11-2020

MASS SPECTROMETRIC DETERMINATION OF RETINOL IN EMIRATI POPULATION

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry

Under the Supervision of Dr. Iltaf Shah

November 2020

Declaration Of Original Work

I, Israa Samir Mohamed Shawky El-Konaissi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Mass Spectrometric Determination of Retinol in Emirati Population",* hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Iltaf Shah, in the College of Science at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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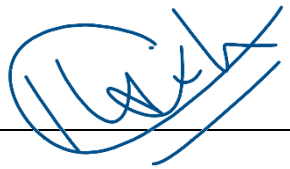
   Signature  Date 3 November 2020

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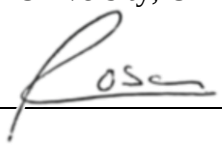
   Signature  Date 3 November 2020

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Abstract

This thesis is concerned with analysing vitamin A deficiency. Vitamin A is crucial for several functions, including vision, growth, and cell differentiation. the main aim is to develop and validate an LC-MS/MS method for the determination of vitamin A in plasma matrix and use the new method for the determination of vitamin A levels in the obese Emirati population. Methods: A positive ion electrospray ionisation (ESI) LC-MS/MS method was used in the Multiple Reaction Monitoring (MRM) mode for quantification. It involved i) LC-MS/MS, ii) a guard column together with C18 Ascentis Express F5 column iii) Internal standard, 25-Hydroxyvitamin D3 (6, 19, 19-d3), and iv) identification via ESI and monitoring of three fragmentation of the parent ion. To demonstrate the practical value of this method, blood samples were collected from 452 Emirati participants (277 obese; 175 healthy). The method was validated according to FDA-US guidelines. The method has been applied on a sample of 277 Emirati obese patients, including 277 baselines, 277 follow-ups, and 175 health samples. The characteristics of the sample included: mixed-gender with 73 males in the age group of (18–82 years) and 204 females in the age group of (18–65 years). The results show that the concentrations of vitamin A for both females and males increased after supplementation (372 ng/mL to 440 ng/mL for female sample, and 438 ng/mL to 540 ng/mL for male sample). This has led to a sufficient level of vitamin A in participants. The new method allowed chromatographic separation and quantification of vitamin A. The new assay could detect 0.48 ng/mL of vitamin A in serum with the calibration curve ranging from 7.8 to 1000 ng/ml. The method validation parameters, including intra and inter-day precision, intra and inter-day accuracy, recovery, linearity, specificity, and stability, were within range. For example, the recovery percentage found were 99%, 96%, and 94% for QCH, QCM, and QCL respectively, while the percentage of change in the stability of vitamin A ranges between 0 and 3%. The applied LC-MS/MS method was intended to accurately detect Vitamin A in human plasma, and has proven to be specific, reliable, and robust. The method can detect low levels of vitamin A. This analytical method does not require time-consuming derivatisation and complex extraction techniques and could prove very useful in clinical studies.

Keywords: Vitamin A, LC-MS/MS, ion dispersion, chromatographic separation.

Title and Abstract (in Arabic)

التحديد الطيفي الشامل للريتانول لسكان دولة الإمارات

الملخص

مقدمة: هذه الرسالة معنية بتحليل نقص فيتامين أ الذي يعد من أهم العناصر الغذائية للعديد من الوظائف ، بما في ذلك الرؤية والنمو وتمايز الخلايا. الأهداف: الهدف الرئيسي هو تطوير والتحقق من صحة طريقة LC-MS / MS لتحديد فيتامين أ في مصفوفة البلازما واستخدام الطريقة الجديدة لتحديد مستويات فيتامين أ في السكان الإماراتيين البدينين. الطرق: تم استخدام طريقة LC-MS / MS - اللوني السائل مع مطياف الكتلة الترادفية - للتأين بالرش الكهربائي الأيوني الموجب في وضع مراقبة التفاعلات المتعددة (MRM) للتقدير الكمي. تضمنت 1) LC-MS / MS، 2) عمود حماية مع عمود C18 Ascentis Express F5 iii) المعيار الداخلي ، 25-Hydroxyvitamin D3 (6، 19 ، 319d-) ، و 4) تحديد عبر ESI ومراقبة ثلاثة تجزئة للأيون الأصل. لإثبات القيمة العملية لهذه الطريقة ، تم جمع عينات دم من 452 مشاركًا إماراتيًا (277 سمينًا ، 175 صحيًا). تم التحقق من صحة الطريقة وفقًا لإرشادات إدارة الغذاء والدواء الأمريكية. النتائج: تم تطبيق الطريقة على عينة من 277 إماراتي مريض يعانون من السمنة المفرطة ، بما في ذلك 277 عينة أساسية ، و 277 تم متابعتهم ، و 175 عينة صحيحة. اشتملت خصائص العينة على: عدد من 73 من الذكور في الفئة العمرية (18-82 سنة) و 204 إناث في الفئة العمرية (18-65 سنة). أظهرت النتائج أن تركيزات فيتامين (أ) لكل من الإناث والذكور زادت بعد المكملات (372 نانوغرام / مل إلى 440 نانوغرام / مل لعينة الإناث ، و 438 نانوغرام / مل إلى 540 نانوغرام / مل لعينة الذكور). وقد أدى ذلك إلى مستوى كافٍ من فيتامين أ في المشاركين. مساهمات كبيرة: سمحت الطريقة الجديدة بالفصل الكروماتوغرافي وتقدير فيتامين أ. يمكن للمقايسة الجديدة اكتشاف 0.48 نانوغرام / مل من فيتامين أ في مصل الدم مع منحنى المعايرة الذي يتراوح من 7.8 إلى 1000 نانوغرام / مل. كانت معلمات التحقق من صحة الطريقة ، بما في ذلك الدقة داخل وبين اليوم ، والدقة داخل وبين اليوم ، والاسترداد ، والخطية ، والخصوصية ، والاستقرار ، ضمن النطاق. على سبيل المثال ، كانت نسبة الاسترداد التي تم العثور عليها 99٪ و 96٪ و 94٪ لـ QCH و QCM و QCL على التوالي ، بينما تتراوح النسبة المئوية للتغير في ثبات فيتامين A بين 0 و 3٪. تم ملء الفجوة: طريقة LC-MS / MS المطبقة كانت تهدف إلى الكشف الدقيق عن فيتامين أ في بلازما الإنسان ، وقد ثبت أنها محددة

وموثوقة وقوية. يمكن لهذه الطريقة الكشف عن المستويات المنخفضة من فيتامين أ. لا تتطلب هذه الطريقة التحليلية اشتقاق تقنيات استخلاص معقدة وتستغرق وقتًا طويلاً ويمكن أن تكون مفيدة جدًا في الدراسات السريرية.

مفاهيم البحث الرئيسية: فيتامين أ، LC-MS/MS، التأين بالترذيذ الأيوني، الفصل الكروماتوغرافي.

Acknowledgements

The author would like to thank Dr Iltaf Shah, the project supervisor. Dr Shah's expertise was invaluable in formulating the research questions and methodology. His insightful feedback has broadened my horizon and improved the quality of my work. This gratitude is also extended to CMHS for providing samples for this study.

Dedication

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parents whose words of encouragement and push for tenacity ring in my ears. My husband and brothers have never left my side and are very special.

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List of Abbreviations

LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
MRM	Multiple Reaction Monitoring
QCH	High Quality Control
QCM	Medium Quality Control
QCL	Lower Quality Control
RDA	Recommended Dietary Allowance
CRBP	Cellular Retinol-Binding Proteins
LpL	Lipoprotein Lipase
RBP	Retinol Binding Protein
ATRA	All-Trans-Retinoic Acid
CRABP	Cellular Retinoic Acid-Binding Protein
RAR	Retinoic Acid Receptors
RXR	Retinoid X Receptors
RAREs	Retinoic Acid Response Elements
SRC	Steroid Receptor Co-Activator
NCoR	Nuclear Receptor Corepressor
HDACs	Histone Deacetylases
PPARs	Peroxisome Proliferator-Activated Receptors
FABP5	The Fatty Acid-Binding Protein 5
PPREs	PPAR Response Elements
ROREs	Retinoid-Related Orphan Receptors
CREB	cAMP Response Element-Binding
LLE	Liquid-Liquid Extraction
SFE	Super Critical Fluid Extraction

UAE	Ultrasonic Assisted Extraction
SPE	Solid-Phase Extraction
DLLME	Dispersive Liquid–Liquid Microextraction
HPLC-MS/MS	High-Performance Liquid Chromatography-Tandem Mass Spectrometry
UHPLC	Ultra-High-Performance Liquid Chromatography
LC	Liquid Chromatography
FLD	Fluorescence Detectors
UV	Ultraviolet
LC-UV	Liquid Chromatography-Ultraviolet Detection
DLLME	Dispersive Liquid–Liquid Microextraction
ELISA	Enzyme-Linked Immunosorbent Assays
DAD	Diode Array Detector
LLE	Liquid–Liquid Extraction
SPE	Solid-Phase Extraction
SDME	Single-Drop Microextraction
HF-LPME	Hollow Fibre Liquid Phase Microextraction
SFC	Supercritical Fluid Chromatography
PDA	Photodiode Array
AA	Ascorbic Acid
ELISA	Enzyme-Linked Immunosorbent Assays
TLC	Thin-Layer Chromatography
CE	Capillary Electrophoresis
MEKC	Micellar Electrokinetic Chromatography

Chapter 1: Introduction

1.1 Background

Many organisms need limited amounts of organic compounds in their body; one of these compounds is vitamins. Vitamins play an essential vital role in the body, like activating an enzyme to catalyse a thousand chemical reactions; so, they are considered as co-factors in enzyme activity [1]. They play a crucial role as antioxidants and pro-hormone agents. Moreover, they are necessary for various physiological, biochemical, and catalytic functions. Ultimately, they are involved in the metabolism of food substances [2]. Vitamins are required in small quantities and, as such, are referred to as micronutrients. The body does not synthesise most vitamins except for vitamin D, which can be obtained by skin exposure to sunlight [3]. Therefore, diet acts as a significant supply source [3]. Each vitamin has its own set of functions; therefore, the body requires different amounts of each vitamin [4]. Vitamin deficiency is associated with several conditions such as pernicious anaemia, scurvy, pellagra, ariboflavinosis, skin conditions, and many others. It is caused by a poor diet, malabsorption, or underlying medical disease [4, 5]. Vitamins' solubility is categorized into two clusters, the first group is water-soluble vitamins while the second group is fat-soluble vitamins. Vitamins B and C are under the group of water soluble vitamins, while vitamins A, D, E, and K are under the group of fat-soluble vitamins [1].

Vitamin A has an organic structure of an 11-carbon side chain and a 6-membered ring. Vitamin A is not a single molecule, it is a name given to a group that contains some related compounds, and they include Retinol (primary molecule, vitamin A alcohol), Retinal and Retinoic acid [6, 7]. Retinol and other compounds, including retinal, retinoic acid, and retinyl esters, have a similar biological activity to vitamin A;

therefore, the term vitamin A is utilised for these compounds as presented in Figure number 1 [7]. Retinoids are a group of chemical compounds that are metabolites of vitamin A and contain four isoprenoid units. These isoprenoid units linked together in a head-to-tail manner to form Vitamin A metabolites as shown in figure number 1 [7, 8].

Retinol can be converted by oxidation to retinal; also, Retinal can be changed to retinol; therefore, these two compounds are interconvertible. Retinal is main source of Retinoic acid which is derived from by oxidation, which is also at the same time a critical signalling molecule; however, retinoic acid cannot be converted to retinal by reduction [9]. Plants contain vitamin A in a pro-vitamin form that is known as β-carotene. β-carotene is derived from carrots because carrots have a rich concentration of vitamin A. β-carotene is an antioxidant which can be converted in the body to vitamin A by an enzyme called β-carotenoid-15,15′-dioxygenase. For instance, this enzyme cleaves β-carotene in a specific region (central region) into two molecules of retinal; then, these two molecules are converted into retinol during the reduction process catalysed by retinal reductase enzyme as appears in the Figure 1 [7, 10, 11].

