DEVELOPMENT OF A METHOD TO DETERMINE VITAMIN A CONTENT IN INFANT FORMULAS

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02/AS/A/001

THIS THESIES IS SUBMITED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE IN PHYSICAL SCIENCES OF THE FACULTY OF APPLIED SCIENCES, SABARAGAMUWA UNIVERSITY OF SRI LANKA

JULY 2006

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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, Head, Department of Physical Sciences of Faculty of Applied Sciences of Sabaragamuwa University of Sri Lanka and Mrs. Sumathi Rajasingham of SGS Lanka (Pvt) Ltd during the industrial training from 03rd April 2006 to 28th July 2006.

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AFFECTIONALY DEDICATED TO MY LOVING FAMILY MEMBERS AND TEACHERS

Acknowledgement

I express my sincere thanks to Dr.Nirmali Wickramaratne, Head, Department of Physical Sciences of Faculty of Applied Sciences of Sabaragamuwa University of Sri Lanka, for her tremendous encouragement, inspiration and guidance through out this project.

My deepest gratitude is extended to my external supervisor Mrs. Sumathy Rajasingham, Manager_laboratory, SGS Lanka (Pvt) Ltd, and Mr.Kolitha Amarasinghe and all laboratory staff for their encouragement and inspiration and constant guidance through out the project.

My sincere thanks to Dr.U.G.Chandrika, Department of Bio Chemistry, Faculty of Medical Sciences of Sri Jayewardenepura University of Sri Lanka, for providing me vitamin A standard solutions.

Abstract

Vitamin A is fortified to dairy products, such as milk, cheese, and ice-cream, infant formulas and many other foods in the form of Retinol or Retinyl Ester and it is important to quantify the amount of vitamin A content in food products because both high intake and low intake of vitamin A causes certain malfunctions in human body. Considering high intake of vitamin A, it may causes transient and disappear, increase the breakdown of our bones, fetal resorption; abortion, birth defects and permanent learning disabilities in the progancy and ect. Low intake if vitamin A can cause fetus resorption, night blindness, loss in the immune response and ect.

Quantification of vitamin A from infant formulas is difficult as it is in minute amount in infants, due to presence of isomers of vitamin A and it's sensitivity to light, air, heat. When extracted from infants or any other food vitamin A tends to degradate in to stable products. So it must be carefully extracted.

We developed a method to quantify the vitamin A content in infant formulas. Major steps involved in the method were saponification, extraction, concentrating, and analysis. Saponification was done using Ethanolic KOH solution and it was extracted to Petether solution, concentrated using both rotary evaporator and Nitrogen flow, and finally analyzed using reversed phase HPLC, using 95% Methanol as mobile phase, at a flow rate of 2 ml/min and the UV-detector at 325nm was used to calculate the peak area. The purity of standard was calculated using maximum UV absorbance at 324-328 nm range. Concentrations of vitamin A in samples were calculated using calibration curve drawn for standard solutions using Minitab software. Four samples were analyzed and two samples showed higher levels of vitamin A compare to that indicated on the label and two samples showed low amount than indicated on the label. Vitamin D is also a fat-soluble vitamin and it is important to develop a method to determine vitamin D content also.

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Chapter 01

1. Introduction

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 unesterified. 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)

The fat soluble vitamins (A, D, E, and K) effect permeability or transport in various cell membranes and 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 effects. So these are added to food products in trace amounts. (David and Michael, 2005). Vitamin A and D are added to fluid milk products. Manufactures of milk products with added vitamins A and/or 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).

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 Infant formulas.

1.2 Objectives

- Development of method to determine vitamin A concentration in infant formulas
- Determine most suitable method to extract vitamin A
- Preparation of secondary working standard of vitamin A using vitamin A capsules
- To analyze whether the samples contains the labeled amount of vitamin A

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.

The precursor molecules 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.

