin D can be extracted by using separate vitamin D2 and D3 standard samples
- ✓ Also it is better vitamin E sample is analyzed as α, β, γ, δ tocopherols by using separate standard solution
- ✓ It is better if carotenoids can be also extracted with vitamin a sample.

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Appendix 01

(Chromatogram for vitamin A in butter sample 1 using Method 1)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Butter sample 4(05-02-09).lcd

Acquired by	: Admin
Sample Name	: Butter sample 4
Sample ID	: Butter sample 4
Tray#	: 1L
Vail #	: 7
Injection Volume	: 20 uL
Data File Name	: Butter sample 4(05-02-09).lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/5/2009 4:26:27 PM
Data Processed	: 2/5/2009 4:36:17 PM

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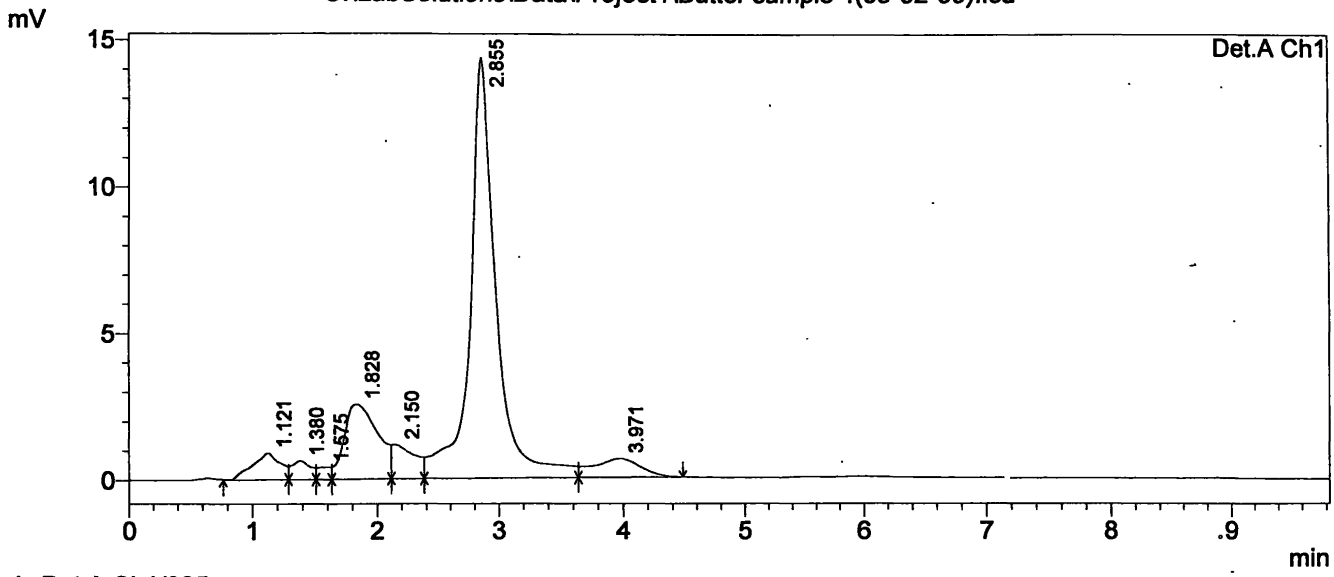
C:\LabSolutions\Data\Project1\Butter sample 4(05-02-09).lcd



1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.121	14553	920	4.576	4.447
2	1.380	6674	641	2.098	3.102
3	1.575	3129	426	0.984	2.058
4	1.828	48362	2550	15.205	12.328
5	2.150	14919	1165	4.691	5.631
6	2.855	213815	14342	67.223	69.346
7	3.971	16615	639	5.224	3.089
Total		318066	20682	100.000	100.000

(Chromatogram for vitamin A in butter sample 2 using Method 1)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Butter sample 3(05-02-09).lcd

Acquired by	: Admin
Sample Name	: Butter sample 3
Sample ID	: Butter sample 3
Tray#	: 1L
Vail #	: 7
Injection Volume	: 20 uL
Data File Name	: Butter sample 3(05-02-09).lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
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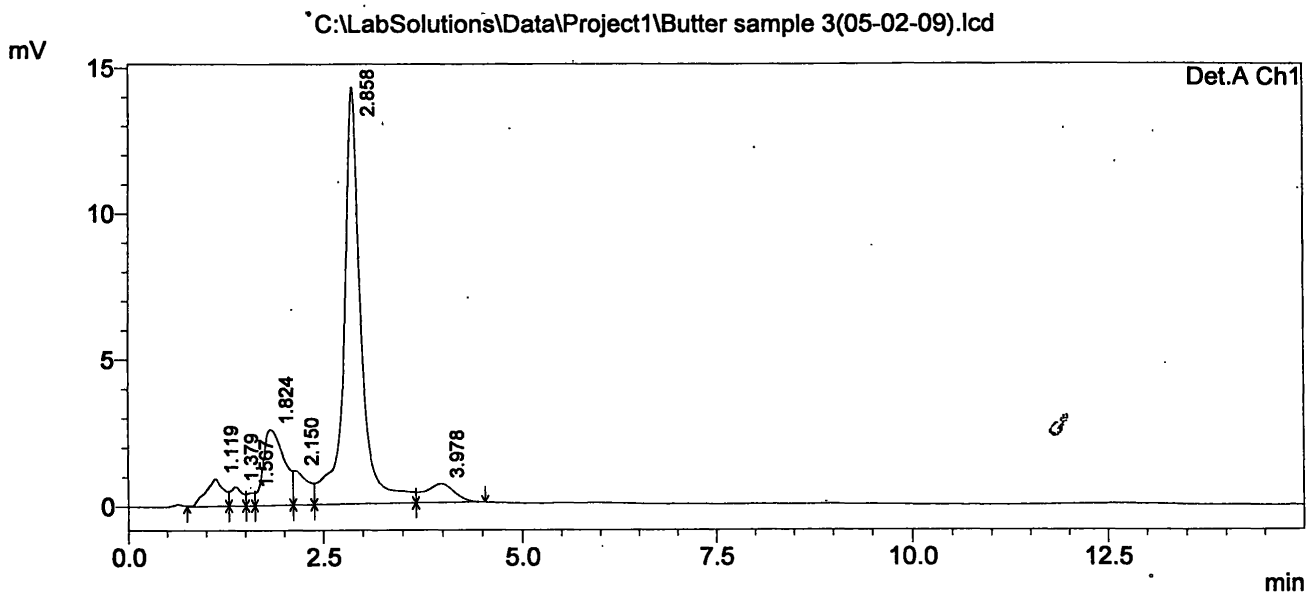

1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.119	14996	934	4.715	4.517
2	1.379	6853	656	2.155	3.176
3	1.567	3004	441	0.944	2.135
4	1.824	48961	2569	15.395	12.429
5	2.150	14952	1162	4.701	5.622
6	2.858	212928	14260	66.950	68.998
7	3.978	16345	645	5.139	3.123
Total		318039	20667	100.000	100.000

(Chromatogram for vitamin A in butter sample 3 using Method 1)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Butter sample 2(05-02-09).lcd

Acquired by	: Admin
Sample Name	: Butter sample 2
Sample ID	: Butter sample 2
Tray#	: 1L
Vail #	: 6
Injection Volume	: 20 uL
Data File Name	: Butter sample 2(05-02-09).lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/5/2009 3:52:10 PM
Data Processed	: 2/5/2009 4:07:14 PM