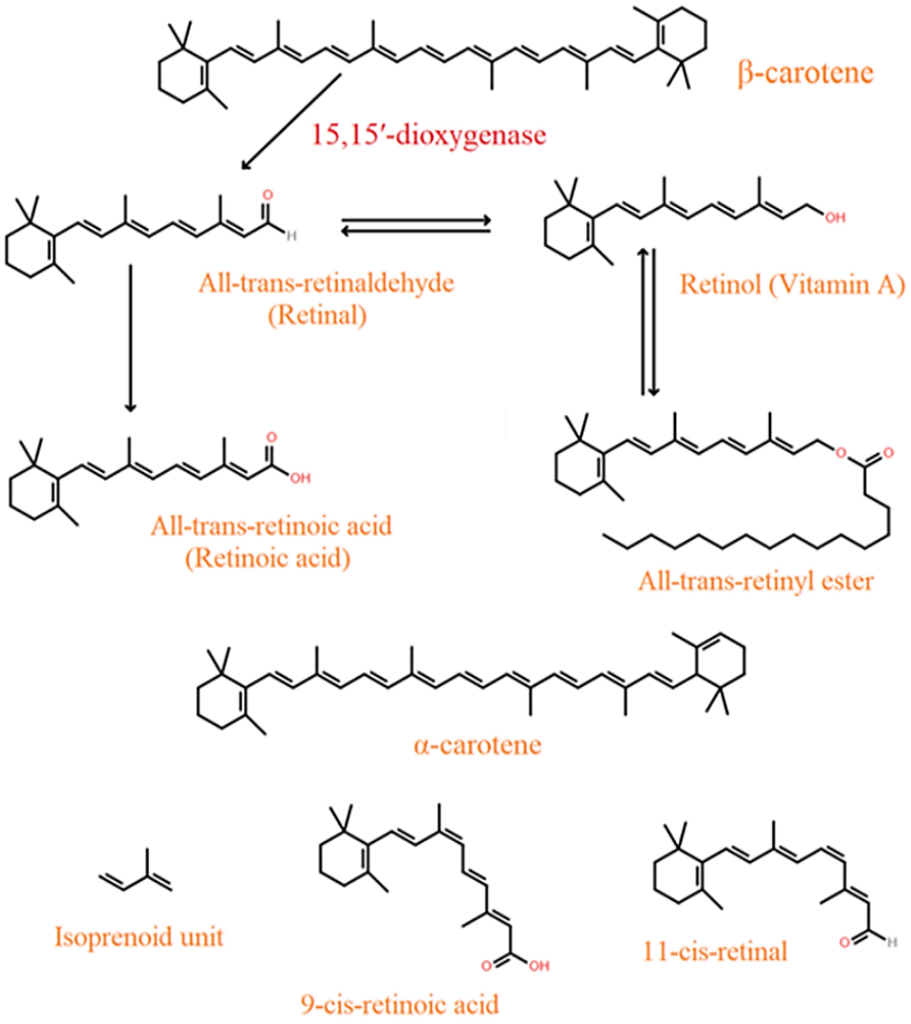


Figure 1: The metabolic relationships between a group of vitamin A compounds including retinal, retinol, β-carotene, retinoic acid, and retinyl ester

Moreover, this figure illustrates the molecular structure of α-carotene, Isoprenoid unit, 11-cis-retinal, and 9-cis-retinoic acid. β-carotenoid-15,15´-dioxygenase enzyme cleaves β-carotene into two molecules of retinal; then, Retinal converted to retinol by retinal reductase enzyme, and there will be interconversion reaction between Retinal and Retinol. Retinoic acid considered the most active biological metabolite of vitamin A produced irreversibly from retinal by an oxidation process [7].

Retinol is considered the common active metabolite biologically present in the tissue of mammals. Retinol solution has a faint yellow colour, and it can be adversely

affected by heat, oxygen, ultraviolet light, and acids [12]. Vitamin A is an essential component needed for healthy vision. There is no need for a prescription to take this vitamin. In the United States, some foods like cereals have a large amount of this vitamin. Undoubtedly, it is proposed that a large amount of vitamin A intake could lead to fracture and osteoporosis in some countries like Scandinavia and the United States, where vitamin A is often consumed highly in food and supplements [7].

The human body must have vitamin A in an adequate amount for many functions, including physical development and growth, vision, regular gene expression, immune function, reproduction, and erythropoiesis [13]. The human body requires a specific amount of vitamins depending on age and gender, as well as other factors such as health status and pregnancy. The Food and Nutrition Board of the Institute of Medicine's Recommended Dietary Allowance (RDA) ranges from 0.3mg- 0.9 mg per day. So the best way to ensure your body gets its daily requirement of essential vitamins is to eat a balanced meal containing a wide variety of fruits, vegetables, whole grains, and dairy products [14].

Various analytical procedures could be utilised in the pre-treatment and determination of vitamins. For pre-treatment, ultrasonic-assisted extraction, liquid-liquid extraction, solid-phase extraction, and dispersive liquid-liquid microextraction are the most common methods. Determination analysis includes chromatography techniques, electrophoretic methods, microbiological assays, immunoassays, biosensors, and several other methods [15-20].

1.2 Aims and objectives of this study

1.2.1 Aim

The aim of this study was to develop and validate an LC-MS/MS method for determination of vitamin A in plasma matrix and use the new method for determination of vitamin A levels in obese Emirati population.

1.2.2 Objectives

1. Develop a sensitive robust, and easy LC-MS/MS-based assay for measuring vitamin A in human plasma.
2. Use the above method to measure plasma vitamin A in healthy and diseased Emirati population.

1.3 Hypothesis

1. Obese Emirati patients have low levels of vitamin A compared to healthy.

1.4 The importance of vitamin A for vision

In the early 1500 BC, vitamin A was significantly important to ancient Egyptians for vision health. As reported by papyrus Ebers (ancient herbal knowledge of medicine), patients who have night blindness disease or nyctalopia patients who were face difficulty in seeing either during the night or in dim light. Those patients were treated by using the specific medication as a topical application in their eye. It is called liver juice or previously cooked ox liver extract [9, 21, 20]. The drops of this medicine contain a large amount of vitamin A (Retinol). They come to the retina through firstly getting in the lachrymal duct and then absorbed through blood circulation [9, 22].

In Figure 1, it is shown that 11-cis-retinal is a Vitamin A derivative that plays a vital role in the rod cells located in the retina of the eye. Rod cells are the photoreceptor cells that have a specific type of vision, which is called scotopic vision; so, it is more for dim light or dark light. Rod cells consist of a specific type of visual pigment, and this pigment is called Rhodopsin. Rhodopsin is made up of two things. The first thing is that retinal, which is 11-cis-retinal, and the second thing is that opsin protein, which is sensitive to the light [9, 10].

When the light rays absorbed by rod cells, they hit the Rhodopsin and change the structure of 11-cis-retinal into a different form, which is called all-*trans*-retinal; therefore, the attraction of this molecule toward opsin protein is loosed which lead to initiate phototransduction process. In the phototransduction process, the light is converted into an electrical signal which is transmitted toward the brain resulting in the vision [9, 23-25]. Vitamin A deficiency precedes to night blindness symptoms. For example, When the amount of 11-cis-retinal is less, the body cannot make Rhodopsin (light-sensitive protein), and a subsequently small amount of light cannot stimulate an adequate response at night [7, 26].

1.5 Dietary sources of vitamin A

Vitamin A can be found in the diet either as pro-vitamin A carotenoids or as preformed vitamin A**.** Retinyl ester is a long-chained fatty acids ester of retinol and considered as major preformed vitamin A. It can be ingested in foods such as liver, milk, butter, eggs, and fortified cereals. Moreover, Vitamin A can be obtained as pro-vitamin A carotenoids, including β-carotene, α-carotene, and β-cryptoxanthin from vegetables such as pumpkins, collards, carrots, squash, and spinach. In the United States, the percentage of β-carotene ingestion exceeds more than 75% compared to the

other pro-vitamin A carotenoids [27, 28]. “The Recommended Dietary Allowance (RDA) for adults for vitamin A is set at 900 ug RAE/day for men and 700 ug RAE/day for women. One ug retinol activity equivalent (ug RAE) is equal to 1 ug all-trans-retinol, 12 ug β-carotene, and 24 ug α-carotene or β-cryptoxanthin” [14]. In the United States, the mean of vitamin A intake is approximately 600 ug RAE/day and preformed vitamin A intake formed 70 -75% of that concertation [27].

1.6 Absorption and transport of vitamin A

The biologically inactive retinyl esters are broken down via hydrolysis with pancreatic and intestinal enzymatic catalysation [29, 30]. The resultant unbound and insoluble retinol is then absorbed by enterocytes and converted from its unbound state to binding with one of the cellular retinol-binding proteins (CRBP). From the six distinct binding proteins, Intestinal Retinol binds preferentially to CRBP type II [31]. In contract to preformed vitamin A, absorption of pro-vitamin A carotenoids occurs either directly by intestinal muscoal cells or by conversion through oxidation to Retinal, followed by reduction to retinol [32]. Retinol esterification occurs with retinol obtained from retinyl esters and pro-vitamin A, through incorporation with long-chain fatty acids. The retinyl esters alongside the unmodified carotenoids are packaged with the rest of dietary lipids into chylomicrons, which are carried by the lymphatic system [33]. There is also a notion that some retinol which has not undertaken esterification is uptaken directly by the hepatic portal system.

Dietary fat plays a fundamental role in the absorption of vitamin A. This occurs through several processes by stimulation of chylomicron production and improving the solubility of retinol and carotenoids in the intestinal mucosa while also initially

aiding in the activation of enzymes acting to performs hydrolysis of dietary retinyl esters [27].

The formation of chylomicron remnants in the bloodstream where retinyl esters are contained occurs as a result of hydrolysis with a function of the enzyme lipoprotein lipase, accompanied by the addition of apolipoprotein. This facilitates hepatic retinol uptake through endocytosis, followed by hydrolysis [31]. It has been proposed that the enzyme lipoprotein lipase (LpL) performs this function in peripheral tissues, facilitating hepatic retinol uptake through endocytosis followed by hydrolysis [34]. Retinol surplus to the body's need is re-esterified and transferred to the hepatic stellate cells for storage.

With 70% storage, the liver is the main tissue site of vitamin A storage in the body. This is in addition to the extrahepatic uptake of chylomicron and remnants, where small portions of retinyl esters and carotenoids are transferred for usage and storage [31] (Figure 2). Once retinol has formed in liver stellate cells from its storage structure of retinyl esters, it binds to a specific transport protein known as Retinol binding protein (RBP) at the hepatocytes. The exact mechanism of which has been the subject of much research. The resultant compound of retinol-RBP then binds to transthyretin in the circulation [35]. This process prevents the renal clearance of retinoids [36]. Immediately following consumption of a meal, retinoid is found in a significant proportion within the circulation in the form of chylomicron retinyl esters while retinol-RBP complex constitutes for the vast majority present in fasting conditions [37].

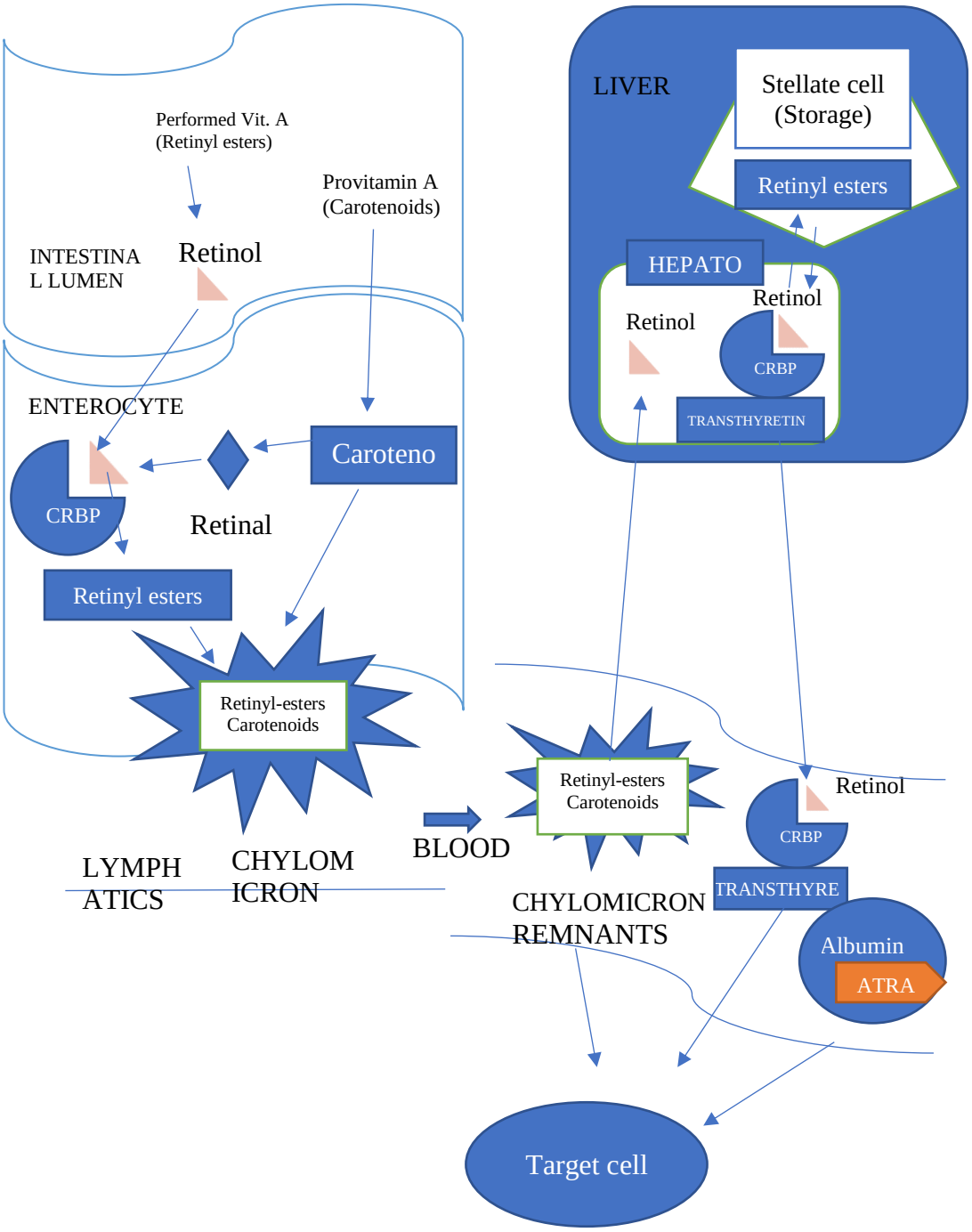


Figure 2: The absorption and transport of vitamin A highlighting the uptake pathway from dietary sources (Dietary uptake and transport of vitamin A) [7]

1.7 Metabolism of vitamin A

Retinoids within the circulation are taken up by peripheral tissue in the form of an all-trans-retinol complex with RBPs. A small proportion of dietary retinoid accounting for 25-33%, however, is taken by other non-hepatic tissue in chylomicrons [31].