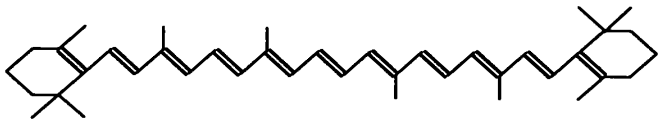

Figure 2.1 The molecule of β-Carotene

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

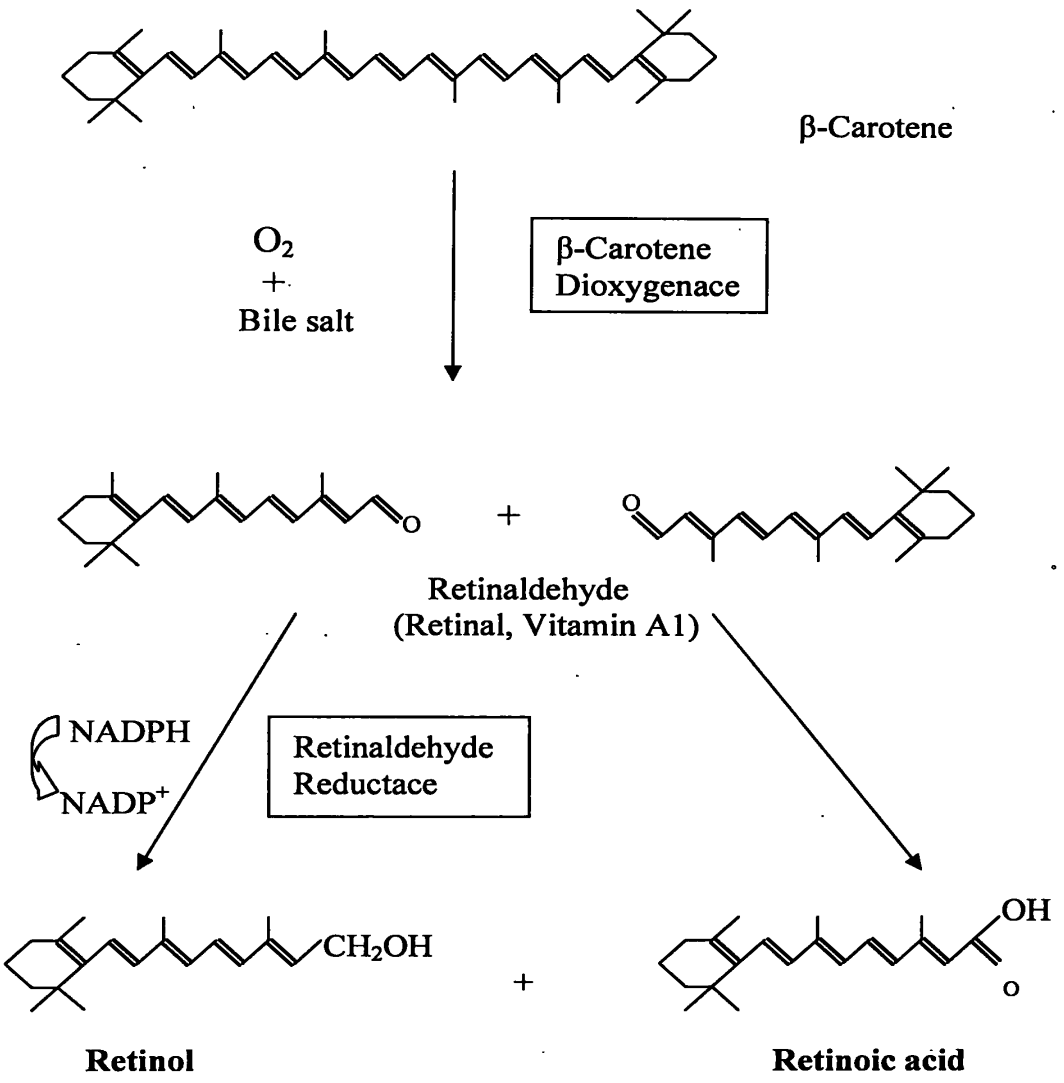

Figure 2.2 β-carotene and its cleavage to retinaldehyde and reduction of Retinaldehyde to retinol and oxidation of retinaldehyde to retinoic acid

The β-Carotene molecule will converted in to 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

1 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
β-CE	Standard Provitamin A
1 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)

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 progancy. 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 Infant formulas

Nutrition is important even before birth. Most newborns will receive adequate levels of vitamins and minerals from their mothers during the pregnancies and from breast milk, infants and/or solid foods during the first year of life.

Basically an Infant formula is a substitute for breast milk for feeding infants. Pediatricians generally advise exclusively breastfeeding (that is, breastfeeding with no formula) for all full term, healthy infants for the first 6 months of life. However, many infants are formula-fed today, at least in part. For infants to achieve normal growth and maintain normal health, infant formulas must include proper amounts of water, carbohydrate, protein, fat, vitamins, and minerals.

The three major classes of infant formulas are:

01) Milk-based formulas prepared from cow milk with added vegetable oils, vitamins, minerals, and iron. These formulas are suitable for most healthy full-term infants.
02) Soy-based formulas made from soy protein with added vegetable oils (for fat calories) and corn syrup and/or sucrose (for carbohydrate). These formulas are suitable for infants who cannot tolerate the lactose in most milk-based formulas or who are allergic to the whole protein in cow milk and milk-based formulas.
03) Special formulas for low birth weight (LBW) infants, low sodium formulas for infants that need to restrict salt intake, and "predigested" protein formulas for infants who cannot tolerate or are allergic to the whole proteins (casein and whey) in cow milk and milk-based formulas.

Nutrition is important even before birth. Most newborns will receive adequate levels of vitamins and minerals from their mothers during the pregnancies and from breast milk, infants and/or solid foods during the first year of life.

The functions and ingredients of breast milk are yet to be defined. But infants are design to function similar to the breast milk and must satisfy the dietary

supplement of breast milk. The recommended amount of vitamin A in infants is about 1500 IU per 100g of formula. Infants are fortified with appropriate levels of vitamins.

2.8 Analysis of vitamin A

Background

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 before the development of final method.

2.8.1 High Performance Liquid Chromatography (HPLC)

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.

2.8.2 Analysis of vitamin A using reversed phase HPLC

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

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 it's heat sensitivity. It will decompose above 65^0C. 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 infant formulas contain residue amount of vitamin A it is better if it can be dissolved in minimum amount of solvent before injecting to the HPLC. After extracting to Petether 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. Finally the analysis part can be done using reversed phase HPLC with the use of LC-18 or LC-8 column.

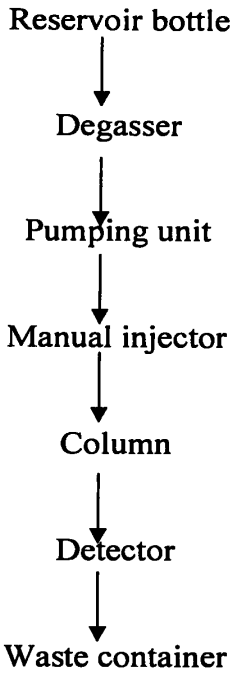

Figure 2.3 Parts of HPLC and solvent system

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

Degasser : 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.

Manual injector : samples are injected to the system via manual injector, with micro syringe (25µl)

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 Other methods for analysis of vitamin A

2.9.1 Colorimetric method

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)

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 concentration of the manufacturer.

