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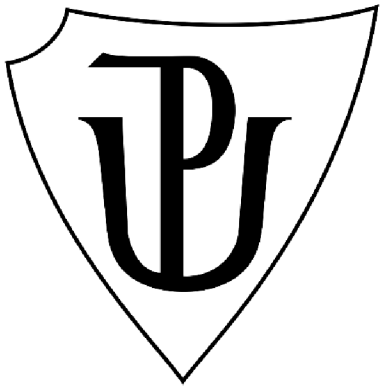

DEVELOPMENT AND VALIDATION OF METHODS FOR ANALYSIS OF FAT-SOLUBLE VITAMINS

DIPLOMOVÁ PRÁCE

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Studijní obor:	Biochemie
Forma studia:	Prezenční
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Rok:	2023

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V Olomouci dne .....

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Podpis studenta

Acknowledgements

I would like to thank my supervisor Lukáš Najdekr PhD. for his patience and guidance throughout my whole thesis. Also, I would like to thank the Institute of Molecular and Translational Medicine (IMTM) management for making my diploma thesis possible. Then I would like to thank the Department of medical chemistry and biochemistry (UP) for letting me analyse some of my samples on their instruments. Lastly, I would like to thank my partner and family members, who were great support for writing the thesis.

Bibliografická identifikace

Jméno a příjmení autora	Bc. Natálie Pokorná BSc.
Název práce	Development and validation of methods for analysis of fat-soluble vitamins
Typ práce	Diplomová
Pracoviště	Katedra biochemie
Vedoucí práce	Najdekr Lukáš Mgr. PhD.
Rok obhajoby práce	2023

Abstrakt

Vitamíny rozpustné v tucích jsou kritické mikronutrienty pro živočichy včetně lidí. Existují čtyři hlavní skupiny vitamínů rozpustných v tucích: A, D, E a K. Vzhledem k charakteristikám vitamínů rozpustných v tucích je důležité stanovit jejich koncentrace v organismu. Avšak je obtížné stanovit všechny v jedné analýze. Pro tuto studii byly vybrány hlavní formy každé skupiny vitamínů: retinol, alfa tokoferol, cholekalciferol, kalcidiol a fylochinon. Cíle práce se zaměřovaly na získání potřebných informací k vývoji a metody na analýzu vitamínů rozpustných v tucích a jejich následnou validaci.

Vývoj metody začal s extrakcí pomocí methanolu, elucí LC gradientu využívá mobilní fáze: (A) Methanol/voda (50/50, v/v) + 0.2% kyselina mravenčí a (B) methanol + 0.2% kyselina mravenčí a detekcí pomocí hmotnostního spektrometru s analýzou pomocí orbitální pasti. Jednofázová extrakce však nebyla dostatečně účinná, přistoupilo se tedy k dvoufázové extrakci pomocí směsi hexanu a chloroformu (90/10, v/v), která byla efektivnější. Avšak analýza orbitální pastí nebyla dostatečně senzitivní pro detekci všech vybraných vitamínů rozpustných v tucích, tedy byla MS detekce přesunuta na kvadrupólový hmotnostní spektrometr s použitím „Multiple reaction monitoring (MRM)“. Příprava vzorku byla optimalizovaná. Finální metoda byla validovaná pro vitamíny A a E pomocí norem evropské medicínské agentury. (EMA)

Klíčová slova	Vitamíny rozpustné v tucích, retinol, vitamín D_3, fylochinon, alfa tokoferol, LC-MS
Počet stran	72
Počet příloh	0
Jazyk	Anglický

Bibliographical identification

Author's first name and surname	Bc. Natálie Pokorná BSc.
Title	Development and validation of methods for analysis of fat-soluble vitamins
Type of thesis	Diploma
Department	Department of biochemistry
Supervisor	Najdekr Lukáš Mgr. PhD.
The year of presentation	2023

Abstract

Fat-soluble vitamins (FSVs) are crucial micronutrients for animals, including humans. Four main FSVs groups exist: A, D, E and K. Due to the nature of FSVs, it is essential to determine the concentrations in the organism, but at the same time, it is challenging to analyse all of the FSVs in one analysis. For this study, only the main forms of each FSV were selected: retinol, alpha-tocopherol, cholecalciferol, calcidiol and phylloquinone. The aims of the work were focused on obtaining the necessary background for the development of the assay analysing FSVs and their following validation.

The assay development started with the extraction using methanol and LC gradient elution applying mobile phases (A) Methanol/water (50/50, v/v) + 0.2% formic acid and (B) methanol + 0.2% formic acid with detection on orbitrap mass spectrometer. The monophasic extraction was not efficient enough. Therefore, biphasic extraction was introduced using a mixture of hexane and chloroform (90/10, v/v), which was the most effective. However, the orbitrap was not sensitive enough to detect all selected FSVs; thus, the MS detection was switched to triple quadrupole (QQQ), applying multiple reaction monitoring (MRM). The sample preparation was modified to obtain better results. The final assay for vitamins A and E was validated according to the European medical agency (EMA) guidelines.

Keywords	Fat-soluble vitamins, retinol, vitamin D_3, phylloquinone, alpha-tocopherol, LC-MS
Number of pages	72
Number of appendices	0
Language	English

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Aims of the work

The first aim was to research fat-soluble vitamins and metabolomics to obtain the necessary academical background for the effective design of the assay. The second aim focused on researching the validation principles, norms, and decrees regarding this process to properly and effectively validate the resulting assay. The third and last aim was to validate the developed LC-MS assay for fat-soluble vitamins analysis.

1. Introduction

Studying the levels of fat-soluble vitamins in blood samples is essential to maintain the population's well-being.