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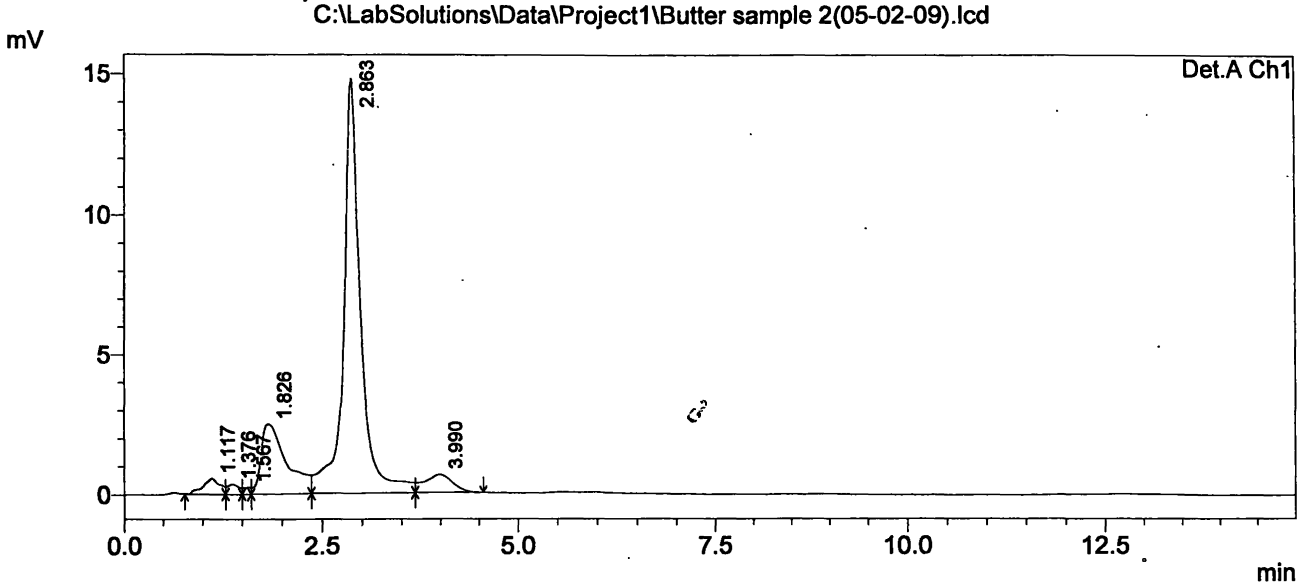

1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.117	8804	573	2.887	2.995
2	1.376	3727	366	1.222	1.914
3	1.567	1675	249	0.549	1.301
4	1.826	55955	2486	18.346	12.996
5	2.863	218586	14813	71.668	77.438
6	3.990	16250	642	5.328	3.357
Total		304997	19129	100.000	100.000

(Chromatogram for vitamin A standard solution using Method 1)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Vitamin A standard(05-02-09).lcd

Acquired by	: Admin
Sample Name	: Vitamin A standard
Sample ID	: Vitamin A standard
Tray#	: 1L
Vail #	: 8
Injection Volume	: 20 uL
Data File Name	: Vitamin A standard(05-02-09).lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
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Data Processed	: 2/5/2009 4:47:00 PM

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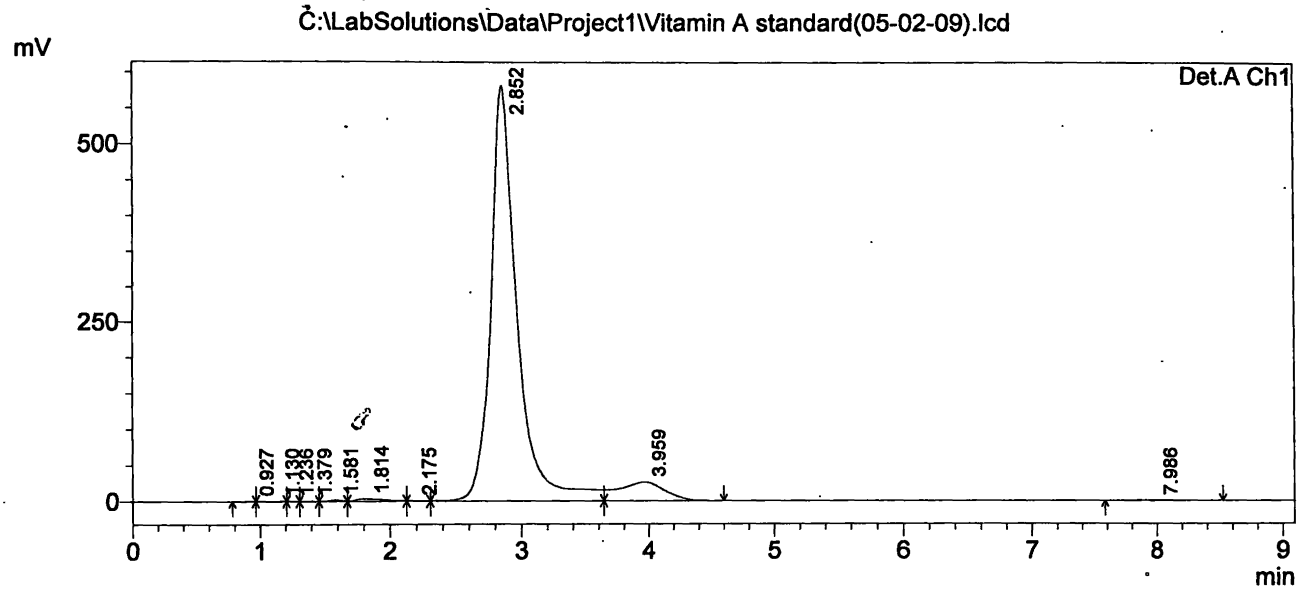

1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.927	1304	201	0.015	0.033
2	1.130	5774	539	0.065	0.087
3	1.236	2474	445	0.028	0.072
4	1.379	3645	462	0.041	0.075
5	1.581	14925	1932	0.168	0.314
6	1.814	54107	3387	0.610	0.550
7	2.175	8880	866	0.100	0.141
8	2.852	8100475	582032	91.371	94.468
9	3.959	668267	26036	7.538	4.226
10	7.986	5637	214	0.064	0.035
Total		8865488	616115	100.000	100.000

Appendix 05

(Chromatogram for vitamin A in butter sample 1 using Method 2)

C:\LabSolutions\Data\Project1\Vitamin A butter (11-02-09)-2.lcd

Acquired by	: Admin
Sample Name	: Vitamin A butter 11-02-09
Sample ID	: Vitamin A butter 11-02-09
Tray#	: 1L
Vail #	: 2
Injection Volume	: 20 uL
Data File Name	: Vitamin A butter (11-02-09)-2.lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/11/2009 3:28:13 PM
Data Processed	: 2/11/2009 3:37:00 PM

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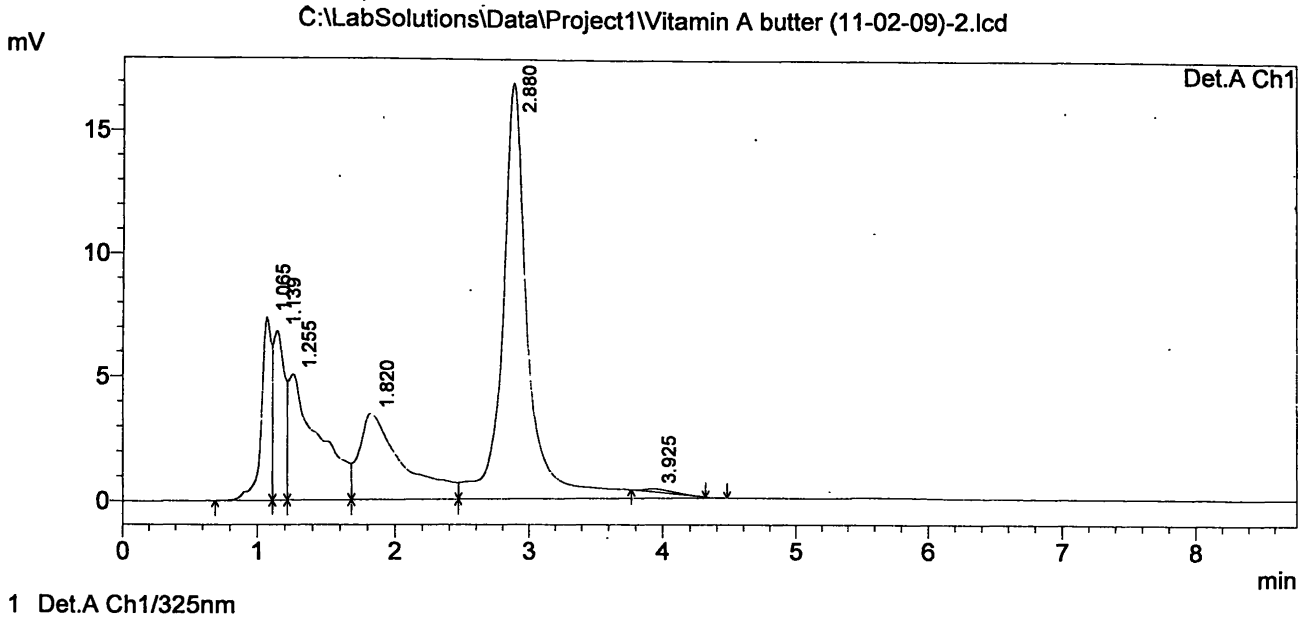