3.2 Materials

3.2.1 Equipment

(a) Flask: Pyrex 50ml, 100ml
(b) Funnels
(c) Cep-pack cartridge: ALLTECH Silica
(d) Cyringer: 25ml (TERUMO Cor. TOKYO, JAPAN)
(e) Measuring cylinder
(f) Vials
(g) Spatula
(h) Analytical balance
(i) Separating funnels
(j) Hot plate
(k) Nitrogen blanket apparatus (liquid Nitrogen tank)
(l) Reflux condenser
(m) Side armed Saponification flask

3.2.2 Instruments

(a) High Performance Liquid Chromatograph (HPLC)

SCL-10A VP System controller

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

HPLC Column: LC-18 Octadecane Silicon (25cm, 4.6mm, 5μm)
LC-8 Octadecane Silicon (25cm, 4.6mm, 5μm)

(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) Acetic acid - Glacial

(c) Methanol - HPLC grade

(d) Ethanol 95%

(e) Tetrahydrofuran

(f) Pyrogallol crystals

(g) KOH solution - 50%

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

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

(j) Hexane solution – Analytical grade

(k) Distilled water

(l) Methylene Chloride – Analytical grade

3.3.2 Mobile phase

(a) Methanol : Water (95:5)

(b) Acetonitrile : Methanol : Water (63:33:4)

3.4 Methodology

As determination of Vitamin A is difficult two standard methods were tried and finaly the new method was developed.

3.4.1 Method 01

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 Preparation of sample and Extraction

A 5g sample of infant formula was dispersed in 30 ml of water using sonicator for 5 min at ambient temperature, fallowed by addition of 30 ml of ethanol containing 0.025% butylated hydroxyl toluene and sonicated (approximately for 10s) to dissolve completely. The solution was quantitatively transferred to a seperatory funnel and it was shaken for 2min with 60ml of Hexane and the hexane phase was decanted. The hexane extraction was repeated 2 more times on the aqueous phase. The hexane phases were combined in new seperatory funnel and washed twice with 140ml aqueous methanol (80%). The hexane phase was collected in a 250ml round bottom flask and evaporated to dryness in a rotary evaporator at 40^0C under vacuum. the residue was dissolved in 2ml of methanol : methylene chloride (50:50)and quantitatively transferred to a 5ml volumetric flask and brought to volume with a polytetrafluoroethylene 0.45μm (PTFE- 0.45μm) filter in to an auto sampler vial for chromatographic determination (Lloyd et al, 2004)

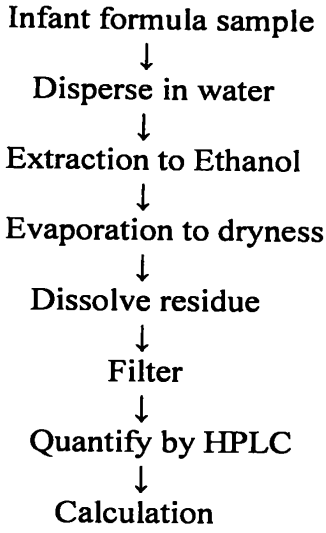

Figure 3.1 Flow chart for method 01

3.4.1.2 Preparation of mobile phase

The mobile phase was prepared using the AOAC international method for vitamin A analysis using HPLC for LC-8 column the mobile phase was a mixture of Acetonitrile: Methanol: Water (63:33:4). The prepared mobile phase filtered through micro filter.

3.4.1.3 Preparation of standard solution

About 50mg of tablet was taken in to a 10ml volumetric flask and it was dissolved in mobile phase and made up to mark with mobile phase.

3.4.1.4 Analysis

Mobile phase was run through column of HPLC. Extract solution was injected at the volume of 25 μm to HPLC 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 injected through injection port.

3.4.2 Method 02

3.4.2.1 Preparation of sample and extraction

About 5g of sample was weighted in to an Erlenmeyer flask and 3ml of water was added to it and dispersed it as slurry and 40ml of ethanol was added to it. Pea-sized pyrogalol acid was added to it and glass beads were added to it to promote even boiling. The flask was swirled well and Nitrogen flow was turned on. After that 10ml of KOH was added and the flask was kept on hot plate and refluxed for 45min.

After refluxing the flask and content were brought to room temperature using ice water bath.10 ml of glacial acetic acid was and flask was cooled to room temperature again. The solution in flask was transferred to 100ml volumetric flask using Ethanol-THF 50:50 mixture and diluted to volume with same solvent. It was kept in refrigerator overnight for fatty acids to precipitate. (AOAC, 2001)

Infant formula sample
↓
Saponify and neutralize
↓
Dilute and convert fat into fatty acids
↓
Converting retinol ester in to retinol
↓
Quantify retinol by HPLC
↓
Calculation of the concentration

Figure 3.2 Flow chart for method 02

3.4.2.2 Preparation of mobile phase

The mobile phase was prepared using the AOAC international method for vitamin A analysis using HPLC for LC-8 column the mobile phase was a mixture of Acetonitrile : Methanol : Water (63:33:4). The prepared mobile phase filtered through micro filter.

3.4.2.3 Preparation of standard solution

Working standard solution was prepared in the fallowing manner. A 50 mg of vitamin A standard was weighed in to a 100ml law actinic volumetric flask and the weight was recorded to nearest 0.1mg and 50mg of pyrogallol was added to it and diluted to volume using Hexane. A 5ml portion of this solution was pipeted in to second low actinic volumetric flask and diluted to volume with 95% alcohol.

To prepare high standard solution, 5 ml of working standard was pipet out in to Erlenmeyer flask and 25ml of ethanol solution was added to it. The same procedure for samples was continued.

To prepare intermediate standard solution, 2 ml of working standard was pipet out in to Erlenmeyer flask and 33ml of ethanol solution was added to it. The same procedure for samples was continued.

To prepare intermediate standard solution, 0.5 ml of working standard was pipet out in to Erlenmeyer flask and 37.5ml of ethanol solution was added to it. The same procedure for samples was continued. (AOAC, 2001)

3.4.2.3 Analysis

Mobile phase was run through HPLC column. Extract solution was injected at the volume of 25 μm to HPLC 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 wavelength of 325nm by UV detector. Standard solution was injected through injection port. (AOAC, 2001)

3.4.3 Method 03

3.4.3.1 Calculating the purity of standard

As the vitamin A standard was an old one it was necessary to calculate the purity of the standard. Exact amount of vitamin A was weighed accurately in to a low actinic volumetric flask and dissolved in Methanol and diluted to volume with Methanol. The maximum absorbance of this solution was obtained at 324-328 nm using 1cm path length cell and Methanol solution was used as the blank.