Vitamins are essential micronutrients for maintaining an organism healthy. They function as cofactors to the crucial processes in the organisms. Their deficiency may cause long-term problems or death, for example, inefficient blood clotting from vitamin K deficiency or disruption of calcium utilisation due to amounts of vitamin D intake. Therefore, the importance of developing robust and sensitive diagnostic assays is rising rapidly.

The robustness and simplicity of the analytical assay are crucial for its expansion to day-to-day in clinical biochemistry laboratories. Simplicity is desired to prevent possible random errors, thus simultaneously helping the results be more accurate and reliable.

Liquid chromatography coupled with mass spectrometry (LC-MS) assays are well suited for fat-soluble vitamin analysis, as they can be used highly for qualitative and quantitative analysis. However, the analytes - fat-soluble vitamins - do not possess high stability and can degrade over time, making them virtually non-detectable in their active form. Determining the stability of fat-soluble vitamins is crucial as it assigns the timespan in which the samples should be analysed to give accurate results. Also, fat-soluble vitamins are present in traceable amounts, and each one has a different concentration range. Therefore, it is challenging to determine all fat-soluble vitamins in one assay. The subsequent problem is also the sensitivity of the detectors, and thus more complicated detection and quantification of vitamins in small amounts in biological samples. One way to overcome such obstacles is to use higher quantities of biological samples and incorporate pre-concentration steps in the sample preparation. Unfortunately, the process's elongation reflects the analyte's low stability and the danger of degradation. Also, more steps in extraction assay can introduce possible human errors, which is undesirable for robust and standard operation procedures.

This issue will need to be addressed in the future. The analysis of degradation products can be one option (especially with dried blood spots (DBS) as a biological material). As the blood dries on the surface, it gets in contact with oxygen, exposing the metabolites in the matrix to chemical changes.

2. The current state of the problem being solved

2.1 Fat-soluble Vitamins

Vitamins are low molecular weight compounds that are important cofactors for essential processes in organisms. The endogenous synthesis of the vitamins is very limited or absent in the organism and requires the intake of these micronutrients. (Verstraete, Boffel, and Stove 2020) Thus, vitamins are a favourable source of information about nutritional health.(Khaksari et al. 2017) The vitamins can be divided into two categories according to their polarity (LogP): (1) water-soluble vitamins and (2) fat-soluble vitamins. All B vitamins and vitamin C belong to the water-soluble group, and vitamins A, D, E, and K belong to the fat-soluble vitamins category.

The absorption process of fat-soluble vitamins consists of emulsification and incorporation into the mixed micelles, which contain cholesterol, phospholipids, and fatty acids. Those micelles are then absorbed into enterocytes, where they are interchanged into chylomicrons. Chylomicrons are then released into the lymphatic system, where enzymes break them down. The fat-soluble vitamins are then stored in the liver or released back into the bloodstream in free form or bound to their specific transport protein. (Bier et al. n.d.) Clinically, vitamins are mainly analysed via immunoassays, which are highly specific but allow only the measurement of single vitamin at once, and they are expensive and time-consuming. Also, those assays require plasma as their sample. Thus, many scientists are trying to shift the assays in different directions, mainly using dried blood spots as a sample and LC-MS combination as an analytical approach. (Garnett et al. 2019)

2.1.1 Analytical assessment of fat-soluble vitamins

The combined analysis of fat-soluble vitamins is nowadays analysed through UHPLC combined with UV and MS detection. (Fanali et al. 2017) Ultra-high-performance liquid chromatography (UHPLC) is a type of high-performance liquid chromatography (HPLC) which uses colons filled with particles smaller than 2 μm. The miniature size of the particles allows for acceleration of the separation but, at the same time, preserves the separation efficiency.(Nováková, Douša, and Česla 2021)

The fat-soluble vitamins can be analysed applying chromatographic separation on the normal phase. It was noted that normal phase chromatography could be used to separate retinols and successfully separate alpha and gamma tocopherols. Normal phase chromatography also has a significant advantage in tolerating high amounts of lipids,

which can be easily removed. In this case, fluorescent detection is the preferred method of detection combined with the normal phase. (Fanali et al. 2017)

On the other hand, reversed phase chromatography is much more robust, has higher reproducibility and has better compatibility with mass spectrometry (using electrospray ionization (ESI) organic solvents evaporating at lower ion source temperature resulting in less in-source fragments and more stable spray). The reverse phase chromatography C_{18} modified silica was noted to be successful in differentiating vitamins D_3 and D_2. However, it is not successful in separating beta and gamma tocopherols. Some assays use C_{30} modified silica but with fat-soluble vitamins, showing less efficient division and broader peaks. Another significant modification of reverse chromatography is a pentafluorophenyl (PFP) modification, which exhibits rigidity and superior selectivity to other modifications. The PFP modification showed efficient separation of alpha and beta tocopherols. Non-aqueous reversed phase (NARP) chromatography was developed precisely to separate low-polar and non-polar compounds. It was successfully used for the separation of fat-soluble vitamins and carotenoids. However, NARP chromatography is hardly compatible with atmospheric pressure ionisation (APCI). It is primarily because of the complicated coordination of all solvents in mobile phases. NARP is needed to find solvents that will effectively support APCI due to acid-base reactions in the gas phase but also have to solubilise the analytes successfully. (De Leenheer et al. 1988)

The direction in which the analysis tends to go is a miniaturisation of the analysis, therefore using smaller amounts of the analyte, mobile phases and short analysis time. This is not only cost-effective but also has a lower impact on patients. One of the options for miniaturisation of analysis is reversed phase UHPLC. The UHPLC columns are characterised by particles smaller than 2 µm. The UHPLC offers excellent advantages such as narrower peaks, more effective separation, low LOD (lower limit of detection), and shorter time of analysis.(Nováková et al. 2021)