1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.065	38879	7383	8.380	18.569
2	1.139	38527	6831	8.304	17.181
3	1.255	78242	5062	16.863	12.731
4	1.820	75972	3460	16.374	8.703
5	2.880	230433	16902	49.665	42.506
6	3.925	1924	124	0.415	0.311
Total		463977	39763	100.000	100.000

Appendix 06

(Chromatogram for vitamin A in butter sample 2 using Method 2)

C:\LabSolutions\Data\Project1\Vitamin A Butter sample(11-02-09)-6.lcd

Acquired by	: Admin
Sample Name	: Vitamin A Butter sample(11-02-09)-5
Sample ID	: Vitamin A Butter sample(11-02-
Tray#	: 1L
Vail #	: 2
Injection Volume	: 20 uL
Data File Name	: Vitamin A Butter sample(11-02-09)-6.lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/11/2009 4:02:15 PM
Data Processed	: 2/11/2009 4:15:40 PM

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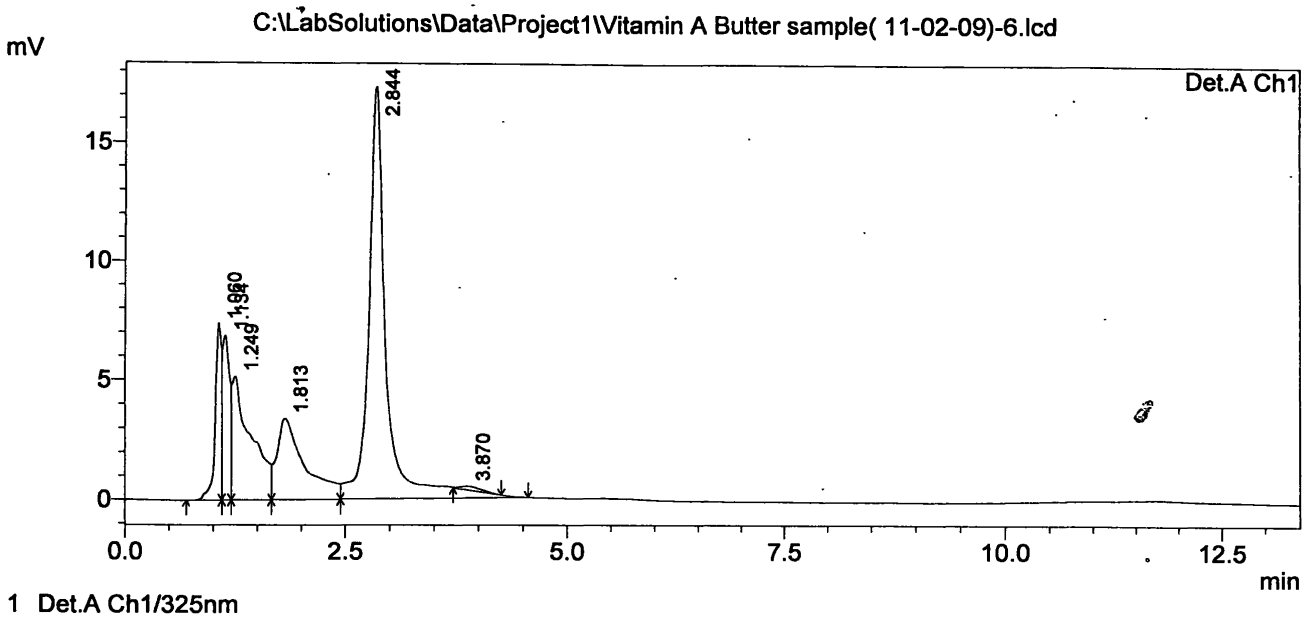

1 Det.A Ch1/325nm

PeakTable

Detector A.Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.060	36530	7404	7.826	18.369
2	1.134	39059	6877	8.368	17.062
3	1.249	78506	5150	16.819	12.778
4	1.813	74547	3392	15.971	8.417
5	2.844	235260	17318	50.401	42.968
6	3.870	2874	164	0.616	0.406
Total		466777	40305	100.000	100.000

(Chromatogram for vitamin A in butter sample 3 using Method 2)

C:\LabSolutions\Data\Project1\Vitamin A Butter sample(11-02-09)-4.lcd

Acquired by	: Admin
Sample Name	: Vitamin A Butter sample(11-02-09)-3
Sample ID	: Vitamin A Butter sample(11-02-
Tray#	: 1L
Vail #	: 2
Injection Volume	: 20 uL
Data File Name	: Vitamin A Butter sample(11-02-09)-4.lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/11/2009 3:49:25 PM
Data Processed	: 2/11/2009 4:00:15 PM

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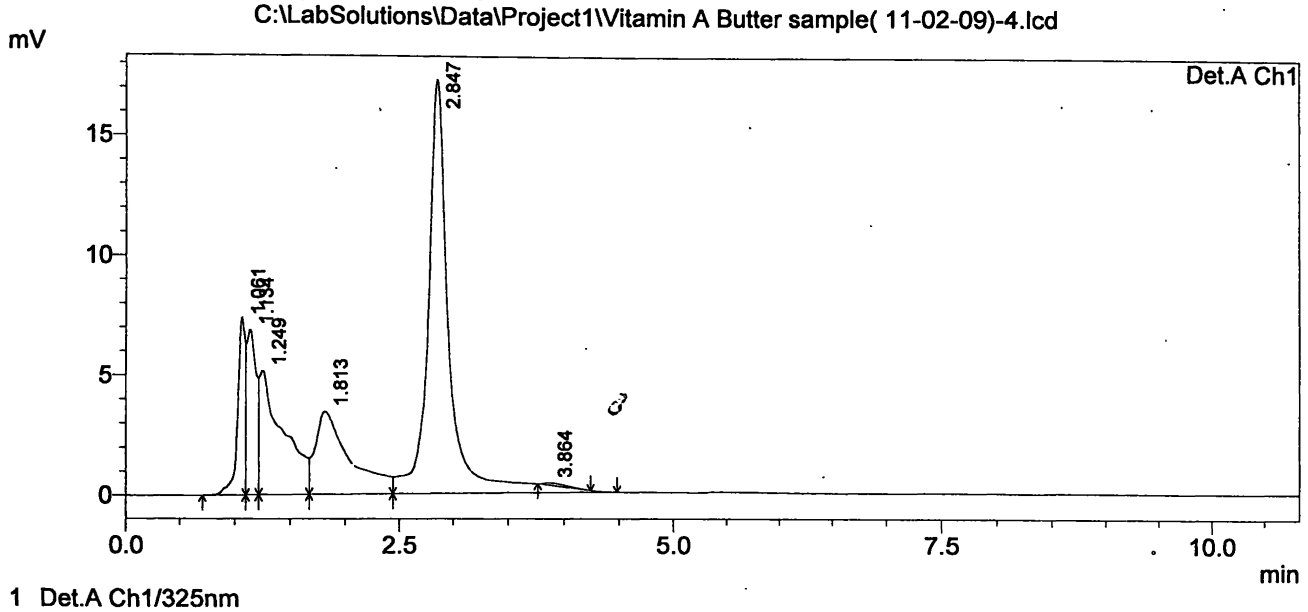