3.4.3.2 Preparation of sample and extraction

About 5-10g of sample was weighed accurately to give about 50µg of vitamin A in to a side armed saponification flask and it was dispersed in small amount of water as slurry using sonicator. Then 40ml of 95% Ethanol was added to it and about 0.2g of pyrogallol was added to it as anti oxidant agent. Glass beads were added to promote boiling and the content was swirled. The Nitrogen flow was turned on and bubbled through the slurry and placed on hot plate. 10 ml of KOH solution was pipette to in the hot plate was immediately switch on. It refluxed for 45 min. and the flask was kept on ice water bath immediately after refluxed. The solution was transferred to a seperatory funnel using 40ml of distilled water and the saponification flask was washed with 150ml of Petether solution and the separating funnel was shaken vigorously for about 3 min. and it was allowed to separate. The bottom layer was collected in to second separating funnel and it was extracted with another portion of Petether. (Ronald et al, 1991)

All the ether extractions were collected in to another separating funnel and it was washed with distilled water until all the KOH in the aqueous phase were neutralized to Phenolphthalein and it was transferred to rotary evaporator flask and 2ml of BHT solution was added to it. The Petether was evaporated using rotary evaporator under vacuum and when it was near to dryness the remaining solution was transferred to 10ml vial and the flask was washed with 2ml of Petether and it was collected in to same vial. Using Nitrogen flow and a water bath of 45^0C-55^0C. The remaining Petether was evaporated and it was continued until the vial was dried and the vial was covered with an Aluminum foil to protect from light.

The residue in the vial was diluted in 1-5 ml of methanol and kept in refrigerator until injected to HPLC.

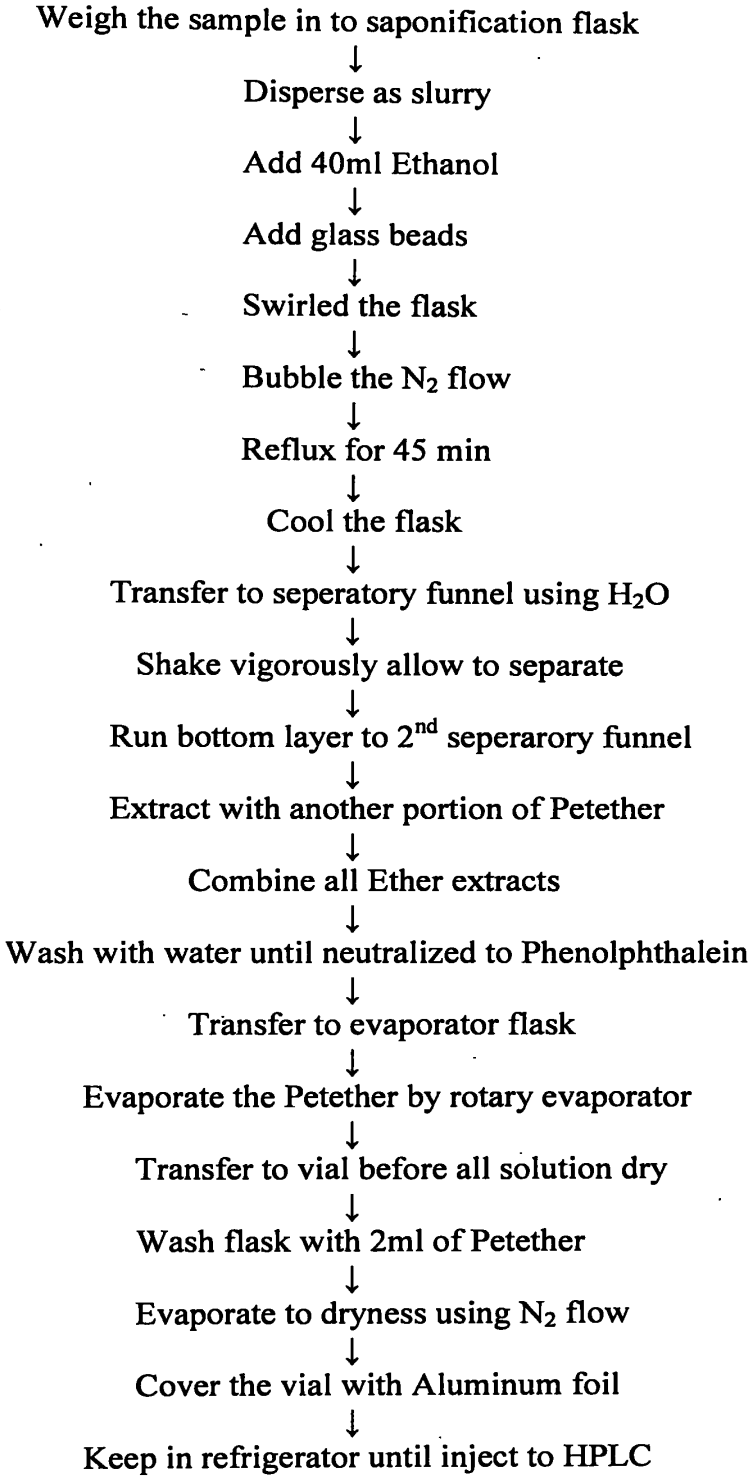

Figure 3.3 Flow chart for method 03

3.4.3.3 Preparation of mobile phase

The mobile phase was prepared by Methanol: water mixture with a proportion of 95:5

3.4.3.4 Preparation of standard solution

Accurately weighed amount of standard retinol (vitamin A) was dissolved in 5ml of Methanol and it can be used up to about two weeks in the refrigerator

3.4.3.5 Recovery test

About 1.5mg of standard was weighed accurately in to 100ml volumetric flask and diluted to volume with Methanol. Blank sample which does not contain vitamin A was taken and test portions of 5g were taken.