There are other different options for fat-soluble vitamins analysis. However, the use of those is relatively rare nowadays. Some examples are nano-LC columns employing monolithic columns, electromigration assays, and supercritical fluid chromatography. (Fanali et al. 2017)

Detection and quantification are possible via mass spectrometry. Usually, they are quantified via multiple reaction monitoring (MRM), where one precursor ion is selected

in the first quadrupole and then fragmented. This results in better selectivity and higher precision while using a small number of precursor ions. (Liu et al. 2021)

2.1.2 Vitamin A

Vitamin A is naturally occurring in food in the form of retinol esters or can be present as provitamin A - carotenoid, which is converted into retinol (9,13-dimethyl-7-(1,1,5-trimethyl-6-cyclohexene-5-yl)-7,9,11,13-nonatetraene-15-ol) – the primary form of vitamin A. (de Leenheer et al. 1988) The conversion rate between provitamin and vitamin varies with the provitamin nature. Vitamin A (retinol or its esters) is mainly obtained from animal sources or fortified food. On the other hand, the provitamins (carotenoids) are primarily obtained from plant sources. (Bier et al. n.d.)

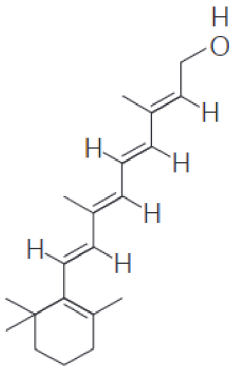

Figure 1: Structure of retinol – Vitamin A (Bier et al. n.d.)

Absorption of vitamin A, mainly retinyl esters, occurs in the intestine, where the esters are hydrolysed, and retinol is absorbed through mucosa cells, where retinol is reesterified. It can either be reesterified with fatty acyls or acetyl, resulting in retinol esters. In the bloodstream, retinol esters can be found incorporated in lipoproteins. The retinol esters are transported form the bloodstream into hepatocytes, where they are stored. The retinol esters are hydrolysed after the mobilisation from hepatocytes. Those can be later stored or transported as retinol or can be again reesterified. The free retinol is then transported in plasma by binding to retinol-binding protein (RBP) or prealbumin; the retinol esters can be transported through lipoproteins. (de Leenheer et al. 1988) The most occurring retinol esters are retinol palmitate, retinol oleate, retinol stearate and retinol linoleate. The esters of retinol do not have any known biological function except the transport and storage form of retinol. (O'Byrne and Blaner 2013) The transport, storage and use of vitamin A are highly dependent on the presence of gastrointestinal disorders and liver diseases. (de Leenheer et al. 1988)

The function of vitamin A is deeply connected with vision in the retina. (Bier et al. n.d.) The retinoids influence mammals' physiology, including growth, immunity maintenance and epithelial barriers. (O'Byrne and Blaner, 2013) Retinoic acid (retinol metabolite) is crucial for embryogenesis and cell differentiation; it also is an essential component in immunity. (Bier et al. n.d.) The concentration of retinoic acid is very low, usually 100 to 1000 less than retinol. In mammalian organisms exist, various types of retinoids, usually at very low concentrations. (O'Byrne and Blaner, 2013) According to the human metabolome database HMDB, the retinal levels in the blood are in the range of 0.155 +/- 0.232 $\mu mol \cdot l^{-1}$, and the levels of retinoic acid are 0.0145 +/- 0.0092 $\mu mol \cdot l^{-1}$ compared to the concentration of all-trans-retinol 1.84 +/- 0.46 $\mu mol \cdot l^{-1}$. (HMDB) Therefore the measurement of blood status for vitamin A is done by measurement of all-trans-retinol. (Huang, Clements, and Gibson 2015)

2.1.2.1 Clinical manifestations

One of the main problems with vitamin A deficiency is vision problems. The problems start with worsened night vision, then lead to soon recovery from flashes of light, thus evolving into permanent blindness if the vitamin A is depleted for too long. The excess of vitamin mainly causes liver damage but can also cause problems with vision and epithelial problems. Dry skin, hair loss. (Bier et al. n.d.)

2.1.2.2 Determinations in biological materials

The detection of retinoids dates back to the 1960s, and gas chromatography applications are highly correlated with trends in analytical chemistry. A combination of gas chromatography with mass spectrometry detection was possible to detect retinoids. Even though electron ionisation brought better results than chemical ionisation, the level of fragmentation was high for all unsaturated conjugated compounds. However, the boom of high-performance liquid chromatography and soft ionisation techniques results in higher quality and more robust analysis. (Fanali et al. 2017)

Regarding the chromatographic systems, the most influencing parts are the mobile and stationary phases. Through time it experimented with a lot of different types of columns. One type with promising results was adsorption chromatography. However, the elution of the retinol was long, and the equilibration of the column took time. The biggest issue with this separation type was the impossibility of using an internal standard, which shifted

columns for adsorption chromatography into the background. However, those columns are used in different forms as SPE columns – solid phase extraction. (Fanali et al. 2017)

Nowadays, reversed-phase columns are mainly used columns. The most common type of reversed-phase column is C_{18}, where the silica is modified with a C_{18} chain. However, the number of differently modified reversed-phase silica columns is significant nowadays.

Detection of retinol is mainly done via UV detection, which can be installed as a part of the HPLC instrument and is overall cheaper than MS detection. UV detection uses wavelengths between 310 – 340 nm. The other option is fluorescent detection, which is based on exciting the retinoids at 325 nm and detection of signals at 470 nm. Nowadays, mass spectrometry is used due to the small amounts of samples, high sensitivity and selectivity and low limit of detection. However, UV detection is still widespread. (de Leenheer et al. 1988)

Routinely blood retinol is analysed with HPLC coupled with UV/Vis detection. However, there are many options for how to perform the analysis. (Albahrani et al. 2016)

2.1.3 Vitamin D

Vitamin D can be obtained from food, vitamin D_3 from animal sources and D_2 from plant sources, or synthesised in skin cells using UVB light. A significant amount of vitamin D in the organism is obtained through synthesis. The biologically inactive form of vitamin D needs to be activated by hydroxylation on carbons with numbers 1 and 25. Calcidiol is the most abundant form of vitamin D in serum (D_3, 25-hydroxyvitamin D). (Bier et al. n.d.)