Research studies in-vivo have found that chylomicrons deliver vitamins to bone-building cells known as osteoblasts making the bone an essential organ in the uptake of chylomicron remnants [38].

Peripheral tissue uptake of chylomicron retinyl esters occur following the hydrolysis of chylomicron retinyl esters by the action of lipoprotein lipase. The uptake Retinol-RBP complex, on the other hand, occurs predominantly via a specific cell surface receptor known as STRA6, which is a multi-transmembrane retinoic acid-responsive gene. Furthermore, the active form of all-trans-retinoic acid (ATRA) is found albumin-bound in small amounts in serum, contributing to tissue uptake of ATRA [39].

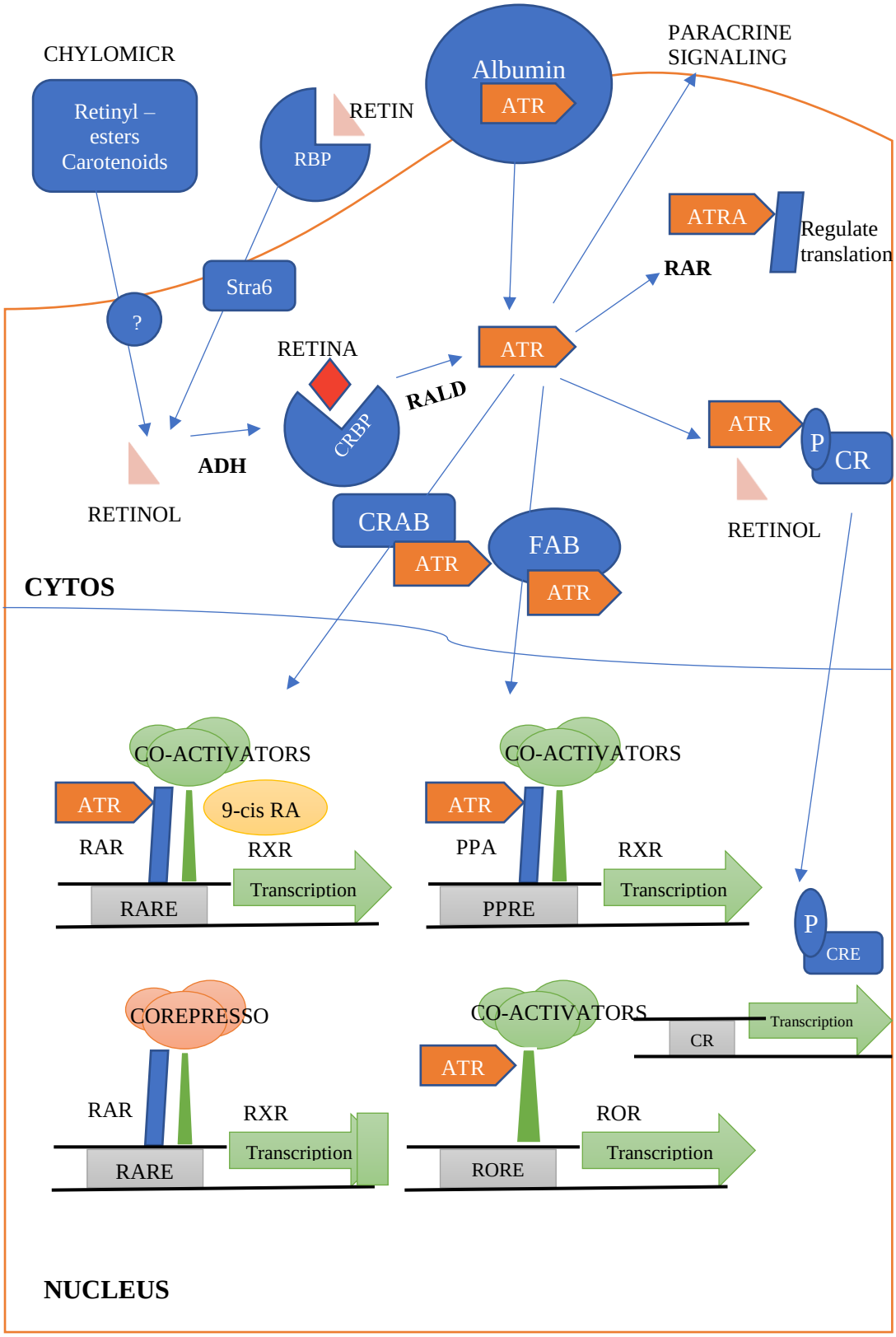


Figure 3: Receptor-mediated cellular uptake of retinoids as well as the signalling by Retinol and its serum binding protein

Immediately post the uptake of retinol by cellular tissues, it is transformed into the all-trans-retinal by oxidation with the action of dehydrogenases within the cytosol. It then binds with CRBP. Following this, the biologically active complex ATRA is formed as all-trans-retinal is oxidised in a process regulated by retinal dehydrogenase. Cellular retinoic acid-binding protein (CRABP) selectively binds ATRA, the most active retinoid metabolite. Retinoids exert their effects through the binding to nuclear receptors, mainly retinoic acid receptors (RAR) and retinoid X receptors (RXR), alongside the action of response elements and multiple core regulators [26] (Figure 3). Both RAR and RXR families exhibit three receptor isotypes (α, β, γ) with at least two different isoforms present for each isotype [40].

Retinoid receptors bind to retinoids in the form of dimers leading to the activation of retinoic acid response elements (RAREs) in the promoter parts of genes at the target site. RARs only work in the form of heterodimers with RXRs (RAR/RXR), with RXRs able to also function as only homodimers (RXR/RXR). RXRs also possess the ability to form heterodimers with various nuclear receptors like vitamin D, demonstrating the involvement of retinoids in various cellular pathways. Research has shown that RARs activation is mediated by ATRA and the isomer9-cis retinoic acid (9-cis RA) *in vitro*, while RXRs bind to 9-cis RA, resulting in its activation. The majority of cellular retinol signaling is believed to occur with the binding of ATRA to RAR in RAR/RXR heterodimers [41].

Active RARs act as ligand transcription factors, binding RAREs in the promoters of target genes [40, 41]. RAREs are made up of hexameric motifs organised as 1, 2, or 5 palindrome pairs DRs or inverted repeats. RA can be shuttled to the nucleus by cellular CRABP-II, which facilitates the movement of ATRA to RARs. Retinoic acid receptors

serve an essential role in the regulation of this pathway through the recruitment of co-activators and corepressor proteins controlling the transcription of target genes depending on the presence of the retinoic acid ligand. In the absence of the ligand, the RAR/RXR complex represses the transcription process by recruiting corepressors, while the binding of ATRA to the RAR/RXR dimer leads to the recruitment of co-activators. Examples of co-activators include steroid receptor co-activator (SRC)/p160 family and p300/CREB-binding protein (CBP) while corepressors include nuclear receptor corepressor (NCoR), silencing mediator of RAR and thyroid hormone receptor (SMRT), mSin3A, and histone deacetylases (HDACs) [40, 42-44] (Figure 3). It has been found that RAR-mediated gene repression is essential in skeletal progenitor differentiation signalling pathways to regulate the emergence of the chondroblast phenotype [45, 46].

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear hormone receptor with three subtypes PPARα, PPARγ, and PPARβ/δ. PPARs dominate the capability to produce heterodimers with RXR, as well as RARs and RXRs. Following heterodimerisation with RXR, PPARs function as a sequence-specific target factor in the promoter region of target genes [47, 48] (Figure 3).

As well as functioning as a ligand RARs, it has been found that ATRA can also bind to PPARβ/δ [43]. The fatty acid-binding protein 5 (FABP5) mediates this pathway by shuttling ATRA to the nucleus, enabling PPARβ/δ activation, allowing the subsequent binding to PPAR response elements (PPREs) to take place [49, 50]. The notion that dual transcriptional activities from alternate activation of two different nuclear receptors (CRABPII/RAR or FABP5/ PPARβ/δ) underlie opposing effects of RA on

cell growth has been shown in keratinocyte proliferation and carcinomas, with a paucity of evidence in bone cells.

Retinoid-related orphan receptors, mainly RORα and RORβ, represent another group of transcription factors that have been found to bind to retinoids [51]. While RARs and PPARs bind RXR is a heterodimer binding mode, RORs undertakes monomeric DNA binding to specific ROR response elements (ROREs) [52]. Recent studies have shown that RORβ has resulted in the failure of mineralisation and osteoclast differentiation [53].

The impact of retinoids at a genomic level can, therefore, be demonstrated through the regulation of multiple physiological processes through the retinoic acid receptor-dependent transcription pathway encompassing the ligation of ATRA to different types of nuclear receptors and the binding to the relevant response elements. In addition to this classical genomic-based mechanism, retinoids can also exert non-genomic effects. These nongenomic effects could either be facilitated by RARs or without RARs involvement.

Non-genomic effects mediated by RARs include RARs inhibitory effect as an RNA-binding protein within the cytoplasm of neuronal cells [54, 55]. The regulation of ERK1/2 kinase phosphorylation is an example where non-genomic effects are displayed independent of RARs. Phosphorylated ERK1/2 leads to phosphorylating various microtubule-based proteins, and it also translocates to the nucleus resulting in the activation of various transcription factors such as cAMP response element-binding (CREB) protein which is important for axonal growth [56] (Figure 3). This action is not only exerted by ATRA but also by retinol [57].

Adipose tissue is a major storage area for retinol, where it has shown a potent inhibitor activity of adipocyte differentiation, as well as inhibiting of PPAR activity, and improves sensitivity to insulin [55]. This finding highlights that retinal could be a further compound derived from vitamin A, which is essential in facilitating a biological function. Unlike Retinol, ATRA acts as a paracrine factor due to its ability to pass through hydrophobic membranes, which is attributed to its partial water solubility. These acts display retinoic acid immersion and engagement acting as a morphogen in early embryonic development.

1.8 Vitamin A deficiency

Safe and optimal vitamin A supplementation has shown a considerable positive influence on improving several physiological functions, including the immune system. With the exception of developing countries where there have been high levels of vitamin A deficiency, this simple and cost-effective approach has had a detrimental impact on reducing mortality worldwide [58]. The application of therapeutically used retinoids extended to reducing acne outbreaks. It has also been integrated into the management of different cancers, including acute promyelocytic leukemia, squamous cell carcinoma of the head and neck as well as ovarian cell carcinoma [59].

There are several factors affecting retinol levels within the body, with increased levels associated with an increase in age as well as high body mass index in children [60, 61]. This could be attributed to increased intestinal uptake and reduced clearance. It has also been found that the level of retinyl esters in the serum is higher in females than males and in those who self-medicate with vitamin supplements [63-66]. Normal values range from 1-3 µmol/L, with suboptimal vitamin A status at <1.05 µmol/L and a deficiency diagnosis with <0.7 µmol/L [60, 61].