Standard was added in the portions of 10ml, 20ml 30ml to blank sample portions and same procedure in method 03 was carried out for each sample separately.

3.4.3.6 Analysis

Mobile phase was run through column of HPLC. Extract solution was injected at the volume of 25 μm to HPLC 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 injected through injection port and the area was compared with it. (Ronald et al, 1991)

3.5 Calculations

Calculating the purity of standard

According to Beer-Lambert law

$$\mathbf{A = \varepsilon \times c \times L}$$

A = maximum absorbance at a wave length of 324- 325 nm
ε = absorbance coefficient of vitamin A
c = concentration of vitamin A
L = path length in cm

If ε is expressed as, ε (1cm, 1%) then the concentration is expressed in grams per 100ml (g/100ml). For vitamin A in methanol ε (1cm, 1%) is equal to 1832. We have diluted the Standard in 100ml. So the value of 'C 'is equal to total amount of Vitamin A in the 100ml solution. (Ronald et al, 1991)

$$\text{Percentage of Vitamin A in standard by mass} = \frac{C \times 100\%}{W}$$

W = Weight of standard taken

C = concentration calculated from Beer-Lambert law in grams per 100 ml

Calculations for recovery test

The percentage loss of vitamin A during the method

$$\text{Loss percentage of the sample} = \frac{\text{Concentration of loss} \times 100\%}{\text{Concentration of standard solution}}$$

Calculation for vitamin A concentration using standard curve

As the first two methods were failed no attempt was taken to calculate the vitamin A content using those methods. The third method had been done successfully and calculations with regarding that method are as fallows.

Calculations were done on the basis of peak area measurements. Amount of vitamin A per 100g of sample in international units (IU) was calculated from the fallowing formula. (Budavari, 1989)

$$\text{Amount of vitamin A} = \frac{Y \text{ ppm} \times V \text{ ml} \times 0.7866 \times 100}{W \text{ g} \times 0.3}$$

Y ppm	=	concentration calculated from the peak area of chromatogram and the regression Equation in parts per million (ppm)
V	=	Final makeup volume in ml
W	=	Weight of sample taken in g's
0.7866	=	Density of Methanol
0.3	=	conversion factor of μg to IU

The standard curve for vitamin A was used in calculating the value of Y and regression equation was obtained using MINITAB release- 14 package.

Chapter 04

4.0 Results and Discussion

The first two methods (method 1 and method 2) were not continued due to some failures and unavailability of some solutions.

In the method one it was necessary to use Tetrahydrofuran (THF) as a solvent to dissolve the extracted vitamin A from samples. But it was a very toxic solvent and also an expensive solvent. It was tried to continue that method without using THF. But the resolution of the peaks of the chromatogram was not sufficient enough to satisfy. So that method was not continued. But in that method we don't have to evaporate the solvent or to dry the extractions. It's a big advantage over other methods. Because, during the evaporation using the rotary evaporator there was a possibility of loosing the extracted vitamin A. figure is a chromatogram obtained for the method 01.

The method 02 was a method developed for non fat dried milk (NFD). It couldn't be directly applied for every infant formula, because most of infant formula contained fat. It was necessary to saponify the sample before the extraction. But from this method a sharp peak for vitamin A was obtained. For the sample an emulsion was formed and it was unable to break the emulsion. It was tried to break the emulsion using Ethanol, Sodium Chloride. But the emulsion wasn't broken and could not continue the method due to loss of vitamin A at the emulsion. The emulsion was occurred most probably due to the fat present in the sample. (Appendix 01)

The third method was the developed one. As the standard solution was a very old standard it was necessary to calculate the purity of standard. When the prepared solution was scanned by the UV spectrophotometer fallowing maximum absorbances were obtained. (Appendix 02)

Table 4.1 The maximum UV absorbance of standard solution

Wave length (nm)	Absorbance
324.5	1.082
247.5	0.529
202.0	0.552

Weight of standard taken = 0.0011g
Volume of MeOH = 100 ml
Maximum absorbance = 1.091
The value of ε (1cm, 1%) = 1832
Content of vitamin A = 5.906×10^{-4} g/100ml

Percentage of vitamin A = 53.69 %

The major objective of this project was to develop a method to determine Vitamin A content in infant formulas.

According to that vitamin A content in formulas were extracted in to methanol after fallowing several steps. Due to the light sensitivity and heat sensitivity of vitamin A it was essential to be careful during the extraction. Otherwise the loss of vitamin A may would have a greater value. So it was necessary to carryout a recovery test for the method. Recovery test was done using a sample, which does not have vitamin A, and standard vitamin a sample was spiked to it and the same procedure for the samples was carried out and fallowing results were obtained. (Appendix 03)

Table 4.2 Results of recovery test

Sample	A	B	C
Amount of standard added(ppm)	00	37.75	75.5
Concentration of final volume	00	24.89	42.4
Percentage loss	00%	35%	44%

According to the results there was a 40% loss of vitamin A during the extraction and the recovery was around 60%.

The second aim of this project was determination of vitamin A content in infant formulas. Mainly the samples obtained from the market were milk samples. Four samples were analyzed and fallowing results were obtained. (Appendix 04)

Table 4.3 Results of sample testing

Sample no:	Indicated amount in packet (IU/100g)	Peak area	Concentration (ppm)	Amount of vitamin A (IU/100g)
1	1500	363754	60.7	1374
2	2500	74266	12.2	2947
3	1250	197758	32.6	2264
4	2500	344017	22.15	2185

According to the results obtained 2 samples have exceeded the labeled amount and two are shorter than indicated amount. The one that contain 2947IU/100g was a nutrition powder.