1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.061	36271	7393	7.784	18.383
2	1.134	41599	6876	8.928	17.096
3	1.249	77454	5151	16.622	12.808
4	1.813	75795	3444	16.266	8.562
5	2.847	233338	17263	50.077	42.921
6	3.864	1504	92	0.323	0.230
Total		465961	40219	100.000	100.000

Appendix 08

(Chromatogram for vitamin A standard solution using Method 2)

C:\LabSolutions\Data\Project1\Vitamin A std 11-02-10.lcd

Acquired by	: Admin
Sample Name	: Vitamin A std 11-02-09
Sample ID	: Vitamin A std 11-02-09
Tray#	: 1L
Vail #	: 3
Injection Volume	: 20 uL
Data File Name	: Vitamin A std 11-02-10.lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
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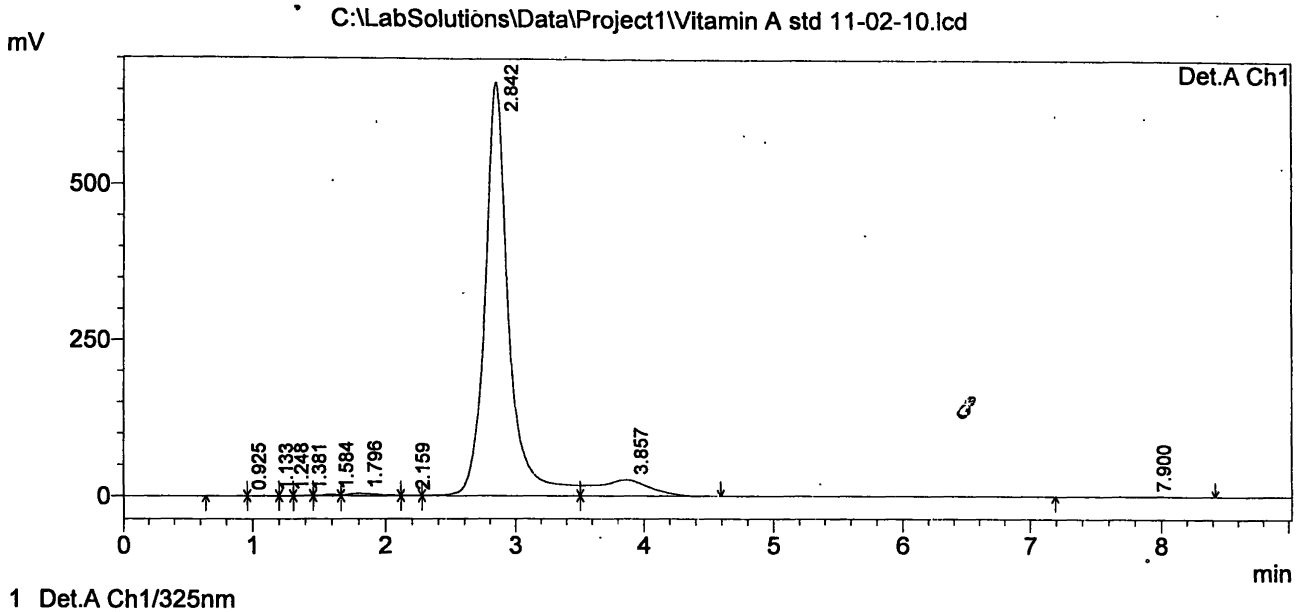

1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.925	1553	235	0.017	0.034
2	1.133	5769	511	0.062	0.073
3	1.248	2898	485	0.031	0.069
4	1.381	4231	551	0.045	0.079
5	1.584	16953	2387	0.181	0.342
6	1.796	60271	3755	0.644	0.537
7	2.159	9784	1089	0.104	0.156
8	2.842	8519330	663827	90.968	94.981
9	3.857	736861	25814	7.868	3.694
10	7.900	7560	251	0.081	0.036
Total		9365210	698905	100.000	100.000

(Chromatogram for vitamin D in butter sample 1 using AOAC method)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Vitamin D Butter sample(12-02-09)-3.lcd

Acquired by	: Admin
Sample Name	: Vitamin D Butter sample(12-02-09)-1
Sample ID	: Vitamin D Butter sample(12-02-
Tray#	: 1L
Vail #	: 2
Injection Volume	: 20 uL
Data File Name	: Vitamin D Butter sample(12-02-09)-3.lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/12/2009 4:01:57 PM
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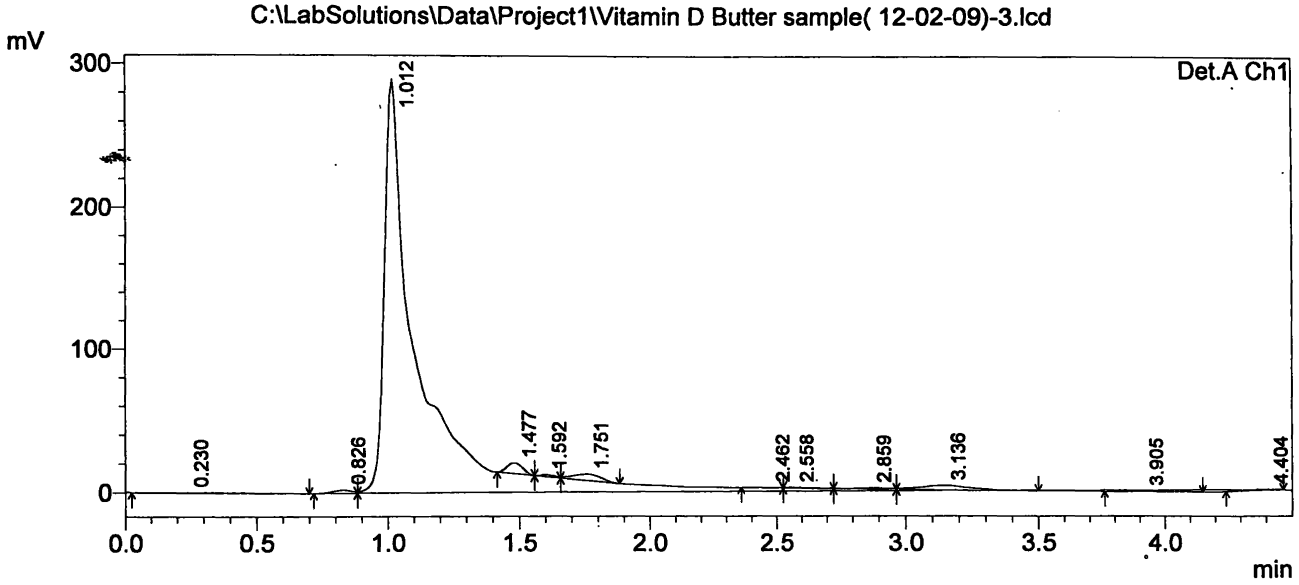

1 Det.A Ch1/254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.230	10386	447	0.369	0.143
2	0.826	11831	2285	0.420	0.732
3	1.012	2651984	289933	94.255	92.906
4	1.477	30425	7526	1.081	2.412
5	1.592	5520	1204	0.196	0.386
6	1.751	30852	4347	1.097	1.393
7	2.462	4031	588	0.143	0.189
8	2.558	6088	674	0.216	0.216
9	2.859	9872	797	0.351	0.255
10	3.136	40958	3024	1.456	0.969
11	3.905	5235	456	0.186	0.146
12	4.404	6449	790	0.229	0.253
Total		2813630	312071	100.000	100.000

(Chromatogram for vitamin A in butter sample 2 using AOAC method)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Vitamin D Butter sample(12-02-09)-4.lcd