Measuring vitamin A levels in the human body has been the subject of much research. Serum retinol and retinyl ester concentrations have been used as commons methods in order to assess the status of vitamin A. However, with the controlled release of vitamin A from its liver storage reserves only when needed, this method is only beneficial at extreme amounts of vitamin A, when these are either dangerously low or high [62]. While serum retinyl esters have been found to be beneficial for chronic cases, other sensitive biochemical methods to marginal changes in vitamin A storage are necessary in order to ascertain clinically significant levels with relevance to toxicity [63]. These encompass dose-response tests and isotope dilution assays [62].

Serum retinyl ester concentrations of 0.2 μmol/L, or more than 10% of total serum vitamin A, including both retinol and retinyl esters, provide an indication of abnormally high and potentially toxic concentrations. The scale of an increased vitamin A level has been highlighted by the Third National Health and Nutrition Examination Survey, where cases of more than 10% of total vitamin A has been significantly high, showing an increased incidence [64].

While a high intake of preformed vitamin A or retinoid derivatives can lead to hypervitaminosis A, pro-vitamin A carotenoids such as B-carotene do not cause hypervitaminosis A, even when consumed in large quantities. This is due to the negative feedback regulation, which limits the conversion of B-carotene to Retinol in order to align with the actual need of the body [65]. Excessive dietary carotenes can cause carotenemia, yellowing of the skin, which is a reversible medical condition on stopping the ingestion of beta-carotene [66].

Evidence suggests that hypervitaminosis A existed a very long time ago with a partial Homo erectus skeleton displaying pathological bony alternations in the outer cortex of

the tibial shaft, corresponding with chronic hypervitaminosis A [67]. It has been suggested that this was a result of the change in the Homo erectus diet at the time to include animal liver, which is a rich source of vitamin A, leading acutely to vertigo, vomiting, diarrhea, headache, seizures, patchy hair loss, and even mortality in some cases. The earliest known description of this illness in the West was documented by Gerrit de Veer, an early Dutch explorer on a 1596 voyage with William Barentsz to find the Northwest Passage [68]. De Veer's diary describes a sickness that occurred after his crew ate polar bear liver—which contains a high concentration of vitamin A. Similarly, in the 1850s, Arctic explorer Elisha Kent Kane initially thought that the widespread caution about polar bear liver was a "vulgar prejudice," but he subsequently changed his mind: "The cub's liver was my supper last night, and today I have the symptoms of poison in full measure-vertigo, diarrhea, and their concomitants" [69]. In the Far Eastern Party of the 1911-1914 Australasian Antarctic Expedition, acute hypervitaminosis A may have caused sickness and death among many polar explorers who ate the livers of Greenland husky sled dogs [70].

Hypervitaminosis A is a rare condition with higher incidence found in children generally attributable to therapeutic retinoids or candy-like chewable vitamin supplements [71]. The rise in misconceptions related to the safe use of vitamins without realisation of the need to ensure consumption is within the recommended daily limit makes this disease of importance in terms of severity and the potential increase in incidence. The highest risk is believed to be related to emulsified, water-soluble, and solid preparations, such as tablets [71].

Hypervitaminosis can lead to skeletal lesions as evident in studies showing a characteristic thinning of the cortex and a decrease in the diameter of the long bones

(Figure 4). Findings have also shown a strong association between excessive vitamin A intake and an increased risk of fracture as exposure to toxic doses of retinoids resulted in spontaneous fractures in rats [72]. A further indication of the skeletal changes associated with hypervitaminosis A reduced endosteal/marrow blood flow accompanied by a higher degree of mineralisation has been shown in rats with hypervitaminosis A. Furthermore, the bone marrow of rats ingested with retinol displayed a high level of osteogenic genes [73].

While most studies have been undertaken on retinoid-treated rats with exposure to excessive amounts that do not lead to toxic levels in humans, mature rats that received considerably lower doses of vitamin A still displayed long thin bones and decreased biomechanical strength [74]. This supports the notion that humans with subclinical elevations in vitamin A are at an increased risk of fractures and osteoporosis.

The skeletal effects studied in humans with an abundant consumption of retinoids found there to be periosteal bone formation characterised by hyperostosis in metatarsals, metacarpals, and the long bones in the body. Other skeletal effects were found comprised of premature epiphyseal closure, skeletal and joint pain, and increased cortical bone resorption [75, 76].

In one microradiography study of a human rib, large consumptions of vitamin A displayed six times more cortical bone resorption as well as suboptimal bone formation (Figure 4) [77]. Even after the restoration of normal vitamin A consumption, humans who previously consumed too much vitamin A had evidence of new subperiosteal bone, particularly in the tibial and fibular shafts [78].

It is worth noting that hypercalcemia has been linked with hypervitaminosis A in numerous case reports, likely because retinoids directly promote bone resorption [79, 80]. This hypercalcemia can be addressed with glucocorticoids, which have been shown *in vitro* to reduce hypercalcaemia by activating the monomeric glucocorticoid receptor and thus opposing bone resorption of ATRA [79-82].

Figure 4 shows that increased vitamin A intake leads to decreased cortical bone mass by increasing the number of osteoclasts on the periosteal surfaces as shown in part A. Part B highlight the controversy of the role of Vitamin A on trabecular bone some studies showing effects on bone mass. In contrast, others provide evidence otherwise. Part C shows the role Vitamin A plays in inhibiting adipocyte differentiation, as shown *in vitro* studies.

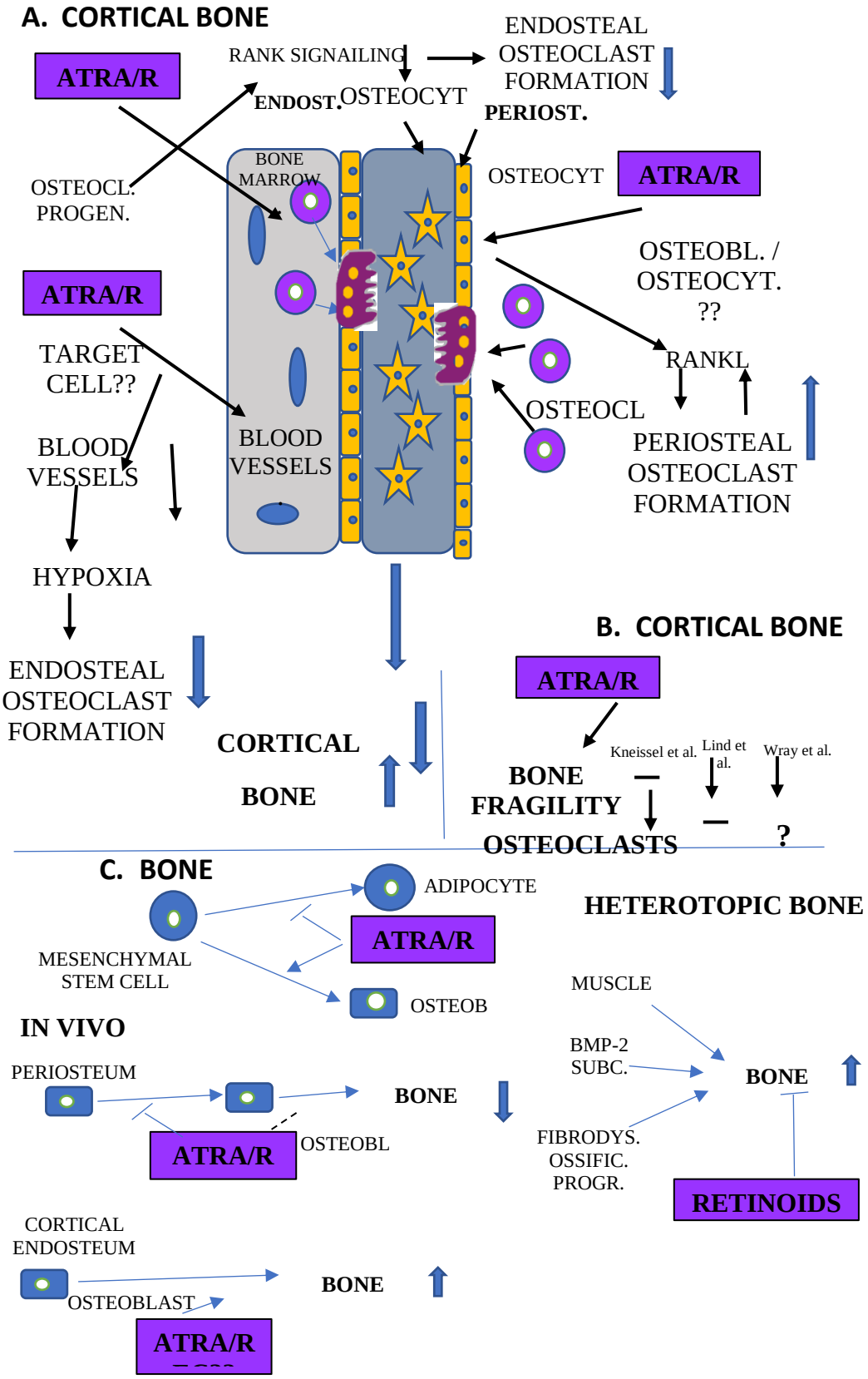


Figure 4: Association of Hypervitaminosis A with reduced cortical bone mass

Table 1 shows examples of HPLC-MS/MS methods for the detection of vitamin A. Pre-treatment and determination methods of vitamins have increasingly been a subject of research interest over the years. Analytical techniques include a pre-treatment process in order to minimise interference of other components and as such enhance the analytical performance. Pre-treatment approaches are varied and include liquid-liquid extraction (LLE), ultrasonic assisted extraction (UAE) and super critical fluid extraction (SFE) alongside other processes (Table 2). Each of these techniques have specific advantages and disadvantages which have been discussed in many studies (Table 3). Depending on the type of sample, UAE, SFE and reflux are the most widely used for solid preparations while LLE, solid-phase extraction (SPE) and Dispersive Liquid–Liquid Microextraction (DLLME) are the techniques of choice in the liquid phase formulations. SFE offers a good efficiency of extraction despite its expensive instruments when compared with UAE. In discussing column passing, processes like SPE can be complex. Nonetheless, SPE provides an ability to prepare several samples at the same time, significantly reducing the total time of the process. In addition, SPE could be coupled with LC to provide analysis within an online platform [20, 83, 84].

As well as the various types of pre-treatment methods, there are many analytical approaches and these include chromatography techniques, electrophoretic methods as well as microbiological and immune assays. The most widely used techniques in the determination of vitamins are those that are based on High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC–MS) or (HPLC-MS/MS) (Table 1). Advances within this technique have led to the development of ultra-high-performance liquid chromatography (UHPLC) which utilises sub-2-μm particle columns with a subsequent enhancement of the chromatographic performance,

particularly within resolution, sensitivity and speed. Combining UHPLC with MS detectors has also shown several advantages [17, 18, 85].

In discussing advantages, Liquid Chromatography (LC) offers a wide variety of qualitative and quantitative analytical benefits in various matrices, including foods and biological preparations, particularly when used with MS. The use of other techniques such as ultraviolet (UV) and Fluorescence Detectors (FLD), has been limited by overlapping peaks with complex samples [17, 18, 85].

The key favourable characteristics of LC-MS/MS is the increased specificity and sensitivity in comparison to other methods. This is because it can easily distinguish between various metabolites through chromatographic separation and mass transition variation. LC-MS/MS methods can separate samples with different polarities and mass to charge (m/z) ratios. In addition, this method posses very Low Limits of Quantitation (LOQ), subsequently providing a higher sensitivity. Therefore, LC-MS/MS provides accuracy and precision [15].

HPLC-MS/MS can also act as a confirmatory technique in the identification of vitamins leading to easily identifying the sample's molecular structure. Utilising MS/MS provides flexibility as complete HPLC separation of the sample constituents would not be essential for selective detection. Key drawbacks for HPLC-MS/MS includes high costs and matrix effects. In essence, matrix effects are when high contents of organic components, from extracts of various matrices, affect the evaporation and ionisation of the analytes [15].

Measuring vitamins in different samples was carried out successfully in many studies utilizing methods based on HPLC-MS/MS. With reference to vitamin A, Midttun and

Ueland [17] quantified three types of vitamins in a small amount of human plasma at the same time. The LOD for trans-retinols were 0.10 μM for 25-OH D2 while 25-OH D3 showed 3.3 nM. Therefore, HPLC-MS/MS can meet the requirements for measuring vitamins and can be applied to preparations of biological nature. The main key advantages involve simplicity with minimal preparation, pace (analysis time does not take longer than 5 min) and high sensitivity [20, 83, 84].