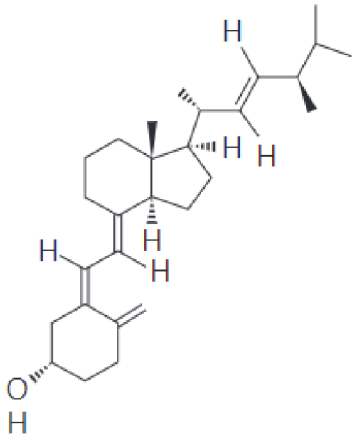

Figure 2: Structure of calcidiol (Vitamin D_3) (Bier et al. n.d.)

This form of vitamin was also reported to be stable in DBS for a long time. Thus, creating a great candidate for quantification of vitamin D.(Binks et al. 2021) Calcitriol (25-OH D_3, 1,25-dihydroxyvitamin D) works as a positive mediator of calcium and phosphorus via intestinal absorption increase or reabsorption of calcium in the kidneys. Added to the regulation of calcium and phosphorus, calcitriol also regulates various processes in the cell, including cell differentiation and regulation of gene expression. Calcitriol has been connected with a reduced risk for autoimmune diseases as well. (De Leenheer et al. 1988)

2.1.3.1 Clinical manifestations

Deficiency in children causes a disease called rickets, which causes insufficient mineralisation of the bones and, thus, changes in the shape of the bones or severe deformity of the bones. On the other hand, vitamin D deficiency in adults likely causes osteomalacia, and long-term lack increases the risk of osteoporosis. (Bier et al. n.d.)

Deficiency can be caused by deficient intake, malabsorption or low exposure to UV light. Low levels of 25-hydroxy vitamin D are caused by decreased hydroxylation due to liver damage (cirrhosis or acute hepatitis). Similarly, as in vitamin A, the overdose is not achieved through ingesting precursors or overexposing to sunlight. It could be obtained only through over-supplementation. The high vitamin D concentration can cause calcification (=the deposition of calcium in soft tissues), which will be the main problem in the kidneys, heart and arteries. Also, the calcium will be withdrawn from bones, leading to fragile bones. (Bierett al. n.d.) As mentioned, the problem may result in rickets type II or sarcoidosis. The 1,25- hydroxylated vitamin D levels are associated with parathyroid issues. (de Leenheer et al. 1988)

2.1.3.2 Determination in biological materials

The most preferred and used assays are radioimmunoassay (RIA), EIA – enzymatic immunoassay or ELISA – enzyme-linked immunosorbent, primarily because of their excellent selectivity and specificity. (Albahrani et al. 2016)

Vitamin D can be determined through gas chromatography (GC); however, the procedure has more negatives than positives. Firstly, due to extremely high temperatures for gasification, vitamin D and its metabolites undergo chemical changes. Secondly, these changes can be prevented by derivatisation but can only be performed on not 25-hydroxylated vitamin D. Thirdly; the MS technique detection depends on

derivatisation. The last and most prevalent negative feature is the destruction of the analyte and adsorption on cholesterol during analysis. The best option is to remove cholesterol from the sample, routinely done via specific SPE. The main reasons for the cholesterol removal are their similar structure and the suppressive effect of cholesterol on vitamin D signals.

Liquid chromatography, primarily HPLC, can be used more successfully to detect vitamin D. Not only for lacking negative features, high temperatures, and requirement for derivatisation. But they also present the possibility of separating all forms of vitamin D and even separating the hydroxylated forms from the non-hydroxylated ones. The detection used for liquid chromatography is usually online connected RIA, UV detection, or MS detection.

Similarly, to vitamin A, the adsorption columns are not widely used nowadays. However, they can be effectively purifying the samples for future analysis. Reversed-phase HPLC is the most popular method for the analysis of vitamin D. The reversed-phase columns used in assays are silica modified with C_{18}, C_8 or PFP. Vitamin D and its metabolites can be detected via UV at 265 nm. However, in this wavelength, many compounds can interfere. This problem can be resolved with a robust purification procedure or derivatisation, which shifts the UV absorption to different wavelengths. Some already published studies about amperometry detection have good results and low detection limits. However, the mass spectrometry detection option exceeds positive features. (de Leenheer et al. 1988)

2.1.4 Vitamin E

Vitamin E is classified as a mixture of tocopherols and tocotrienols forms — the most abundant – alpha-tocopherol, gamma-tocopherol, alpha-tocotrienol and gamma-tocotrienol.

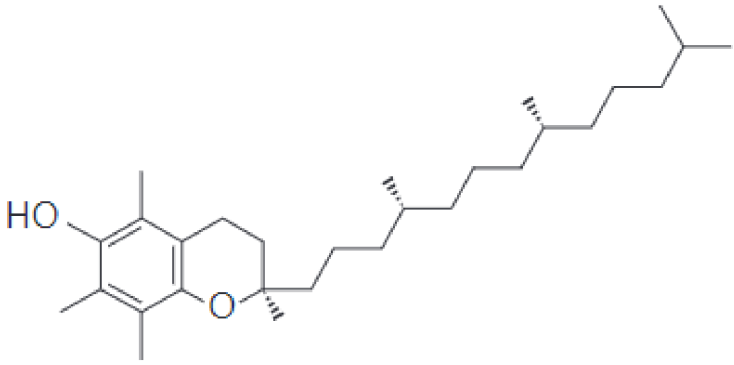

Figure 3: Structure of alpha-tocopherol (Bier et al. n.d.)