Acquired by	: Admin
Sample Name	: Vitamin D Butter sample(12-02-09)-2
Sample ID	: Vitamin D Butter sample(12-02-
Tray#	: 1L
Vail #	: 2
Injection Volume	: 20 uL
Data File Name	: Vitamin D Butter sample(12-02-09)-4.lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/12/2009 4:07:26 PM
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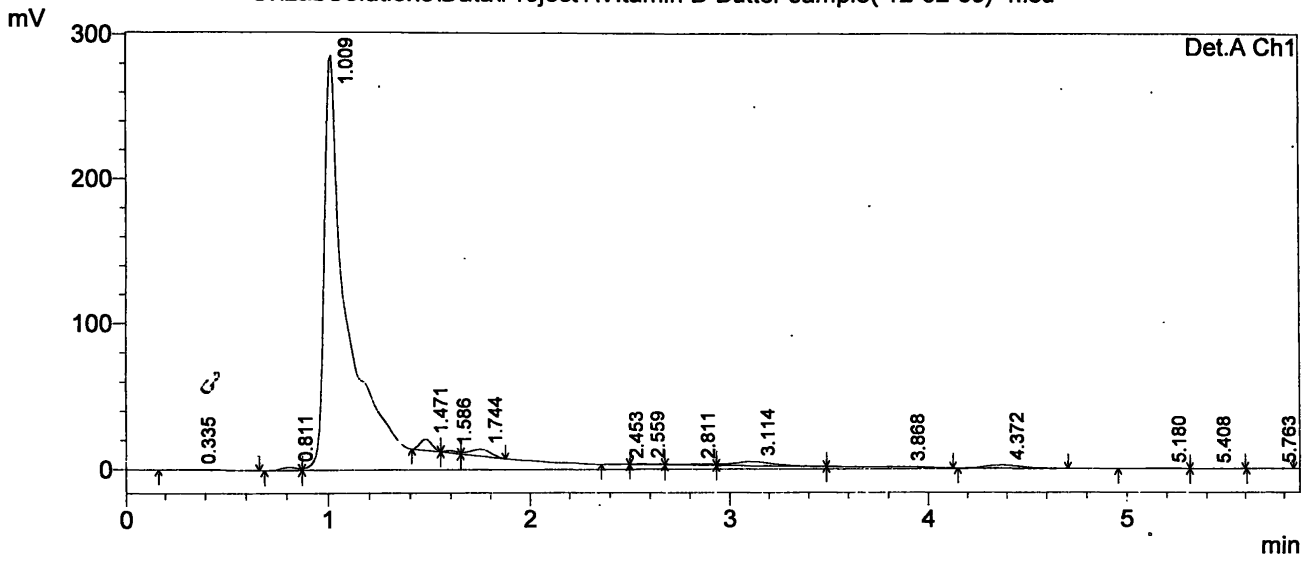

1 Det.A Ch1/254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.335	6477	423	0.208	0.136
2	0.811	11824	2205	0.379	0.710
3	1.009	2921225	286074	93.638	92.112
4	1.471	31659	7665	1.015	2.468
5	1.586	6632	1384	0.213	0.446
6	1.744	33086	4550	1.061	1.465
7	2.453	2681	462	0.086	0.149
8	2.559	6474	683	0.208	0.220
9	2.811	13526	930	0.434	0.299
10	3.114	43611	3032	1.398	0.976
11	3.868	7436	491	0.238	0.158
12	4.372	28015	2080	0.898	0.670
13	5.180	3618	248	0.116	0.080
14	5.408	2133	188	0.068	0.061
15	5.763	1309	156	0.042	0.050
Total		3119705	310571	100.000	100.000

(Chromatogram for vitamin A in butter sample 3 using AOAC method)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Vitamin D Butter sample(12-02-09)-5.lcd

Acquired by	: Admin
Sample Name	: Vitamin D Butter sample(12-02-09)-3
Sample ID	: Vitamin D Butter sample(12-02-
Tray#	: 1L
Vail #	: 2
Injection Volume	: 20 uL
Data File Name	: Vitamin D Butter sample(12-02-09)-5.lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/12/2009 4:14:23 PM
Data Processed	: 2/12/2009 4:29:25 PM

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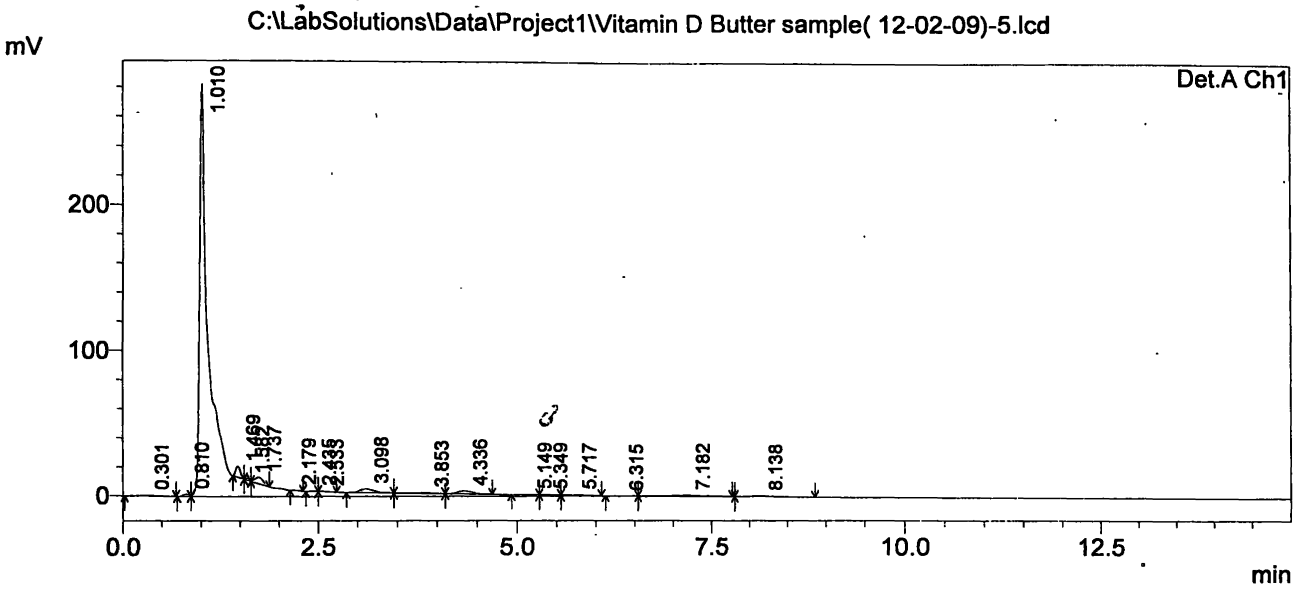


1 Det.A Ch1/254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.301	10505	504	0.314	0.164
2	0.810	8891	1614	0.266	0.524
3	1.010	3092879	282614	92.421	91.769
4	1.469	30974	7567	0.926	2.457
5	1.582	5791	1225	0.173	0.398
6	1.737	32660	4433	0.976	1.439
7	2.179	1294	211	0.039	0.069
8	2.435	3433	502	0.103	0.163
9	2.533	3998	603	0.119	0.196
10	3.098	35193	2690	1.052	0.874
11	3.853	14051	731	0.420	0.237
12	4.336	30460	2156	0.910	0.700
13	5.149	4638	305	0.139	0.099
14	5.349	5108	359	0.153	0.116
15	5.717	9299	531	0.278	0.172
16	6.315	3212	176	0.096	0.057
17	7.182	33435	909	0.999	0.295
18	8.138	20689	833	0.618	0.270
Total		3346511	307963	100.000	100.000