Table 1: Examples of HPLC-MS/MS methods for the detection of vitamin A

Instrument Analysis Methods	Limit of Quantification (LOQ)	Limit of Detection (LOD)	Mobile Phase	Column	Analysis Time (min)	Ref.
High Performance Liquid Chromatography–Ion Trap Mass Spectrometry (HPLC–Msn)	0.1 μg/100 mL for all trans-retinol and α-tocopherol and 1 μg/100 mL for β-carotene	no report	A: water; B: methanol. Gradient	Polaris C_{18} column (2.1 × 150 mm, 5 μm)	26 min	[83]
Liquid Chromatography/Tandem Mass Spectrometry	no report	0.1 μM for all-trans retinol, 3.3 nM for 25-OH VD_2 and 25-OH VD_3	A: Ammonium formate in MeOH; B: H_2O. Gradient	Ascentis Express C_{18} column (4.6 × 50 mm, 2.7 μm)	6 min	[17]
HPLC-MS/MS	0.2–520 ng/mL	0.07–170 ng/mL	A: 10 mM ammonium acetate solution (pH 4.5); B: MeOH with 0.1% acetic acid; C: MeOH with 0.3% acetic acid. Gradient	ACE-100 C_{18} (2.1 × 100 mm, 3 μm)	30 min	[18]
LC-DAD-MS/MS	2.7–46.8 μg/L	0.9–15.6 μg/L	A: Methanol; B: isopropanol/hexane (50:50, *v*/*v*). Gradient	a Supelcosil C_{18} (4.6 mm × 50 mm, 5 μm) and an Alltima C_{18} (4.6 mm × 250 mm, 5 μm) for fat-soluble vitamins, ProntoSIL C_{30} column (4.6 × 250 mm, 3 μm) for carotenoids	30 min	[85]
LC-MS/MS	n.d.	n.d.	A: 20 mM ammonium formate (pH 4.0); B: methanol. Gradient	Cadenza CD-C_{18} stationary phase (4.6 × 250 mm, 3 μm particles)	45 min	[86]
SPLC-MS/MS	n.d.	n.d.	(retinol-d6 and α-tocopherol-d6) in 70% methanol in acetonitrile	Cyclone P (5 × 50 mm) TurboFlow™ column (Thermo-Scientific, Waltham, MA). Shimazu, Kyoto, Japan) equipped with a Brownlee Spheri-5 RP-18 (4.6 × 250 mm) column.	7 min	[87]
LC-MS method	1.4 ng	1.6 ng	Mobile phases A and B for fat-soluble vitamin method were 9:1 (v/v) ACN/water and 100% MeOH, both containing 5 mM ammonium formate	Fat-soluble vitamins and their ISs were retained in the chromatography column	18 min	[88]
LC–MS/MS	0.00972 mg/L	n.d.	Mobile phase A contained water with 0.1% formic acid, and phase B consisted of methanol.	BEH Phenyl column (2.1 mm × 100 mm, 1.7 μm).	4.5 min	[89]
LC–MS/MS	0.1 μmol/L	0.1 μmol/L	Mobile phases A (MpA) 0.1% formic acid and 2% methanol in milli-Q water	column (Pursuit Pentafluorophenyl (PFP) (150 mm × 2 mm × 3 μm, Cat no. A3051150 × 020)	10 min	[90]
LC-MS/MS	n.d.	n.d.	Mobile phases B (MpB) 0.1% Formic acid in methanol Mobile phase additives, formic acid and ammonium formate, LC-MS grade water and methanol, Acetone.	A Restek Raptor Biphenyl (2.7 μm dp, 2.1 mm ID, 100 mm length; Bellefonte, PA) analytical column and a Shim-pack MAYI-C4(HP) (10 mm × 4.6 mm, 50 μm dp) high pressure restricted access media trap column from Shimadzu were used	12 min	[91]

Table 2: Pre-treatment methods, sample matrices and targets of the recent articles

Pre-treatments	**Determination Methods**	**Sample Matrix**	**Analytes**	**Ref.**
liquid-liquid extraction (LLE)	liquid chromatography-ultraviolet detection (LC-UV)	Human serum	Vitamins A (retinol, retinyl esters), E (α- and γ-tocopherol) and D (25-OH vitamin D)	[92]
LLE	LC-UV	Human serum	All-trans-retinol, retinyl acetate, a-tocopherol, a-tocopheryl acetate	[93]
UAE	LC-UV	Vitamin tablets	10 vitamins (7 water-soluble and 3 fat-soluble)	[94]
LLE	LC-UV	Pharmaceutical formulations	Fat-soluble vitamins	[95]
UAE	LC-UV	Food samples, human plasma and human adipose tissue	Retinol, tocopherols, coenzyme Q_{10} and carotenoids	[96]
LLE	LC-MS	Bovine milk	Vitamins A, E and b-carotene	[83]
LLE	LC-MS	Human plasma	Vitamins A, D and E	[97]
UAE	LC-DAD-MS	Green leafy vegetables	Fat and water-soluble vitamins	[18]
UAE	LC-MS	Nutritional formulations	Fat- and water-soluble vitamins	[85]
UAE	MEKC	Commercial multivitamin pharmaceutical formulation	Water- and fat-soluble vitamins	[86]
UAE	MEKC	Multivitamin formulation	Water- and fat-soluble vitamins	[98]
LLE	MEKC	Multivitamin tablets and vitamin E soft capsules	Fat-soluble vitamins	[19]
Dilute and shoot	Spectrofluorimetry	Multivitamin drugs, food additives and energy drinks	Fat- and water-soluble vitamins	[99]
Centrifugation	HPLC, ELISA	Serum	Vitamins A, C and D	[100]
SPE	LC-UV	Plasma	Retinol and α-tocopherol	[19]

Table 3: Determination methods advantages and disadvantages

Determination Methods	**Advantages**	**Disadvantage**
LC-UV [94]	• Nondestructive, very reliable, and easy to use. • Near-universal detection of organics at low UV wavelength; compatible with gradient analysis.	• Analytes must have chromophoric activity. • The mobile phase must be transparent to UV to have acceptable sensitivity and linearity
LC-MS [97]	• Simultaneous multianalyte analysis • The ability to detect sensitivity, mass accuracy, and resolution • Good capability of separating complex samples	• Expensive • Not portable • Requires an experienced technician
MEKC [98]	• limited elution time means relatively short separation time at high efficiencies • high separation power • separates ionic and neutral compounds • Only requires a few nanoliters of sample • Can separate small molecules	• limited elution time limits peak capacity of technique • generally limited to compounds which are reasonably soluble in the mobile phase • low sensitivity in low concentrations
HPLC, ELISA [100]	• Simple procedure • High specificity and sensitivity Easy to perform with simple procedure	• It required effort and time and expensive to prepare antibody • The used culture media is expensive as well as it requires sophisticated techniques. • High possibility of false positive/negative • Antibody instability

Chapter 2: Materials and Methods

2.1 Materials

Standard metabolites and other chemical solvents for this project were obtained from different sellers' company. Vitamin A standard in the form of Retinol, 25-Hydroxyvitamin D3 (6, 19, 19-d3), Albumin from human serum was purchased from Green Oasis Medical Equipment Installation and maintenance (Gomet). Phosphate buffered saline, and other solvents such as methanol, deionised water, hexane, ethyl acetate, formic acid, ammonium format, LC-MS-grade water, and LC-MS-grade methanol were purchased from Emirates Scientific and Technical Supplies L.L.C in Abu Dhabi (ESTS). Ascentis Express F5 column (dimensions: 150 mm x 2.1 mm x 2.7 um) was purchased from LABCO LLC.

2.2 Methods

2.2.1 Preparation of standard solutions

The ethanol has been used to prepare the stock solution of Vitamin A (Retinol) and 25-Hydroxyvitamin D3 (6, 19, 19-d3) (Internal Standard) at a concentration of 1mg /ml (1000 ug/ml). Amber vials have been used to store the stock solutions at -20°C to avoid degradation. The diluting and mixing stock solutions in methanol solvent have been used to prepare the working solutions of Vitamin A and Internal Standard by using the methanol solvent to reach a desired concentration for the analysis. The preparation of working solutions was protected from exposure to the light during the laboratory process to minimise vitamin degradation.

2.2.2 Collection of blood samples

Blood samples (1 mL) were attained from 277 Emirati individuals from obese Emirati patients including 277 baseline, 277 follow-ups, while the study covered additional 175 healthy participants. The characteristics of the sample as follows: mixed-gender with 73 males in the age group of (18–82 years) and 204 females in the age group of (18–65 years). While in the health sample the number of males is 8 while the number of females is 167. A centrifugation process to separate serum with a scale of 1500x has been implemented for the collected blood samples, and the resulting serum was extracted. Afterwards, a freezing process was completed by freeze the serum samples individually in labelled plastic amber vials. The ethics approval has been granted and approved by UAEU research ethics committee under ethics approval of Protocol number (AAHEC-3-17-055) for recent Vitamin D study samples.

2.2.3 Extraction method

The human plasma samples were stored in the refrigerator at -80oC. Once the analysis of these samples begins, they were thawed to room temperature. 500 µl of serum samples were treated into Culture Tube, Borosilicate Glass, 16 x 100mm, 14mL. Subsequently, A 20 µl of Internal Standard were added with a concentration of 1 µg/ml to spike the samples. After that, Internal Standard and an aliquot of plasma sample solution were vortexed for 1 minute. Then, 1.0 mL of hexane: ethyl acetate (9:1) mixture has been added in the process to extract vitamin A and Internal Standard in the plasma samples and later vortexed for a few seconds. After vortexing the samples, they were centrifuged at 4000 rpm (round per minute) for 20 minutes at regular conditions. After that, the resulting supernatant was separated using Pasteur pipettes into a new set of a borosilicate culture tube glass. The remaining lower layer was

further extracted twice by repeating the same steps mentioned above, starting from 1.0 mL of hexane: ethyl acetate (9:1) mixture. A further and additional extraction has been conducted in which the resulted extracts were combined together into a new test tube; then dried using room temperature under a flow of air in a sample concentrator. The dried samples were then re-concentrated by adding 100 µl of LC-MS/MS grade methanol. Finally, the samples were transferred into LC-MS/MS amber vials and placed in an autosampler where the samples were kept at 4°C. A 5 ul in LC-MS/MS instrument has been set under the injection volume of sample extract.

2.2.4 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

The Chromatogram of vitamin A was achieved with the Nexera ultra-high-pressure liquid chromatography (UHPLC) system (Shimadzu, Japan) on a reversed-phase column Ascentis Express F5 column (dimensions: 2.7 µm particle size, L x I.D. 15cm x 2.1 mm). The F5 column for physical filtration has been connected to the guard column. The temperature for the column was kept at 30°C with the mobile phase flow rate of 0.5 ml/min in the UHPLC system. There are two mobile phases that were utilised in the system (Figure 5):

- Mobile phase A is the LC-MS/MS grade water with 0.1% formic acid or 5 mM ammonium format.
- Mobile phase B is LC-MS/MS grade methanol with 0.1% formic acid or 5 mM ammonium format.

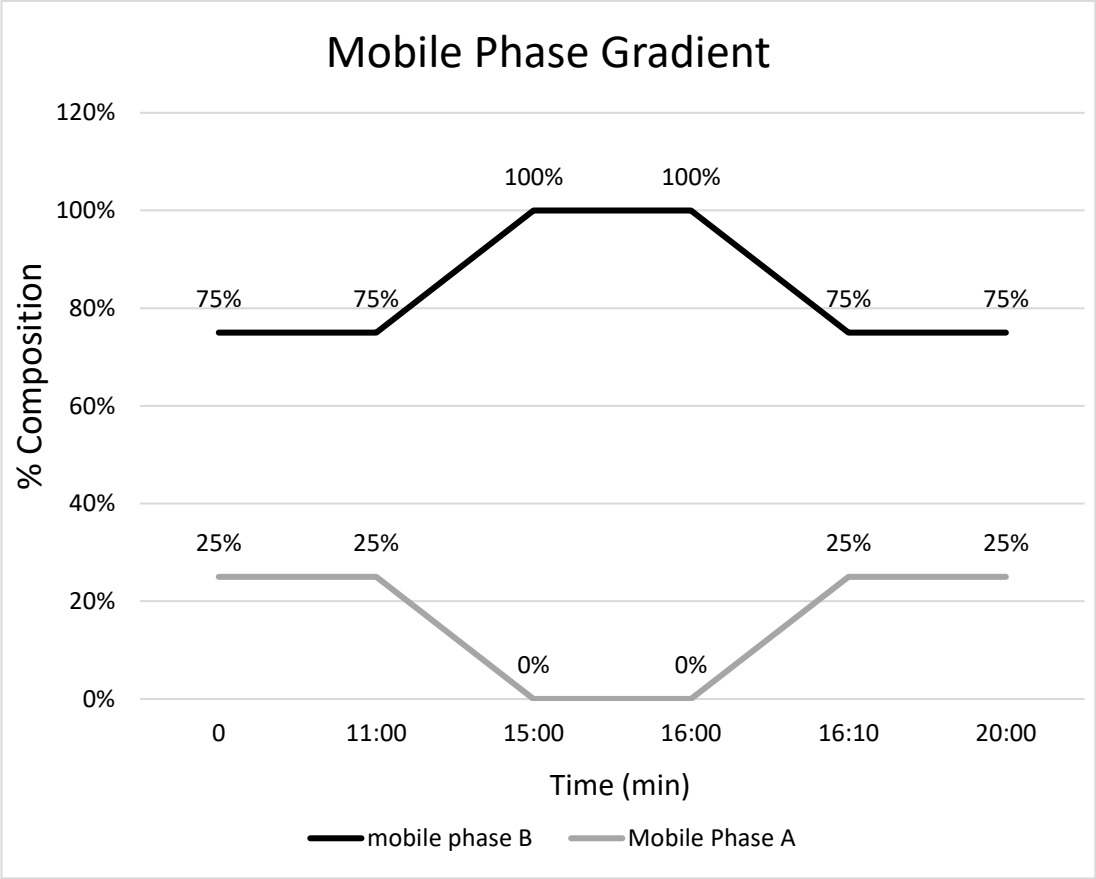


Figure 5: Mobile phase gradient elution profile used for the LC-MS experiments

Mobile phase A and B are presented in the upper and lower line, respectively. The amount of Mobile phase A presented in water with 5mM ammonium format, while the amount of Mobile phase B presented in methanol with 5mM ammonium format. As shown in the figure, the upper line begins at 80% of the composition and steadily increases to 100% before decreasing to 80% at the time of 16:10. At the same time, the lower line begins at 20% and steadily decreases down to 0% before increasing to 20% at the time of 16:10.