However, alpha-tocopherol is the main form of vitamin E stored and used in the human organism. Alpha-tocopherol is naturally found in plant oils. (Bier et al. n.d.) Tocopherol synthesis occurs only in photosynthetic organisms in the inner envelope of chloroplasts, mainly to protect chloroplasts against oxygen toxicity. Thus, vitamin E can be obtained only from food, preferably plant sources. (Colombo 2010) The function of vitamin E is an antioxidant, thus preventing the oxidative damage of lipid membranes and circulating lipoproteins. Therefore, preserving cellular integrity. Vitamin E has to be recycled through other antioxidants (mainly vitamin C) or freshly ingested to maintain the antioxidative function. (Bier et al. n.d.) The subgroup of tocotrienols has powerful neuroprotective, cholesterol-lowering and anticancer properties, which are not usually demonstrated by tocopherols. (Colombo 2010) Unfortunately, simple measurements of tocopherol will not cover total vitamin E concentration. The determination of tocopherols in plasma can be inaccurate due to the storage of tocopherols in the biomembranes; thus, the measurement from the whole blood would be more proper. (De Leenheer et al. 1988)

2.1.4.1 Clinical manifestations

Vitamin E's antioxidant properties can identify the main deficiency symptoms as oxidative damage of cells and breakdown of cell membranes. As a result of this haemolysis, degeneration of axons and peripheral neuropathy can occur. (Bier et al. n.d.) For those reasons, vitamin E was tested and approved as an antioxidant necessary for the human body. (Clement et al. 2012) Vitamin E is also associated with pathological conditions due to its antioxidant properties. Those pathological conditions include chronic lung disease (CLD), retrolental fibroplasia and haemolytic anaemia in premature infants—serum concentrations below eight $\mu mol \cdot l^{-1}$ show hazardous symptoms such as peripheral neuropathy and spinocerebellar ataxia. Lowered concentrations of vitamin E are also linked with Alzheimer's disease. Overdose can be caused only by ingesting too

much vitamin E supplement. (Clement et al. 2012) Overdosing can lead to blood coagulation problems. (Bier et al. n.d.)

2.1.4.1 Determination in biological materials

Detection of the vitamin by GC is possible and gives robust results. Previously, the combination of packed GC column and flame-induced detection (FID) or electron capture detection (ECD) was used. Nowadays, if GC is used, it is mainly coupled with MS, as this detection method is cheaper, more robust and has a lower detection limit. Also, using derivatisation and the internal standards gives an excellent opportunity to quantify alpha-tocopherol robustly. Detection of vitamin E with LC-MS is preferable nowadays, primarily due to better separation properties and no need for derivatisation. There are studies using adsorption chromatography, and they show modest results. The most significant advantage is the effective separation of alpha, beta and gamma tocopherols. However, the separation and resolution are better when using a reversed-phase column. LC-MS using the C_{18} column allows incorporating the vitamin E analysis into the metabolomics assays.

Detection can be practically drawn in two ways. One way of detection is UV, where adsorption is monitored at 292 nm or 280 nm. The measurement at 280 nm gives a more robust detection and a better signal-to-noise ratio. However, this detection method has a huge limitation: a high lower detection limit in tenths of nanograms. Therefore, detecting physiological levels of Vitamin E in plasma is not easy, and the preconcentration step must be done. The second possibility for detecting vitamin E is fluorescent detection, where vitamin E is excited at 205 and 295 nm and emits radiation at 330 nm. Fluorescent detection has higher sensitivity and selectivity compared with UV detection. The best option is the third detection method, which is MS. This detection method has high sensitivity and selectivity, has a low detection limit, is cheap and can be used in the combined analysis, where not only tocopherols are detected. (De Leenheer et al. 1988)

Routinely tocopherols, similarly retinols are analysed with HPLC combined with UV/Vis detection. (Albahrani et al. 2016)

2.1.5 Vitamin K

Vitamin K is similar to Vitamins D and E, classified as a group of molecules – Phylloquinone and Menaquinones. Phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone) is the main form of vitamin K, also called vitamin K_1. (Bier et al. n.d.)

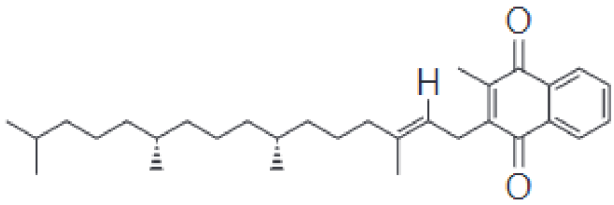

Figure 4: Structure of phylloquinone (Bier et al. n.d.)

Vitamin K_1 is usually ingested with food with a small amount of menaquinones. The main part of menaquinone is synthesised from phylloquinone. The colon microbiome synthesises menaquinones with long chains. The primary source of phylloquinone is green vegetables. (Bier et al. n.d.) Other molecules also considered vitamin K are menaquinone (K_2) and menadione (K_3). Menaquinone can be found in fermented food or is synthesised by gut microflora. Various types of menaquinone vary with the number of isoprenoid chains. The primary vital forms for the human body are those with 4 and 7 isoprenoid residues. (Klapkova et al. 2018)

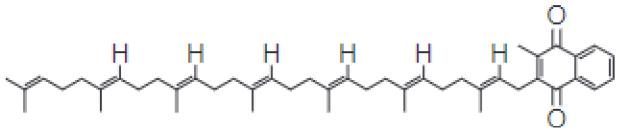

Fig 5: Structure of Menaquinone (Bier et al. n.d.)