Concentration of vitamin A was calculated using a standard curve (calibration curve) of vitamin A. calibration curve was everyday tested using vitamin A standard solution and the calibration curve was drawn by the use of three different Vitamin A standard solutions and the MINITAB software. (Appendix 05)

Table 4.4 Resulted peak areas for Vitamin A standard solutions

Concentration of vitamin A standard (ppm)	0	547.2	1578.9	2402.7
Result (peak area)	0	2856384	11757637	12537325

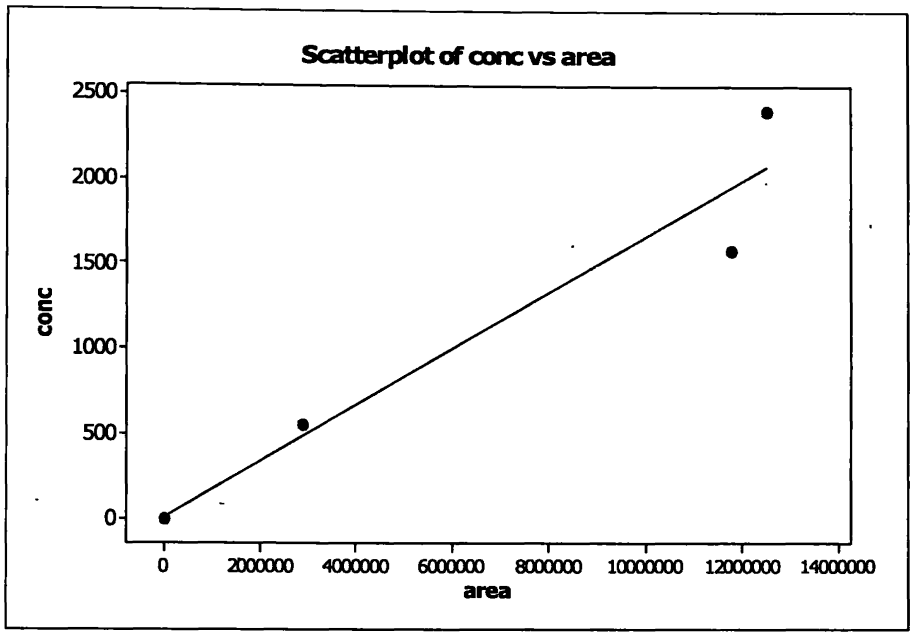

Figure 4.1 Standard curve for vitamin A

Regression equation

$$\text{Concentration (ppm)} = 0.000167 \times \text{peak area}$$

Chapter 05

5.1 Conclusion

Extraction method was developed according to the facilities that SGS laboratory does have. But the recovery was around 60% and there was a lost of 40%. 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.

According to the results 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 infants and it must be careful, as some formulas contained excess amount of vitamin A than the recommended intake. It will usually effect 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 40%. 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.

Two samples (sample2 and sample 3) analyzed were actually nutrition powders. They must be fed to the infants as prescribed by the physician. When that sort of nutrition powders are taking, only the prescribed amount must be fed to infants. Otherwise health problems may be occurred. Manufactures must be careful to fortify only the recommended amount of vitamins, and other minerals for foods.

When analyzing the standard solution gave two peaks in the chromatogram and the peak which has the higher area was taken as the peak for vitamin A. When the standard was opened to air and light the area of that peak had a lower area than the first injection. So it can be thought, that peak represents the elute of vitamin A from the column.

Until the standard was obtained it was tried to use a vitamin A tablet as an secondary standard. But the purity of that tablet was very low and contained binding agents. So the peak of vitamin A could not be identified from that.

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.

The second method may be more accurate if the sample is saponified before the extraction part and the chromatogram of that method would yield a sharp peak for vitamin A.

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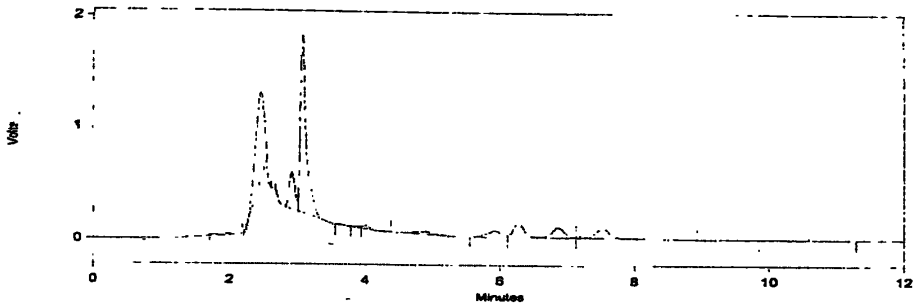
Appendix 01

Chromatogram of sample testing

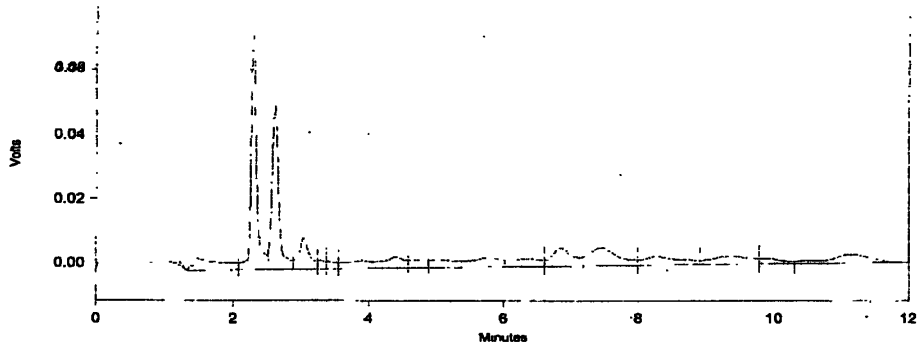
Method 01



Detector A
(280nm)