The mobile phase gradient was set as follows: The mobile phase B was set at 75% for 11 min, while mobile phase A was set at 25%. Afterwards, the mobile phase B was increased to 100% from 11-15 min and retained the same percentage up until to 16 min whereas mobile phase A reduced to 0% for the duration of that period. Then, the

percentage of mobile phase A increased to 25% while the percentage of mobile phase B was reduced to 75% from 16:00 to 16:10 min and maintain the percentage of both mobile phases consistent to 20:00 min, where the mobile phase gradient ends. The mass spectrometry analysis was conducted on Shimadzu, model 8060, with a triple quadrupole mass spectrometry system that is operated in positive electrospray ionisation (ESI) mode (Figure 6).

Figure 6: LC-MS/MS - Shimadzu, model 8060 - UAEU laboratory

The Nebulising gas flow was set at 2 L/min while drying, and heating gas flow was 8 L/min at 300°C of interface temperature (Table 4). It has been shown that the increased flow rate results in reduced signal intensities. This decrease in signal intensity could be attributed to the loading of plasma with the gas which may change its nature through various reactions eventually leading to interference with the signal intensity [15]. The positive electrospray ionisation (ESI) mode has been used to function the mass

spectrometer (MS); it was beneficial due to the achievement of enhancement of ion signal intensity and reducing noise. The favour goes to the newly developed UF-Qarray ion guide technology that increases sensitivity. A new level of sensitivity is presented in the used instrument of LCMS-8060, where data is controlled, functioned and analysed using the Shimadzu's Lab Solutions software.

Table 4: MS parameters for nebulising, drying, and heating gas flow, followed by interface and heating block temperature

Ms Parameters	**Ms Conditions**
Nebulising gas flow	2 L/min
Drying gas flow	8 L/min
Heating gas flow	8 L/min
Interface temp.	300°C
Heating block temp.	400°C

2.2.5 Validation method

The validation has been implemented based on the requirements and recommendations of the US Food and Drug Administration (FDA) guidelines for method validation. Accordingly, the validation has been conducted for the purpose of stability, recovery, accuracy, specificity, precision, and linearity [101]. There are four control limits have been stated (QCs) in four different concentrations as following: higher quality control limit (QCH) with a concentration of 800 ng/mL, (QCM) medium quality control limit with 400 ng/mL and (QCL) lower quality control limit with a concentration of 200 ng/mL, and finally, (LLOQ) with a concentration of 100 ng/mL. The stated quality

controls have been analysed to calculate the linearity, intra-day and inter-day precision, and accuracy of the vitamin A assays. The analysis, in each validation, includes the six controls at each concentration level (QCH, QCM, QCL, and LLOQ) together with a calibration curve. A blank serum has been used to prepare the calibration curves and all quality control samples. A published recipe has been used to make the blank serum, in which the human serum albumin is mixed with phosphate-buffered saline at a concentration of 50 g/L [102]. The recovery was calculated in the recovery experiment, where the six quality control samples at four different concentrations (800 ng/mL, 400 ng/mL, 200 ng/mL, and 100 ng/mL) were spiked with methanol. A repeated experiment with four similar concentrations was used, with blank serum samples spiked with quality controls, and the recovery was calculated and compared using the peak area results (or area under the normal curve). Then a stability experiment was measured and checked by examining six controls at each concentration level (800 ng/mL, 400 ng/mL, 200 ng/mL, and 100 ng/mL), executing three freeze/thaw cycles of the spiked quality controls at 0 h, 24 h, 48 h, and 72h.

The representative chromatogram of vitamin A is spiked and taken out using the retention time and multiple reaction monitoring (MRMs) for the accurate quantification of small molecules in serum. As mentioned above in the validation approach followed in the study there are six QC samples were analysed at four different levels of concentration (QCH = 800 ng/mL, QCM = 400 ng/mL, QCL = 200 ng/mL, LLOQ = 100 ng/mL). These standards, as shown in Table 5, are used to gauge the average, intra/inter-day accuracy, specificity, recovery, linearity, stability, intra/inter-day precision. Each value in those parameters was calculated based on approved equations. The equation of [(mean value/nominal value)×100] has been used

to calculate the percentage of intra/inter-day accuracy of the assay of the QC values. While the equation of [(standard deviation/mean)×100] has been used to calculate the percentage of intra/inter-day precision (% CV) values from QC values. The equation of [(mean unextracted QC values/mean extracted QC values)×100] has been used to calculate the percentage of absolute recoveries. The lower limit of detection (LOD), which is LOD is the lowest analyte concentration can be reliably detected from the limit of blank and at which detection is feasible. It is calculated based on the Signal-to-Noise approach by correlating the signal-noise ratio to the analytes lowest concentration, decided by reducing the analyte concentrations until a response equivalent to three times "3x" the detected background level. The generally acceptable detection limit for the single/noise ratio equals 3 "three".

Chapter 3: Results and Discussion

3.1 Validity results

The favourable linear range for all the analytes was 7.8125–1000 ng/mL, with a very good regression value (R^2 = 0.999). Finally, as appearing in Figure 7, the specificity of the validation was calculated by coating the peaks to check for any interfering/co-eluting peaks at the particular retention times of the analytes. The specificity calculation based on the outcomes achieved by extracting and analysing many blank serum QC samples.

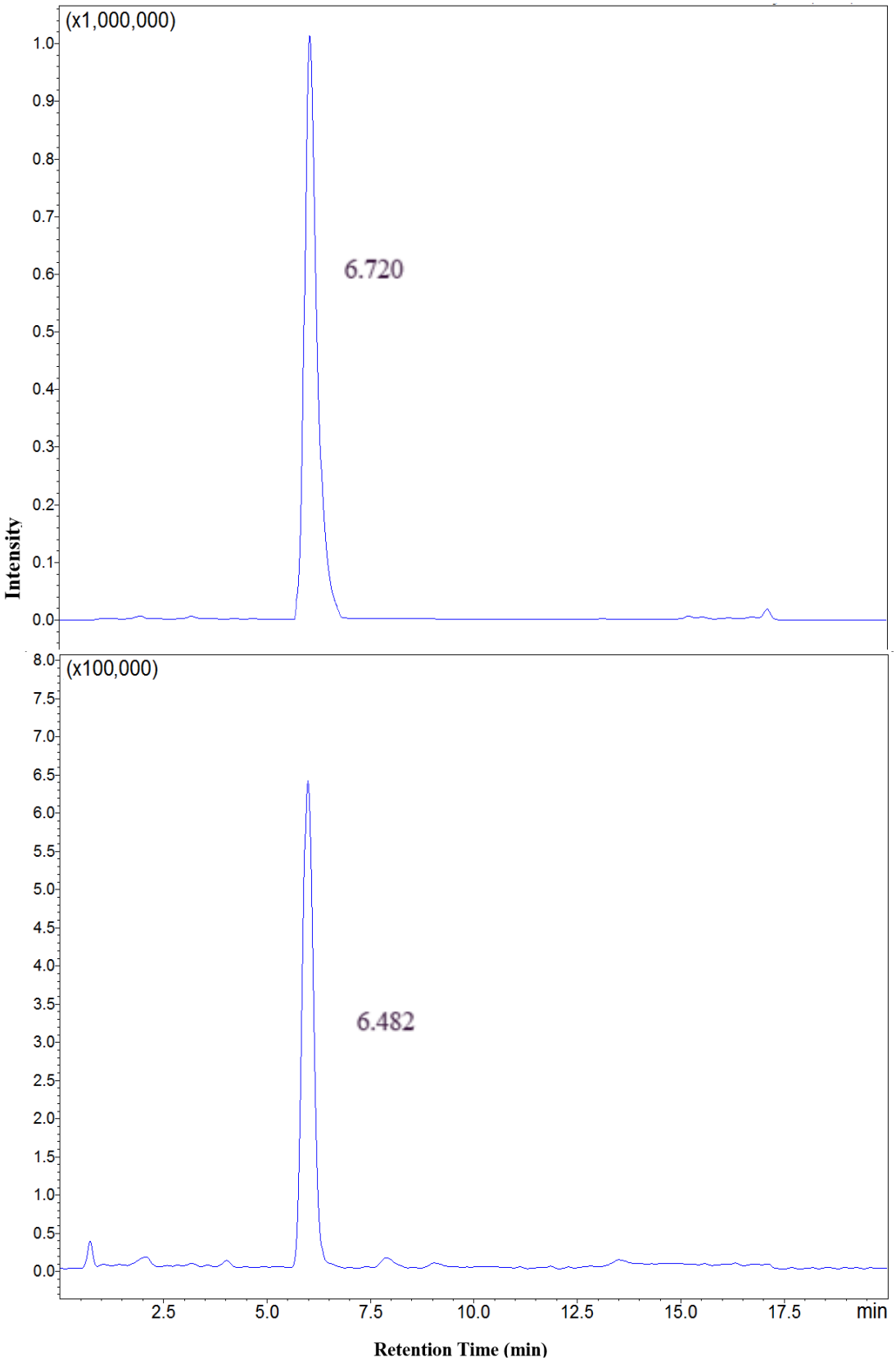


Figure 7: A spiked and extracted representative chromatogram of vitamin A along with the retention time and multiple reaction monitoring (MRMs) with internal standard 25-Hydroxyvitamin D3 (6, 19, 19-d3)

Shimadzu 8060, which represents a novel instrument that has been found to offer a high degree of sensitivity, was selected to ensure accurate detection of analyte ions over a 20-min run. The MRM transitions were selected from the [M+H]+ protonated molecules in the electrospray ionisation source. The MRM mode was used to monitor the most sensitive precursor ions, which were predominantly seen after the retinol Vitamin molecule lost a water molecule and the most sensitive product ions. A direct infusion of standard compound mixtures and individual solutions was used to optimise the MRM parameters of each analyte. The selected MRM transitions were confirmed using the Shimadzu 8060 optimiser.

- Calibration Curve

A blank serum has been used to prepare the calibration curve and all quality control samples. The human serum albumin is mixed with phosphate buffered saline at concentration of (50 g/ml). The favorable linear range for all the analytes was 7.8125–1000 ng/mL with a very good regression value (R^2 = 0.999) (Figure 8).