Unlike other fat-soluble vitamins, most vitamin K is stored in adipose tissues. The primary function of vitamin K is vitamin K-dependent blood coagulation. (Bier et al. n.d.)

2.1.5.1 Clinical manifestations

As vitamin K is tightly bound with blood coagulation, the deficiency symptoms are decreased ability to coagulate blood, thus a high risk of haemorrhaging. Due to this significant role in blood coagulation and insufficient transport of Vitamin K through the placenta, newborns are given vitamin K injections soon after birth. (Bier et al. n.d.) Two main groups are tended to have vitamin K deficiency, and those are newborns, which are in a higher risk group for K_1 deficiency due to the complicated transport of the vitamin through the placenta. The second group are post-menopausal women in a risk group for K_2 deficiency. (Klapkova et al. 2018)

2.1.5.2 Determination in biological materials

It is challenging to measure vitamin K using GC, mainly due to the on-column degradation of the analyte. One GC assay for the quantitation of vitamin K from Berchtold and Jähnchem successfully avoided the degradation of the analyte. However, this method's downside is the inability to quantify endogenous vitamin K.

Liquid chromatography, especially HPLC, has a significant advantage: the low risk of analyte degradation. The second significant advantage is spectrophotometric detection, which is sensitivity and cheap. However, the detection is not as sensitive as the use of mass spectrometry.

Similarly, as for most fat-soluble vitamins, adsorption chromatography is an excellent option for extracting and purifying the vitamin or all compounds that fall under one vitamin group. Adsorption columns are also great for lipid removal, as some lipids may have similar polarity, are common interferents and may alter the analysis results.

A better option is reversed-phase chromatography. Reversed-phase chromatography is cheaper and quicker than adsorption and effectively separates vitamin K and fatty acids. Detection by UV is most commonly used to detect vitamin K, as it is affordable, robust and compatible with most systems. However, it comes with lower sensitivity and selectivity. The lower detection limit hangs around 0.5 ng, which helps to detect even minimal amounts of vitamins. Another possibility is amperometric, reductive electrochemical and fluorescent detection. However, vitamin K, in its native form, does not possess fluorescent properties. Only the reduced-form – hydroquinones can be detected. Therefore, one extra step must be taken after the analysis, the post-column reduction and detection system. This can bring some systematic errors into the analysis. Another possibility is a wet-chemical post-column reaction with tetramethylammonium octahydridotriborate. (De Leenheer et al. 1988)

As stated by Klapkova et al. 2018, nowadays most common way to analyse vitamin K levels is HPLC with fluorescent detection and post column zinc-reduction. This method was also used in their assay with a specified excitation wavelength of 246 nm and emission wavelength of 430 nm. The samples were extracted through liquid-liquid extraction with ethanol and hexane. (Klapkova et al. 2018)

2.2 Biological materials

A wide variety of biological materials can be collected and used depending on the goals of the analysis. One of the primary materials is blood and blood fractions - plasma, serum, red blood cells and buffy coat (white blood cells and platelets). Other commonly used biological samples are tissue samples, urine, and saliva. The storage and collection procedures and conditions are specific for each sample type. (Vaught and Henderson n.d.)

2.2.1 Dried blood spots (DBS)

DBS were in 1963 proposed as a sampling method in paediatrics. There are four blood sample types – (1) whole blood with anticoagulant, (2) plasma, (3) serum and (4) dried blood spots. The main advantage of DBS is its low-invasive collection properties (only a small quantity is needed). The blood drop is pushed on filtration paper by a pin prick technique. These properties are a significant advantage in paediatrics, where invasive methods and withdrawing high amounts of blood can be dangerous or sometimes impossible.

The making process of the DBS is also straightforward. The process consists of puncture of the heel (in case of newborn screening) or finger with a sterile lancet and depositing a few drops of blood on filter paper (in card format may be called "Guthrie cards" after one of the authors of the article propagating the DBS). (Wagner et al. 2016) The process's simplicity gives a non-trained person the option to withdraw the sample, or the patient can take the sample. For example, patients living in a senior home can send their DBS by regular post to the hospital for analysis. It also brought the opportunity for metabolomic studies in the population. DBS are easy to store, can be stored for a long time and have little damage effect on the human organism. (Palmer 2019) The next great advantage is the stability of the DBS, which is essential for transporting the samples. Thus, obtaining cheaper transportation without requiring shipment in dried ice or cooled packages. However, some substantive analytes in the blood are not majorly stable ex-vivo. Luckily the stability of those analytes may be enhanced using antioxidants or other stabilising molecules. Also, DBS samples significantly reduce biohazard risks, usually high in whole blood or plasma. (Wagner et al. 2016)

2.2.2 Plasma and serum

Plasma and serum are widely used samples. The serum is prepared by blood collection, followed by blood clotting; the resulting liquid does not contain blood cells nor fibrinogen

(for collecting, no-additive tubes are used). Serums have lower protein and peptide concentrations, therefore, beneficial for analysing low-molecular-weight compounds. Excess of protein can interfere with the analysis, which can reduce the sensitivity of the assay. However, better reproducibility was found in plasma samples, as the sample preparation process was more unified.

Plasma is prepared by collecting blood and adding anticoagulants, followed by centrifugation. The resulting liquid does not contain any blood cells. Plasma does have a significant advantage in quick processing. Compared to the usual clotting time of serum, which is 30 to 60 minutes. Also, the necessity of room temperature for clotting may alter the metabolite composition; some labile metabolites may be lost due to oxidation and enzymatic degradation. However, the anticoagulant molecules may also alter the composition of the plasma and have to be carefully chosen regarding the clinical analysis.

Consistency is the key to obtaining good results regardless if serum or plasma is used for biochemical/metabolomic assay.