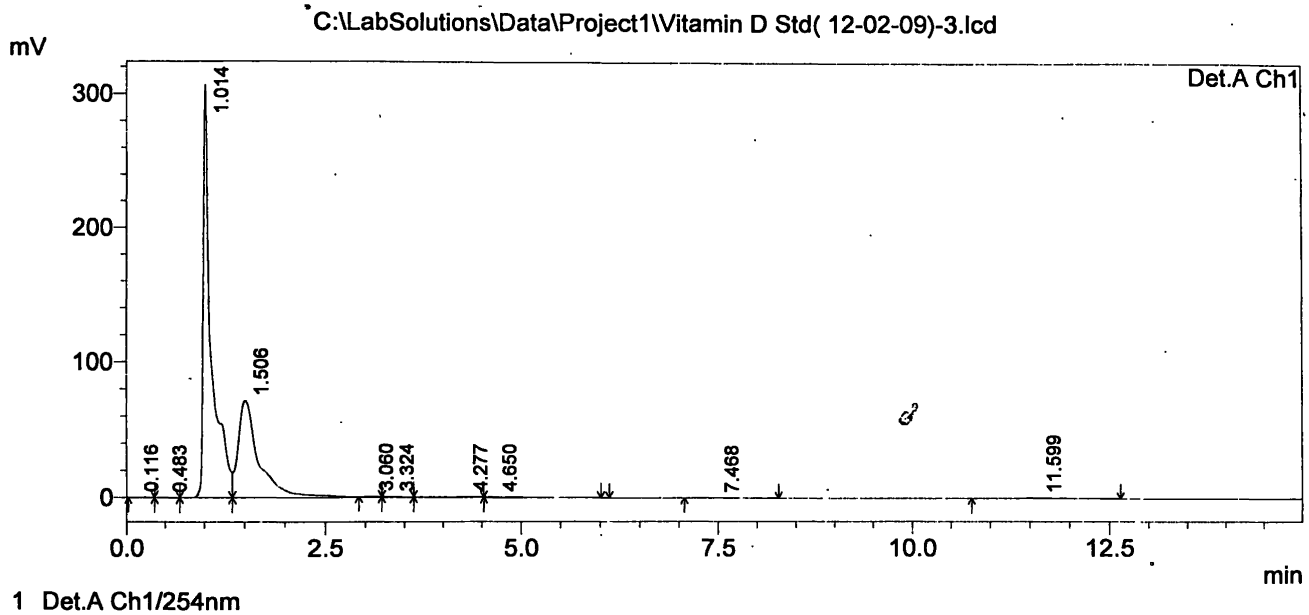
(Chromatogram for vitamin A standard solution using AOAC method)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Vitamin D Std(12-02-09)-3.lcd

Acquired by	: Admin
Sample Name	: Vitamin D Std(12-02-09)-2
Sample ID	: Vitamin D Std(12-02-09)-2
Tray#	: 1L
Vail #	: 3
Injection Volume	: 20 uL
Data File Name	: Vitamin D Std(12-02-09)-3.lcd
Method File Name	: vitamin A trial 1.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/12/2009 4:40:53 PM
Data Processed	: 2/12/2009 4:55:55 PM

<Chromatogram>



1 Det.A Ch1/254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.116	2223	143	0.062	0.038
2	0.483	1425	115	0.040	0.030
3	1.014	2165431	306665	60.128	80.518
4	1.506	1372765	71696	38.118	18.824
5	3.060	3631	339	0.101	0.089
6	3.324	4003	261	0.111	0.069
7	4.277	5598	336	0.155	0.088
8	4.650	11900	347	0.330	0.091
9	7.468	12997	409	0.361	0.107
10	11.599	21421	555	0.595	0.146
Total		3601395	380867	100.000	100.000

Appendix 13

(Chromatogram for vitamin A recovery using Method 1)

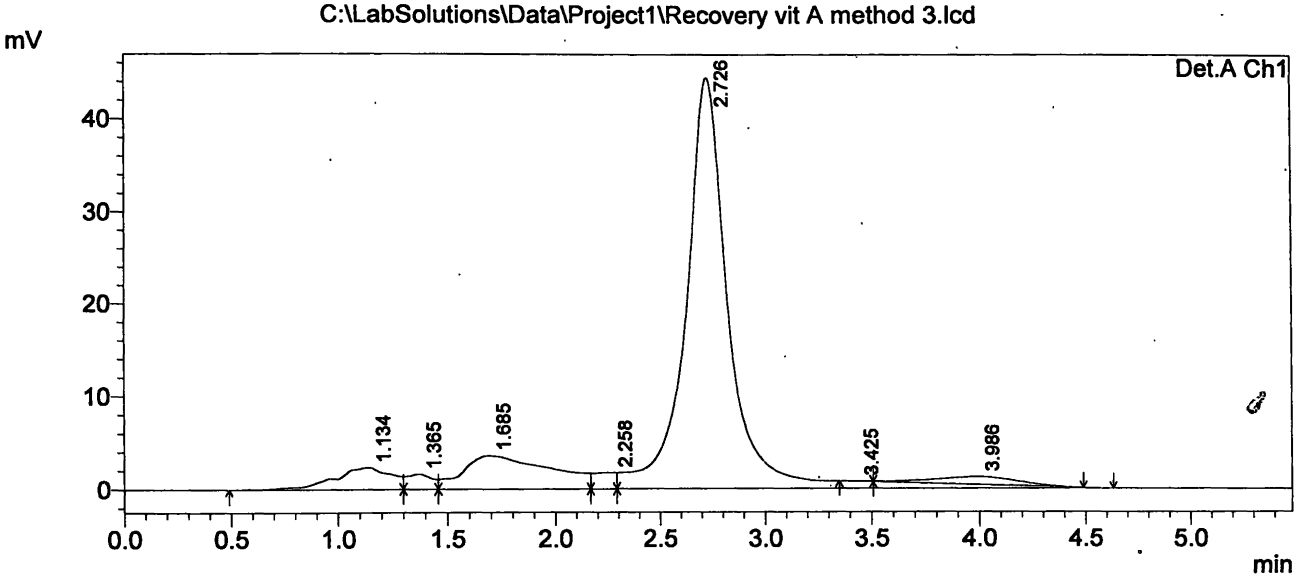
==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Recovery vit A method 3.lcd

Acquired by	: Admin
Sample Name	: Recovery method 2
Sample ID	: Recovery method 2
Tray#	: 1L
Vail #	: 4
Injection Volume	: 20 uL
Data File Name	: Recovery vit A method 3.lcd
Method File Name	: Vitamin A Analysis method.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/16/2009 4:19:21 PM
Data Processed	: 2/16/2009 4:49:33 PM

<Chromatogram>

C:\LabSolutions\Data\Project1\Recovery vit A method 3.lcd



1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.134	44023	2364	5.246	4.326
2	1.365	13120	1633	1.563	2.989
3	1.685	100869	3570	12.019	6.534
4	2.258	12919	1745	1.539	3.194
5	2.726	644614	44422	76.810	81.299
6	3.425	466	51	0.055	0.094
7	3.986	23217	855	2.767	1.564
Total		839227	54640	100.000	100.000

(Chromatogram for vitamin A recovery using Method 2)

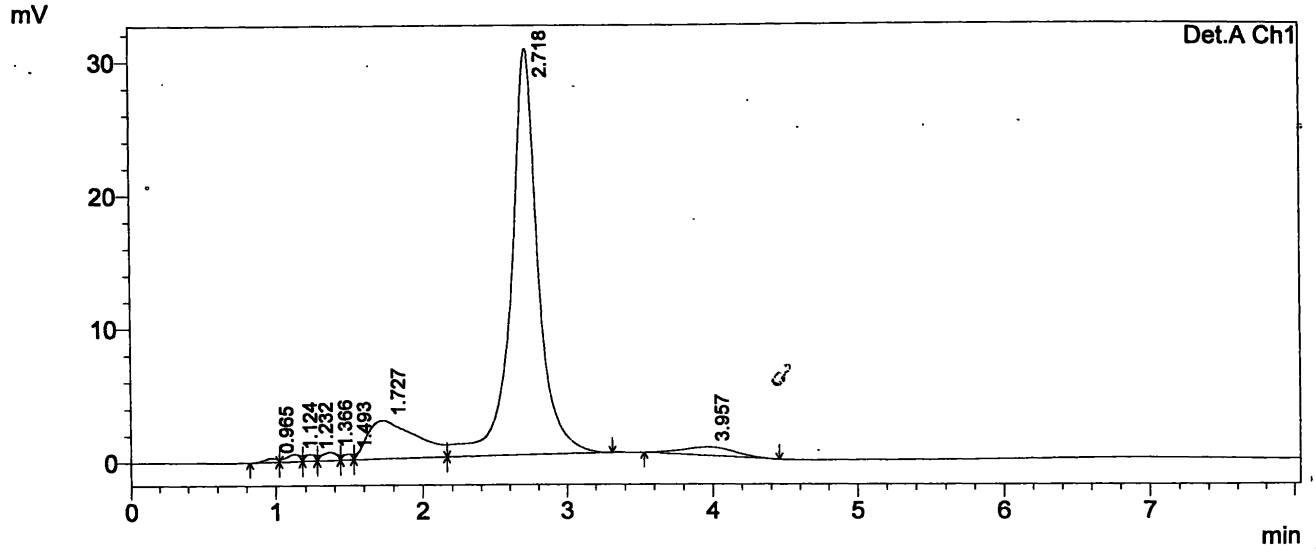
==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Recovery method 2.lcd