Pk #	Name	Retention Time	Area	Units
6	Vitamin A	3.067	9620394	IU
Totals			9620394	

Method 02

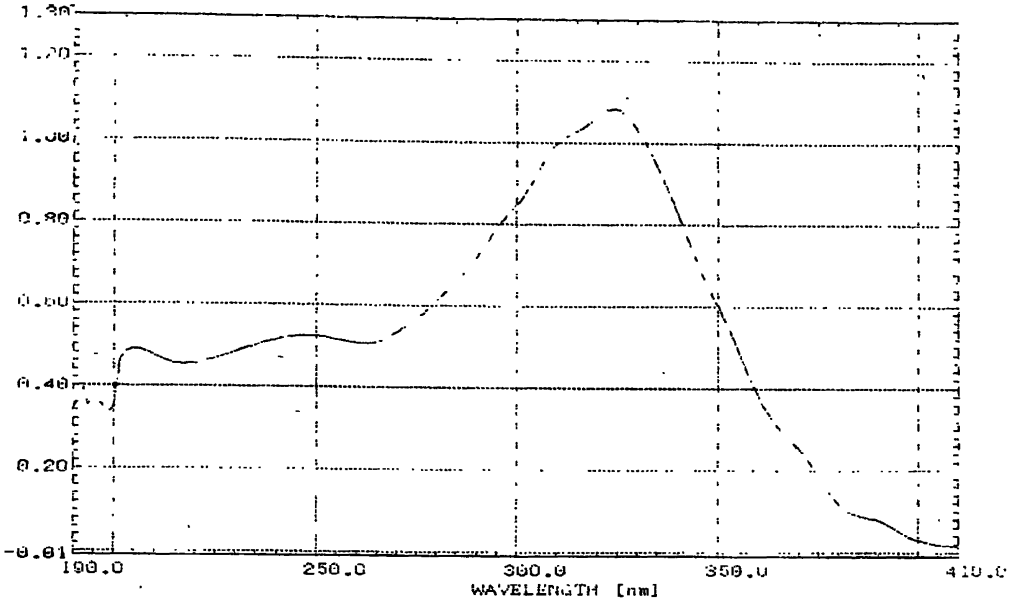


Detector A
(280nm)

Pk #	Name	Retention Time	Area	Units
5	Vitamin A	2.613	362314	IU
Totals			362314	

Appendix 02

Calculating the purity of standard – UV spectrogram

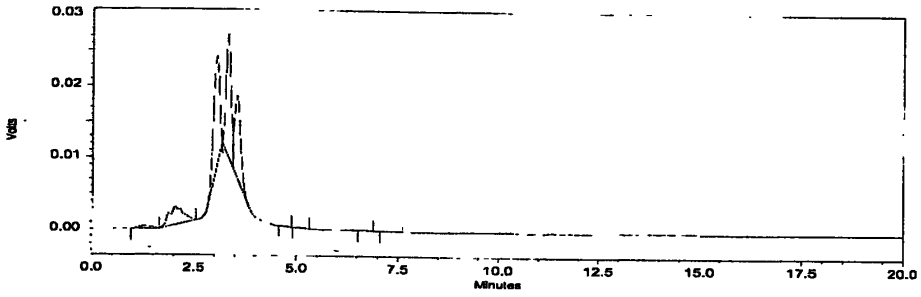


Wave length (nm)	Absorbance
324.5	1.082
247.5	0.529
202.0	0.552

Appendix 03

Chromatograms of recovery test of the method 03

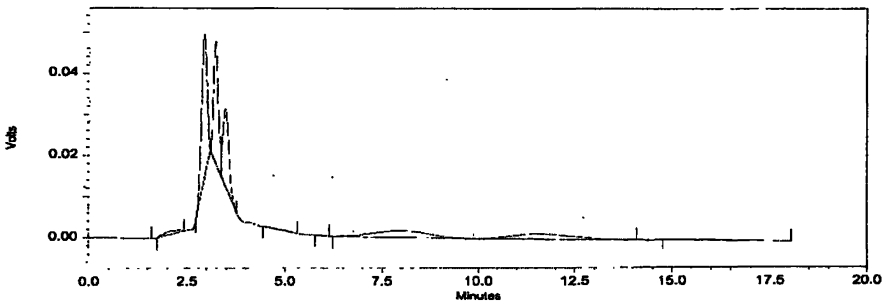
Sample B



Detector A
(325nm)

Pk #	Name	Retention Time	Area	Units
4	Vitamin A	3.320	143953	IU
Totals			143953	

Sample C



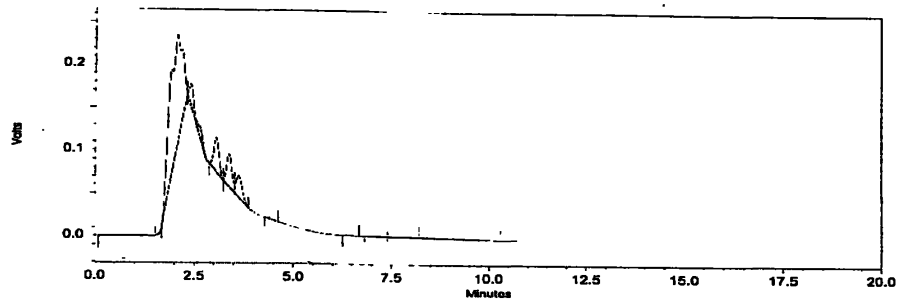
Detector A
(325nm)