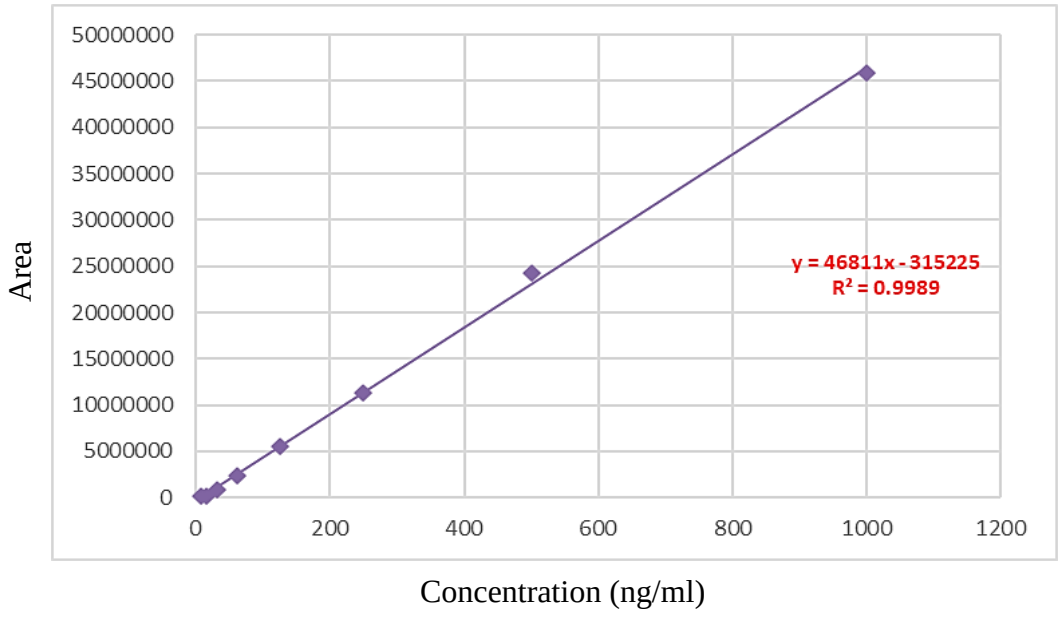


Figure 8: Calibration curve to determine the quality of the samples

Table 5: The values of method validation parameters for each quality control level

N	Analytes	Conc. (ng/mL)	Intra-day			Inter-day			% Recovery	LOD (ng/m)	Linear range (ng/mL)
			Precision (% CV)	% Accuracy	SD	Precision (% CV)	% Accuracy	SD			
1	Vitamin A	QCH 800	0.07	100.09	0.52	0.09	100.09	0.69	99	0.488	7.81-1000
		QCM 400	0.09	100.09	0.38	0.06	100.09	0.23	96	0.488	
		QCL 200	0.17	100.28	0.35	0.06	100.24	0.12	94	0.488	
		LLOQ 100	0.29	100.73	0.3	0.27	100.81	0.27		0.488	

Table 5 shows the names of the analytes and concentrations at the four quality control levels (QCH, QCM, QCL, LLOQ), the criteria presented: the intra-day and inter-day precision and accuracy results with the standard deviation, per cent recovery, the limit of detection (LOD) values, and linear range in ng/mL. Table 5 presents an overview of the selected MRM transitions together with their respective parameters. The validation method parameters are in acceptable range for the three QC levels. For example, the accuracy and precision indicators signify that the goodness of the method used to analyse vitamin A metabolites for intra and inter-day, with accuracy significantly close to 100% and precision ranged from 0.07 to 0.29 for the four concentration. Increasingly, as presented in Table 5, the calculated recovery percentage shows that the extraction method is efficient in extracting vitamin A metabolites from serum samples. The literature has shown different values in terms of the LOD and LOQ values. For example, Plozza et al. reported the LOQ for 0.1 μg/100 mL for all trans-retinol and α-tocopherol and 1 μg/100 mL for β-carotene [83]. Furthermore, Midttun and Uelan reported the LOD 0.1 μM for all-trans retinol [97]. In another study for Santos et al., the LOD and LOQ were reported 0.07–170 ng/mL and 0.2–520 ng/mL respectively [18]. Moreover, Gentili et al. have reported for LOD

0.9–15.6 μg/L and 2.7–46.8 μg/L for LOQ [85]. Table 5 shows the values of LOD and LOQ for vitamin A by LC-MS/MS 8060, which explain that the method is extremely sensitive. The table also presents the intra and inter-day coefficient of variation and accuracy marks among four quality control level. The recovery percentage were calculated by comparing areas under the normal curve in the chromatogram for the extracted and unextracted quality control samples. The LOD was concluded from the lowest concentration of vitamin A metabolite that can be detected by the instrument.

Many blank serum QC samples have been used to calculate the specificity of the validation. This calculation has been done by the extracting and analysing the blank serum QC sample. The peaks were overlaid to check for any interfering/co-eluting peaks at the respective retention times of the analytes, as shown in the following Figure 9.

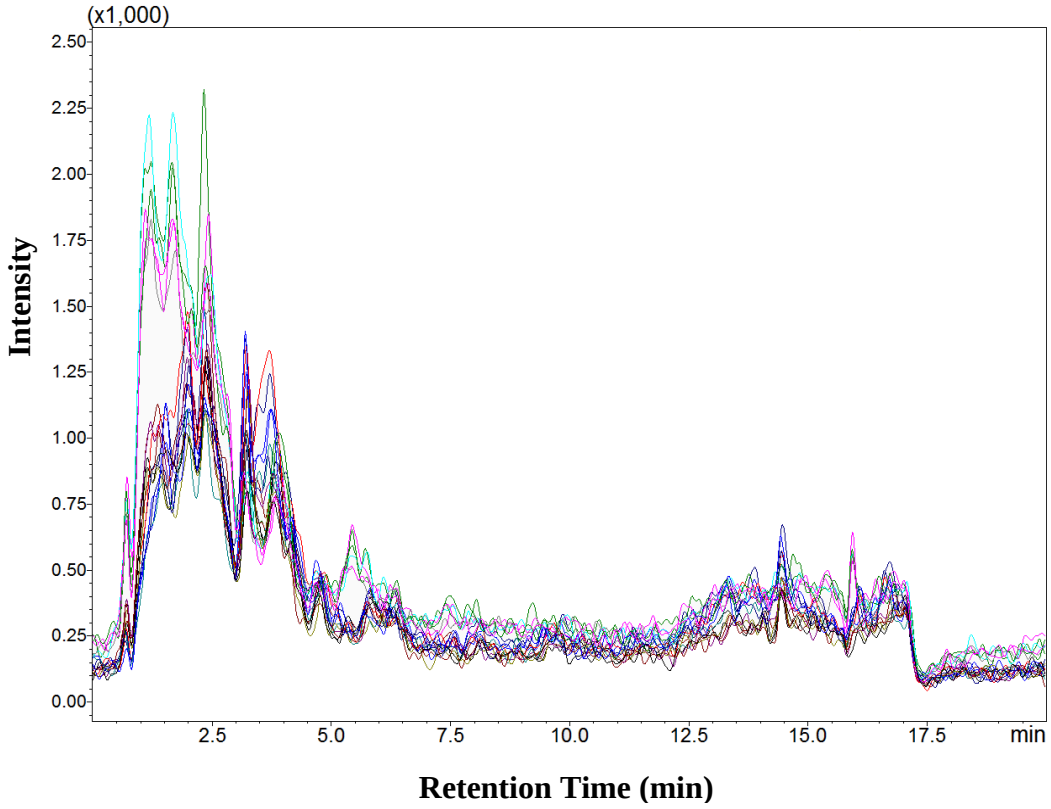
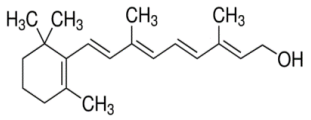
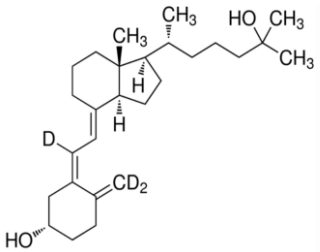


Figure 9: An overlay of six chromatographic peaks showing the specificity of the assay. No interfering co-eluting peaks were found for vitamin A.

Checking the internal standard response of each sample within a batch is also a useful way of picking up problems with individual samples. The precursor and product ions of vitamin A and internal standard with their collision energies are illustrated in Table 6. The table shows optimum MS/MS parameters: precursor ion (m/z) 269.25, product ion 93.05 at collision energy -22 V, 91.10 m/z at -43.0 V and 95.15 at a collision energy of -15.0 V. The table also presents the chemical structure of the retinol with a retention time of 6.720 minutes and mass of 286.45 g/mol.

Table 6: Names, structure, mass, precursor and product ions along with collision energies

Name	**Structure**	**Retention time**	**Mass** (g/mol)	**Precursor ion** (m/z)	**Product ion** (m/z)	**Collision energy** (eV)
Retinol (Vitamin A)	 **$C_{20}H_{30}O$**	6.720	286.45	269.25	93.05 91.10 95.15	-22.0 -43.0 -15.0
25-Hydroxyvitamin D3 (6, 19, 19-d3)	 **$C_{27}D_3H_{41}O_2$**	6.482	403.66	386.35	368.25 257.2 95.2	-15 -183 -35

As shown in Figure 10 Stability tests were operated over 24 h, 48 h, and 72 h, where the outcomes were compared with time zero. The percentage of change from time = 0 is presented in the figure for the four used concentration levels of 800 ng/mL, 400 ng/mL, 200 ng/mL, and 100 ng/mL, where they are used for gauging the stability for Vitamin A. As appearing in the graph, Vitamin A shows a percentage of change up to 0.08% in 24 h of the freeze/thaw cycle, while the % change was within 0.16% for 48 and 0.50% for 72 h in a concentration level of 400 ng/mL. While in the concentration level of 800 ng/mL the % change has fluctuated between -0.05% in 72 h and 0.02 in 48 h and -0.02 in 24 h. The % of the change in the concentration level of 100 ng/mL has shown -0.15% in 24 h, -0.13% in 48 h, and 2.96% in 72 h. It appears from the figure that the Vitamin A is stable when exposed to continuous freezing and thawing cycles. The LC–MS/MS method has proven its reliability, accuracy, reproducibility, specificity and robust.

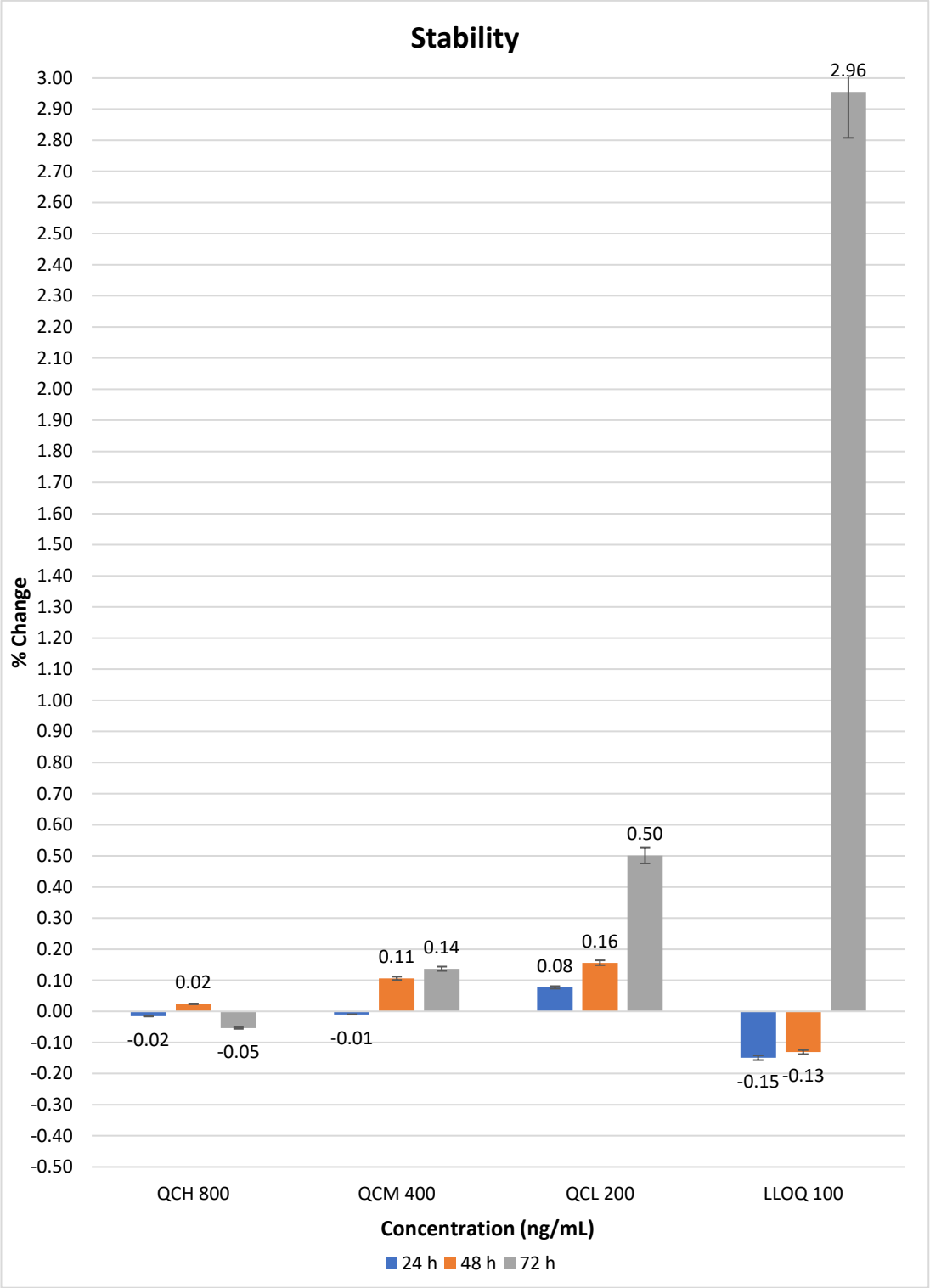


Figure 10: The stability of vitamin A in serum after three freeze/thaw cycles

The blue, orange and grey bars represent the % change in concentration of a particular vitamin A at 24 h, 48 h, and 72 h' intervals, respectively, with the use of quality

controls at four different concentration levels, LLOQ-100 ng/ml, QCL-200 NG/mL, QCM-400 ng/ml, and QCH-800 ng/ml. The percentage of change in the stability of vitamin A ranges between 0 and 3%. The error bars in the graph represent the standard errors of the mean.