A critical preanalytical error is haemolysis. It is recognised by the characteristic release of intracellular contents of erythrocytes into the plasma matrix. Interferences caused by haemolysis alter the metabolic profile of the sample, and some of the values obtained from haemolytic samples cannot be used (e.g. Ions, haemoglobin, Acid-base homeostasis, enzymes). (Hernandes, Barbas, and Dudzik 2017)

2.3 Liquid chromatography-mass spectrometry (LC-MS) systems

2.3.1 High-performance liquid chromatography (HPLC)

HPLC can be called the evolution of liquid chromatography. It is the most used type of liquid chromatography nowadays. It does have advantages compared to basic column chromatography. HPLC is quicker than basic column chromatography, mainly due to the higher pressure of the mobile phase. There is a wide variety of stationary phases. Then due to smaller particle size, resolution and sensitivity are improved. (Snyder, Kirkland, and Dolan 2010)

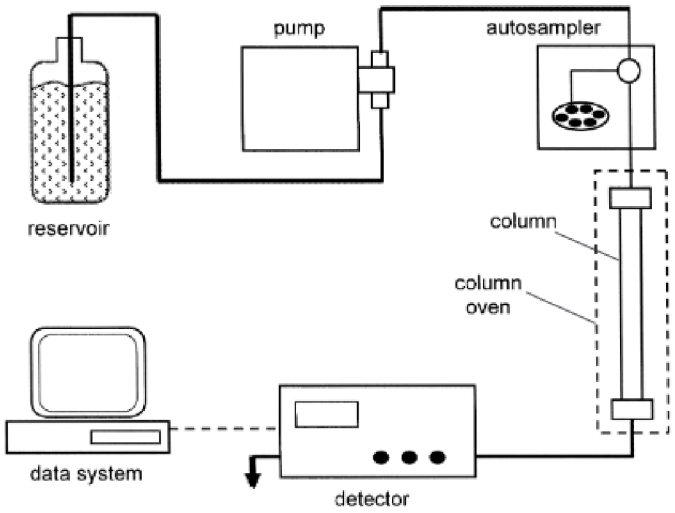

Figure 6: Scheme of HPLC system (Snyder et al. 2010)

The HPLC setup can be divided into five essential functional parts: storage and transport of the mobile phase, dosing of the sample, separation of the compounds, detection of the separated compounds and data recording station. The storage and transport of mobile phases include stock of mobile phases and the pumps. (Nováková et al. 2021) The pumps can be different types but have a similar flow rate range. If an HPLC system is built for a gradient system, the mixer must be present; it could be present before or after the pumps. (Snyder et al. 2010) There are two basic types of gradient systems: quaternary and binary. The quaternary system is a low-pressure system. It can mix up to 4 components of the mobile phase. The mixer, followed by the degasser, is usually placed before the pumps. This creates the dwell time – delay of the gradient. The binary system is a high-pressure system where only two components of the mobile phase can be pooled; the components of the mobile phase are mixed in a mixer and placed after two independent pumps. (Nováková et al. 2021)

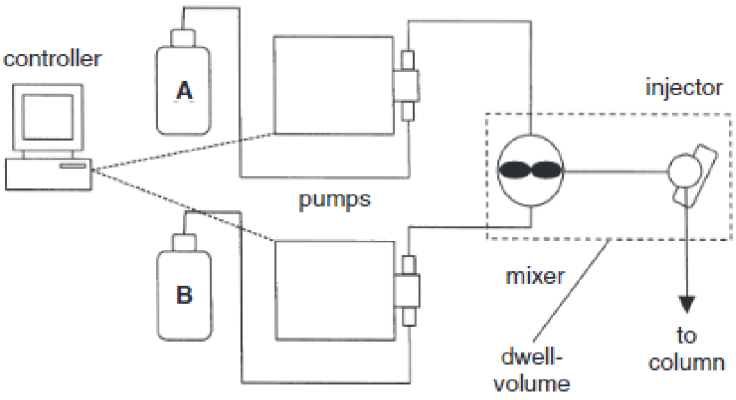

Figure 7: HPLC system with high-pressure mixing – binary system (Snyder et al. 2010)

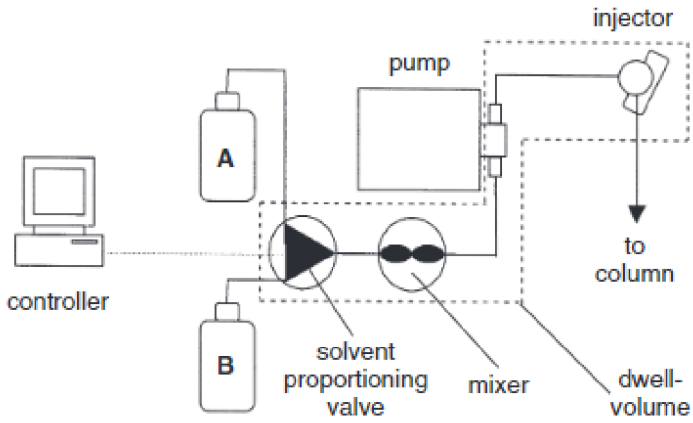

Figure 8: HPLC system with low-pressure mixing – quarternary system (Snyder et al. 2010)