Pk #	Name	Retention Time	Area	Units
4	Vitamin A	3.227	257036	IU
Totals			257036	

Appendix 04

Chromatograms for test samples - Method 03

Sample 01

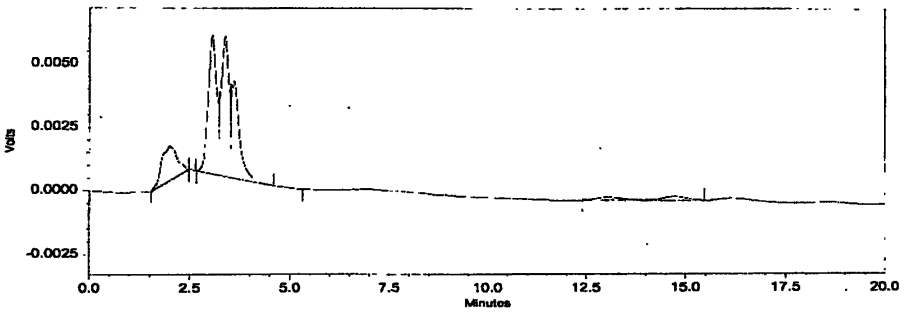


Detector A
(325nm)

Pk #	Name	Retention Time	Area	Units
5	Vitamin A	3.387	363754	IU

Totals			363754	

Sample 02

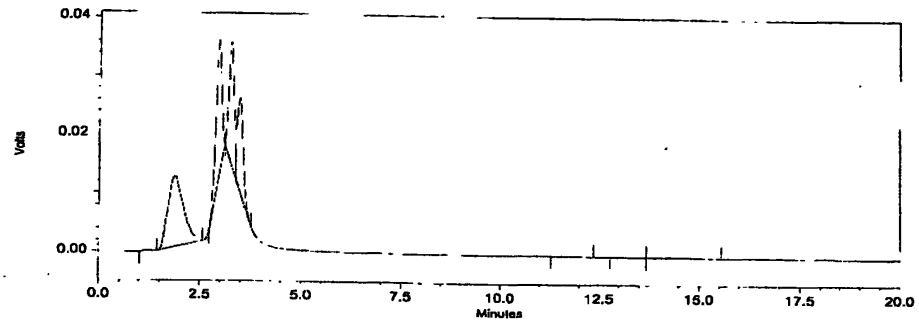


Detector A
(325nm)

Pk #	Name	Retention Time	Area	Units
4	Vitamin A	3.387	74266	IU

Totals			74266	

Sample 03

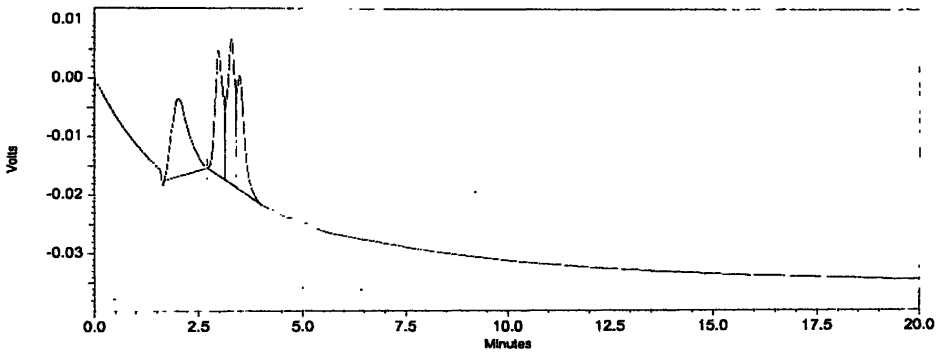


Detector A
(325nm)

Pk #	Name	Retention Time	Area	Units
4	Vitamin A	3.253	197758	IU

Totals			197758	

Sample 04



Detector A
(325nm)

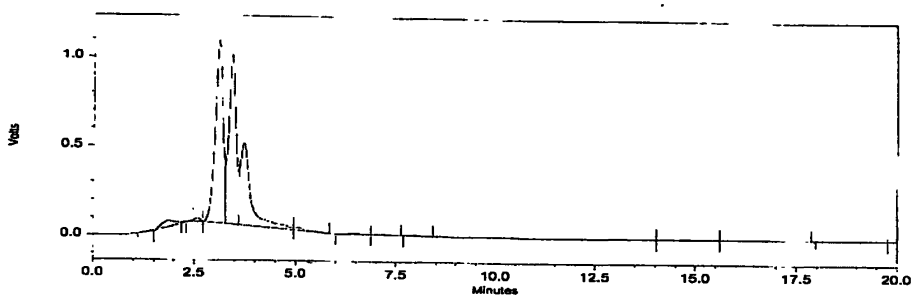
Pk #	Name	Retention Time	Area	Units
3	Vitamin A	3.307	304017	IU

Totals			304017	

Appendix 05

Chromatogram for standard solutions in method-03

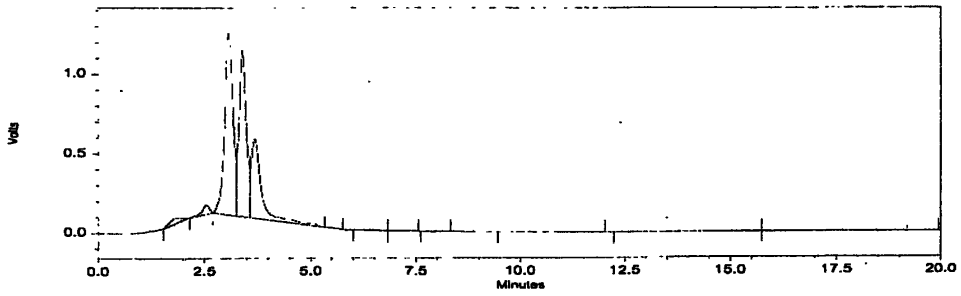
Standard solution 1578.9 ppm



Detector A
(325nm)

Pk #	Name	Retention Time	Area	Units
7	Vitamin A	3.413	11757637	IU
Totals			11757637	

Standard solution 2402.7 ppm



Detector A
(325nm)

Pk #	Name	Retention Time	Area	Units
5	Vitamin A	3.400	12537325	IU
Totals			12537325	

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