Acquired by	: Admin
Sample Name	: Recovery method 1
Sample ID	: Recovery method 1
Tray#	: 1L
Vail #	: 3
Injection Volume	: 20 uL
Data File Name	: Recovery method 2.lcd
Method File Name	: Vitamin A Analysis method.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/16/2009 4:10:17 PM
Data Processed	: 2/16/2009 4:25:05 PM

<Chromatogram>

C:\LabSolutions\Data\Project1\Recovery method 2.lcd



1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.965	1999	304	0.403	0.836
2	1.124	3994	556	0.806	1.529
3	1.232	2758	506	0.556	1.391
4	1.366	4716	633	0.951	1.740
5	1.493	2200	443	0.444	1.217
6	1.727	67471	2863	13.607	7.867
7	2.718	396901	30441	80.044	83.656
8	3.957	15814	642	3.189	1.764
Total		495854	36389	100.000	100.000

(recovery vitamin A standard)

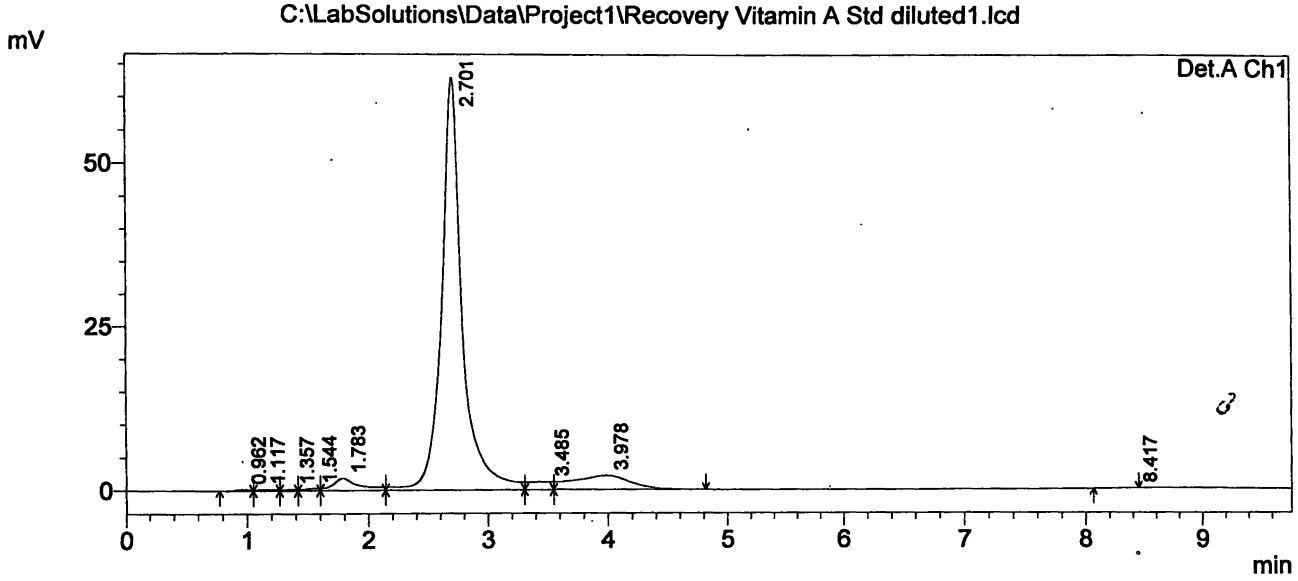
==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Recovery Vitamin A Std diluted1.lcd

Acquired by	: Admin
Sample Name	: Recovery Vitamin A Std diluted
Sample ID	: Recovery Vitamin A Std diluted
Tray#	: 1L
Vail #	: 2
Injection Volume	: 20 uL
Data File Name	: Recovery Vitamin A Std diluted1.lcd
Method File Name	: Vitamin A Analysis method.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/16/2009 3:59:07 PM
Data Processed	: 2/16/2009 4:13:35 PM

<Chromatogram>

C:\LabSolutions\Data\Project1\Recovery Vitamin A Std diluted1.lcd



1 Det.A Ch1/325nm

PeakTable

Detector A Ch1 325nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.962	2141	233	0.242	0.337
2	1.117	2538	239	0.287	0.345
3	1.357	1745	214	0.197	0.309
4	1.544	3146	364	0.355	0.526
5	1.783	28198	1864	3.186	2.690
6	2.701	758221	62986	85.663	90.925
7	3.485	17186	1204	1.942	1.739
8	3.978	71801	2159	8.112	3.116
9	8.417	142	9	0.016	0.013
Total		885117	69273	100.000	100.000

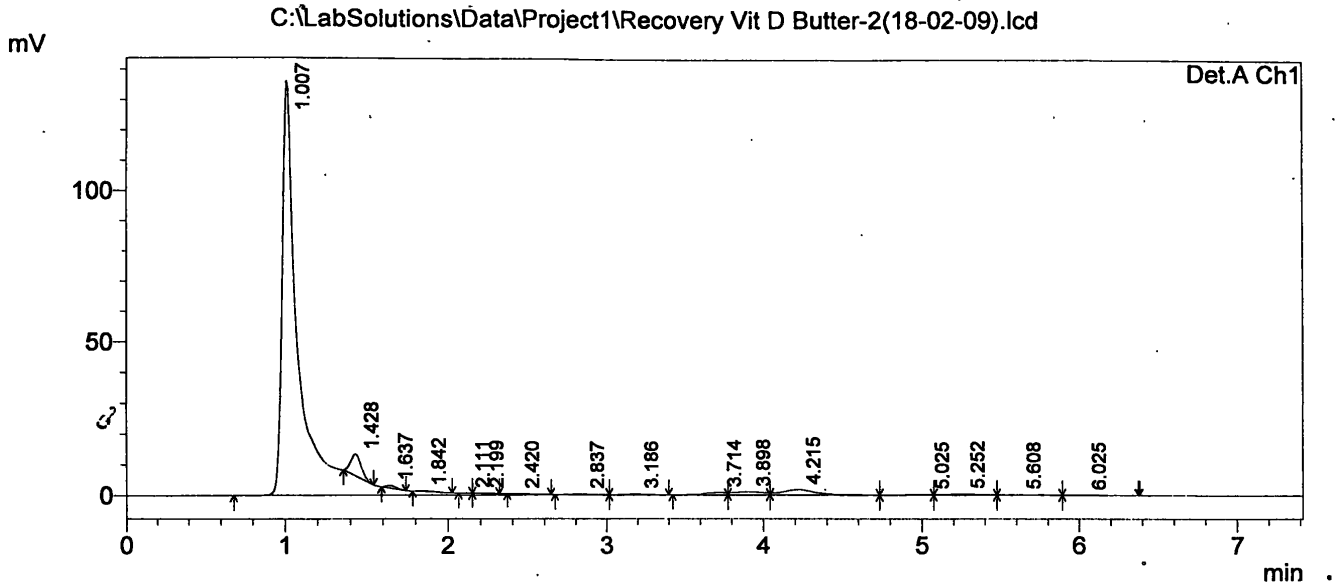
(recovery vitamin D sample)

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Recovery Vit D Butter-2(18-02-09).lcd

Acquired by	: Admin
Sample Name	: Recovery Vit D Butter-2(18-02-09)
Sample ID	: Recovery Vit D Butter-2(18-02-0
Tray#	: 1L
Vail #	: 3
Injection Volume	: 20 uL
Data File Name	: Recovery Vit D Butter-2(18-02-09).lcd
Method File Name	: Vitamin A Analysis method.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/18/2009 3:50:40 PM
Data Processed	: 2/18/2009 4:06:39 PM