3.2 Results of obese Emirati samples

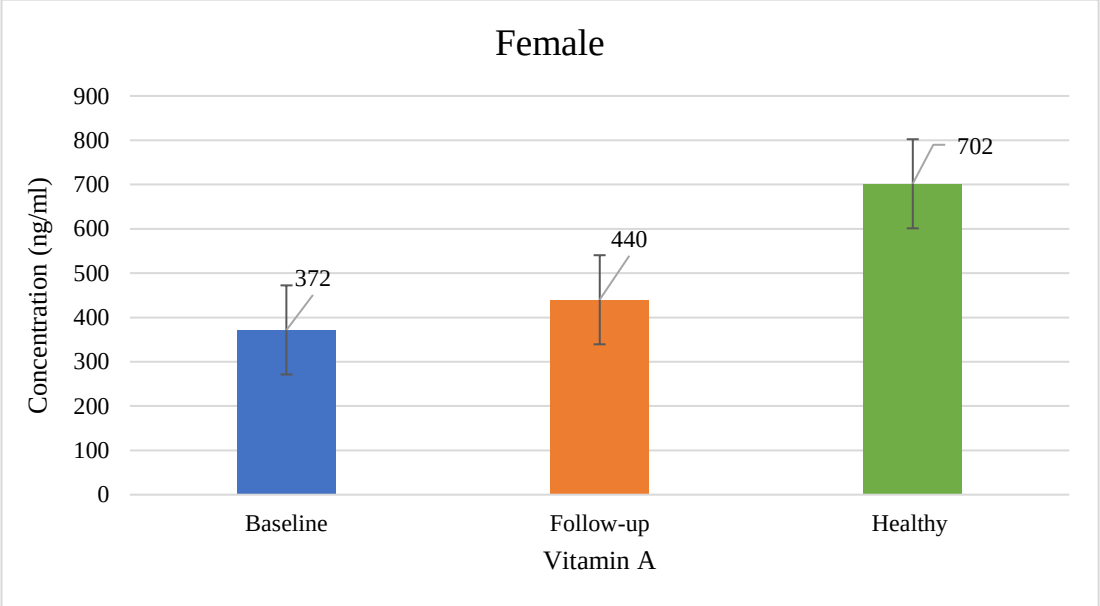


Figure 11: A comparative account of vitamin A concentration in Emirati female blood samples

From left to right, baseline concentrations of vitamin A, and follow-up, and healthy volunteers are shown by blue and orange and green bars, respectively. Vitamin A deficiency is a clinical condition (dryness in the eyes, poor growth of bones, and some specific skin conditions, etc.). In the laboratory, vitamin A deficiency is defined as a level below 20 micrograms/dL (.7 micromole/L). The upper limit of normal vitamin A is 60 micrograms/dL.

Table 7: Vitamin A indicators

Condition	ng/ml
Deficient	< 200
Optimal	≥ 200 to ≤ 600
High	≥ 600 to < 1000
Hypervitaminotic	≥ 1000

Table 7 [103] shows the vitamin A range guideline from the literature. This range has been followed in the result and discussion part by comparing the achieved findings with these indicators.

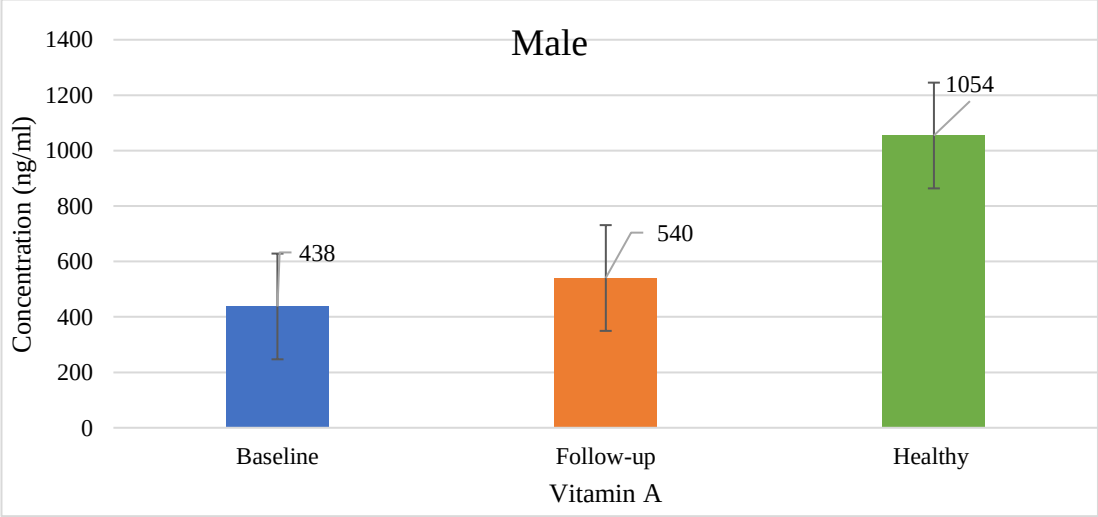


Figure 12: The comparative accounts of vitamin A concentrations in Emirati male blood samples. From left to right, the concentrations of vitamin A in baseline and follow-up, and healthy volunteers are shown by blue and orange and green bars, respectively.

Figures 11 and 12 show the analysis of blood samples from a sample of the Emirati population in the UAE for both female and male patients, respectively using the developed and validated LC–MS/MS method. The proportion of female sample to male sample is (2.8:1) in baseline and follow sample, as also the female patients

usually return for follow-up sessions compared to the male patients. The level of vitamin A deficiency appears in the baseline volunteers in the female sample which presented in the blue bar in the female results, which is higher than the lower acceptable value of (600 ng/mL >372 ng/ mL > 200 ng/ mL). This result has increased upon supplementation in the follow-up samples to reach 440 ng/mL. On one hand, the male results were within the acceptable upper and lower limits (600 ng/mL> 438 ng/mL > 200 ng/mL, 200 ng/mL< 540 ng/mL < 600 ng/mL). The healthy sample has achieved an extremely high number for male to achieve 1045 ng/mL, while in female this reached 702 ng/mL. The healthy sample size is lower than the baseline and follow-up sample with 175 participants whom age rages between (18:65 years). The results clearly show that the concentrations of vitamin A for both females and males increased after supplementation (372 ng/mL to 440 ng/mL for female sample, and 438 ng/mL to 540 ng/mL for male sample). The result shows a sufficient level of vitamin A in the participants.

Table 8: A comparative account of the vitamin A in the baseline, and follow-up samples. The average concentration of vitamin A is given in the last column (in ng/ml) along with the standard deviation (SD). N signifies the number of samples analysed in this study from baseline and follow-up.

Vitamin	Sample Type	N	Mean ± SD
Vitamin A	Baseline	277	389±215
	Follow Up	277	466 ± 232
	Healthy	175	1054±344

As appears in Table 8 for vitamin A with a total sample of 277, including the male and female, the calculated mean for the baseline is 389 plus and minus the standard error. This increased in the supplementations to achieve 466 plus and minus the standard

error. Surprisingly, the healthy sample has shown a dramatic increase to reach 1054 plus and minus the standard error.

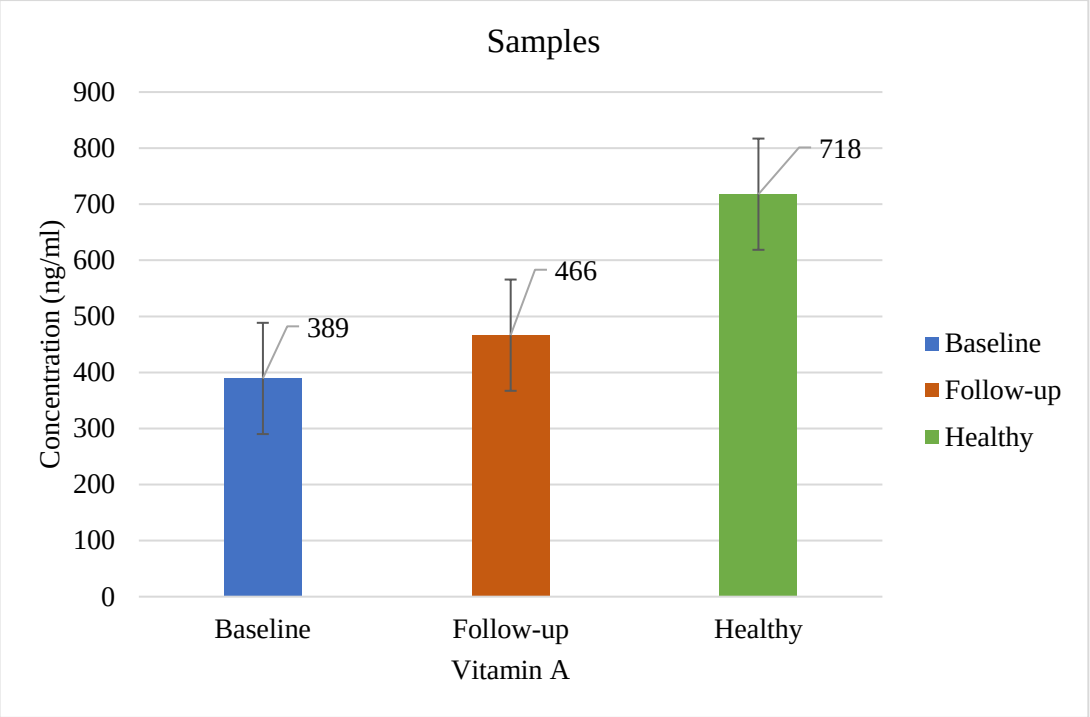


Figure 13: A comparative account for vitamin A concentration at baseline, follow-up and healthy samples. The error bars in the graph represent the standard errors of the mean.

Figure 13 shows the combined results for both female and male participants where the baseline samples for obese Emirati patients were above the optimal levels of vitamin A (389 ng/mL > 200 ng/mL), and, similarly, the follow-up sample results showed higher and acceptable results for vitamin A (200 ng/mL > 466 ng/mL > 600 ng/mL). While the healthy sample has achieved 7018 ng/mL. The result from the current analysis shows that vitamin A is present in the blood with acceptable limits according to the approved guidelines (Figure 7). Additionally, the perceived results also suggest that the current supplementation strategy for vitamin A in the UAE for the Emirati population may is acceptable and favourable.

Chapter 4: Conclusions

In summary, the current research intended to develop a LC-MS/MS-based assay for quantifying vitamin A in human plasma in healthy and diseased Emirati population. The reached findings show the suitability and robust LC-MS based assay for the simultaneous quantification of vitamers in human plasma where the stability of vitamin A ranges between 0 and 3%, and optimum MS/MS parameters: precursor ion (m/z) 269.25, product ion 93.05 at collision energy -22 V, 91.10 m/z at -43.0 V and 95.15 at a collision energy of -15.0 V. The research methodology has followed the preparation procedures of standard solutions, and collection of blood samples and ethical approval number. The MRM-based LC-MS/MS method has been used to extract and analyse the vitamin A from the human plasma. The method has been applied on a sample of 277 Emirati obese patients for the baseline and the patients have been followed-up, while new 175 healthy control volunteers participated. The characteristics of the sample as follows: mixed-gender with 73 males in the age group of (18–82 years) and 204 females in the age group of (18–65 years). The level of vitamin A appears in the baseline volunteers in the female sample which is higher than the 200 ng/ mL and less than the 600 ng/mL. This perceived result has increased upon supplementation in the follow-up samples to reach 440 ng/mL. While the male results are within the acceptable limits upper and lower limits for the (600 ng/mL> 438 ng/mL > 200 ng/mL, 200 ng/mL< 540 ng/mL < 600 ng/mL). The healthy sample showed 718 ng/mL combining the female and male. The results clearly show that the concentrations of vitamin A for both females and males increased after supplementation (372 ng/mL to 440 ng/mL for female sample, and 438 ng/mL to 540 ng/mL for male sample), this increase has made the values become within the optimal

range as shown in the Table 6. The result shows a sufficient level of vitamin A in the participants.

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