<Chromatogram>



1 Det.A Ch1/254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.007	1041508	136013	90.392	90.950
2	1.428	31247	7059	2.712	4.720
3	1.637	3101	761	0.269	0.509
4	1.842	2276	297	0.198	0.198
5	2.111	249	63	0.022	0.042
6	2.199	770	149	0.067	0.100
7	2.420	480	56	0.042	0.037
8	2.837	2677	289	0.232	0.193
9	3.186	2573	285	0.223	0.190
10	3.714	7653	747	0.664	0.499
11	3.898	13438	1021	1.166	0.682
12	4.215	26863	1728	2.331	1.156
13	5.025	3262	234	0.283	0.156
14	5.252	7730	412	0.671	0.275
15	5.608	5321	244	0.462	0.163
16	6.025	3071	191	0.267	0.128
Total		1152219	149546	100.000	100.000

(recovery vitamin D standard)

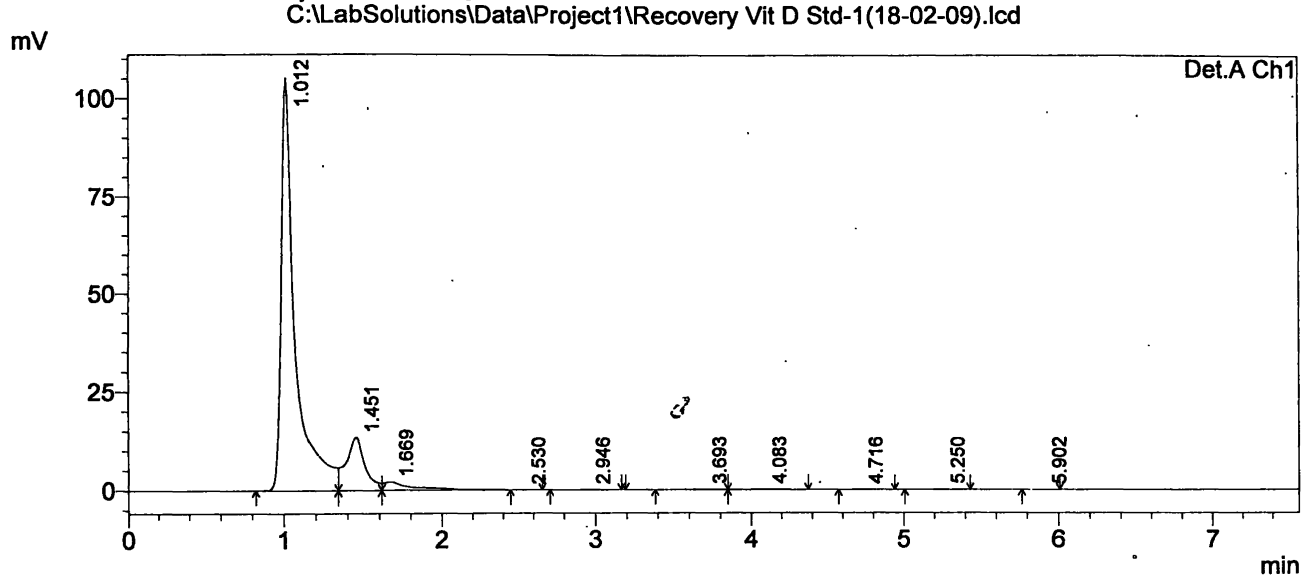
==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\Recovery Vit D Std-1(18-02-09).lcd

Acquired by	: Admin
Sample Name	: Recovery Vit D Std-1(18-02-09)
Sample ID	: Recovery Vit D Std-1(18-02-09)
Tray#	: 1L
Vail #	: 2
Injection Volume	: 20 uL
Data File Name	: Recovery Vit D Std-1(18-02-09).lcd
Method File Name	: Vitamin A Analysis method.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 2/18/2009 3:19:54 PM
Data Processed	: 2/18/2009 3:42:35 PM

<Chromatogram>

C:\LabSolutions\Data\Project1\Recovery Vit D Std-1(18-02-09).lcd



1 Det.A Ch1/254nm

PeakTable

Detector A Ch1 254nm

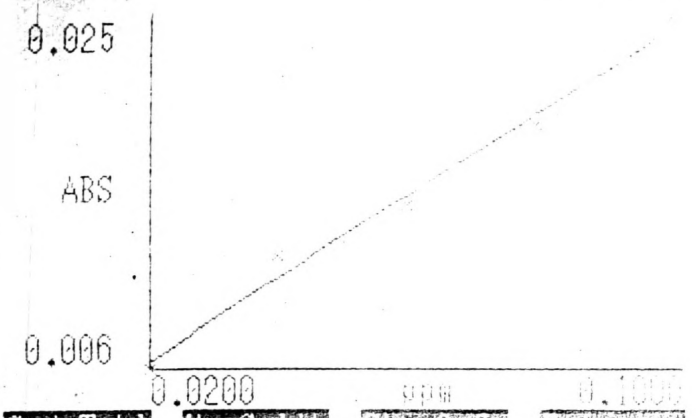
Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.012	634097	105247	81.240	86.768
2	1.451	109737	13408	14.059	11.054
3	1.669	29996	2117	3.843	1.745
4	2.530	177	26	0.023	0.022
5	2.946	733	74	0.094	0.061
6	3.693	1980	148	0.254	0.122
7	4.083	2438	156	0.312	0.128
8	4.716	595	55	0.076	0.045
9	5.250	654	51	0.084	0.042
10	5.902	119	16	0.015	0.013
Total		780526	121298	100.000	100.000

Appendix 18

(recovery vitamin E standard curve)

15/Mar/11 09:41:47

CalCurve



Std.Tabl NewCalib

15/Mar/11 09:42:32

Std.Tabl

470.0nm 0.01454

No.	Conc.	ABS	No.	ABS
1	0.0200	0.006		
2	0.0400	0.012		
3	0.0600	0.015		
4	0.0800	0.019		
5	0.1000	0.025		

CalCurve Change Delete

15/Mar/11 09:43:14

Calib. curve equation

$ABS = K3C^3 + K2C^2 + K1C + K0$

K3 = 0.0000

K2 = 0.0000

K1 = 0.2301

K0 = 0.0015

r^2 = 0.9883

(concentration in vitamin E sample 1)

22/Mar/11 12:

Quantitation

470.0nm= 0.4464

Smpl No.	A B S	Conc.(ppm)
1 - 1	0.4464	1.9335
1 - 2	0.4464	1.9335
1 - 3	0.4464	1.9335
1 - m	0.4464	1.9335
2 - 1		

Smpl No. DataFile

Appendix 20

(concentration in vitamin E sample 2)

23/Mar/11 11:47:

Quantitation 470.0nm 0.40672

Smpl No.	A B S	Conc. (ppm)
1 - 1	0.4071	1.7627
1 - 2	0.4066	1.7606
1 - 3	0.4067	1.7611
1 - m	0.4068	1.7615
2 - 1		

Smpl No. DataFile

Appendix 21

(concentration in vitamin E sample 3)

22/Mar/11 10:50:58

Quantitation

d7fi,0nm= 0.0048

Smpl No.	A B S	Conc.(ppm)
1 - 1	0.4214	1.8248
1 - 2	0.4214	1.8248
1 - 3	0.4214	1.8248
1 - m	0.4214	1.8248
2 - 1		

Smpl No. DataFile DataList

Apendix 22

(recovery vitamin E concentration

22/Mar/11 14:10:18

Quantitation	470.0 nm	0.0090A

Sample No.	A B S	Conc. (ppm)
2-1	0.9766	4.2375
2-2	0.9766	4.2375
2-3	0.9766	4.2375
2-m	0.9766	4.2375
3-1	0.2598	1.1224
3-2	0.2596	1.1219
3-3	0.2594	1.1208
3-m	0.2590	1.1217

Smpl No. | Data File | Data Disp | Equation

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