The sample dosage includes automatic valve injectors or manual dosage valves. (Nováková et al. 2021) The injectors in most HPLC systems are automatic valve injectors which load the samples into external loops from where there flushed into the column. The critical part of the HPLC platform is the compound separation functional part, where the column and column thermostat belong. (Nováková et al. 2021) The column is the medium where the compounds are separated, and its composition influences the separation process. The outside of the columns is mainly made out of stainless steel. The resin of the column, which is inside of the column, is highly diverse in size of the particles, its material and particle surface modifications. Dividing according to particle size can be made into two groups: HPLC columns (> 2 µm) and UHPLC columns (with < 2 µm). Other categories are divided according to the material or surface modification of the particles. In modern HPLC columns, the particles are primarily porous silica with a modified surface. The type of surface modification affects the separation of the analytes, and

columns are usually chosen according to that. Commonly used surface modifications are C_{18}, C_{30}, and C_8, which are alkyl residues bound to silica, also called the reversed phase. The last part of the HPLC system is the functional detection part, where detectors are widely used UV/Vis spectrometers. Those are divided into three categories: fixed wavelength, variable wavelength, and DAD (diode array detection). The most universal is DAD, as it could detect all absorption spectra from 200 nm to 700 nm. Other commonly used detectors are fluorescent, electrochemical, and mass spectrometers. Although the most common detector, other detectors exist. Some other widely used detectors are fluorescent, refractive, amperometric, conductivity, optical rotation and mass spectrometry-based detectors. (Snyder et al. 2010) The last part of the HPLC setup is data from the recording station, which nowadays is a computer. (Nováková et al. 2021)

2.3.2 Mass spectrometry

Mass spectrometry has gained popularity since the 1970s, primarily due to the invention of the "soft" ionisation methods, which allowed chromatographic techniques to connect.

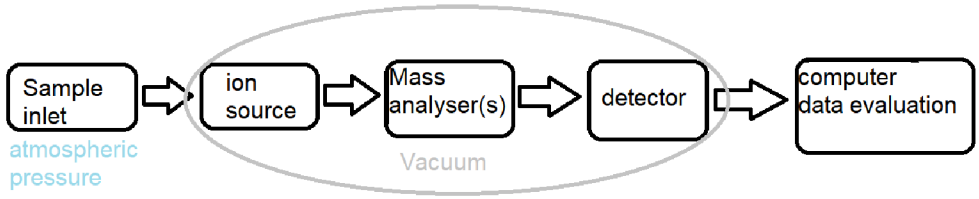

Figure 9: Scheme of Mass spectrometry system

Mass spectrometry works on the principle of the ionisation of the molecule and the separation of the ions according to their mass-to-charge ratio (*m/z*). Separation takes place in a mass analyser. The principle mainly depends on the type of analyser. From the mass analyser, the separated ions continue to the detector. The resulting electric signal from the detector creates the mass spectrum of the analyte, and due to the unique *m/z* compound can be identified. However, as the spectrum shows separated compounds based on their *m/z,* the isomers cannot be differentiated. Therefore, the structure identification of the analyte based on molecule fragmentation is necessary. In these steps, ions are fragmented primarily using collision with other molecules. The change from *m/z* of the ion and the *m/z* of the produced fragments can be labelled as a transition. A set of two or more transitions can be used as an isomer differentiation tool or to identify the analytes in the sample. If ion fragmentation is used in mass spectrometry, it is called tandem mass spectrometry or MS/MS.

The crucial part of mass spectrometry is the ionisation of the sample. There are different types of ion sources, and their use highly depends on the situation. For example, electrospray ionisation (ESI (DOLE, COX, and GIENIEC 1973)) can be used with LC systems and does not ionise the analyte in the vacuum. Another widely used type is atmospheric pressure chemical ionisation (APCI (MItchum and Korfmacher 1983)), which is compatible with the LC system and softer than ESI, thus preventing unwanted in-source fragmentation of the samples.

The second part of the mass spectrometer is a mass analyser, which separates the ions according to their mass-to-charge ratio (*m/z*); the analysers are limited by mass range, resolution, accuracy and sensitivity. The type of mass analyser can be selected by choosing the main characteristic. There are basic mass analysers and a combination of those analysers, which are called hybrid mass analysers. Commonly used mass analysers are ion trap, time of flight or quadrupole analysers.

Tandem mass spectrometry allows characterisation and quantification at much higher sensitivity and specificity than conventional mass spectrometry. There are two basic types of collisions in tandem mass spectrometry: (1) collision-induced dissociation (CID(Cody and Freiser 1982)), (2) collision-activated dissociation (CAD) and other types of collision used. Tandem mass spectrometry is usually run-on a triple quadrupole (QQQ) mass spectrometer allowing selected reaction monitoring (SRM) or multiple reaction monitoring (MRM). The process starts in the first quadrupole, where the ions are filtered according to the preselected *m/z*; in the second quadrupole = the collision cell, the preselected ions are fragmented, and in the third quadrupole, the fragments are separated according to their *m/z*. (Anon n.d.; Nielsen n.d.)

2.4. Method validation guidelines

According to EU and Czech legislation, a new method for clinical biochemistry must be fully validated, whether entirely new or based on the literature. The partial validation of the methods is performed when minor changes are applied, equipment is changed etc. Cross-validation is essential for validating the data obtained at different laboratories and is vital for the quality control of all clinical laboratories.

The main objective of full validation is to demonstrate the reliability of the results of a particular method run with a particular sample in specific conditions. The validation of the bioanalytical methods has some essential characteristics which demonstrate the

reliability of the results; (1) selectivity, (2) lower limit of quantification (LOQ), (3) calibration curve performance, (4) precision, (5) accuracy, (6) matrix effect, (7) stability of the analytes, (8) matrix, (9) internal standards and solutions of analytes and (10) internal standards in a matrix in various concentrations.

At the beginning of planning the validation process, it is crucial to select the reference standards. Those must have exceptional quality from the manufacturer, as lower quality may significantly alter the results. The nature of the standard highly depends on the quantification method. If absorption spectroscopy is applied, the standard has to have a changed retention time but the same spectroscopic properties as the analyte. If Mass spectrometry is used, the stable isotope labelled standard has to be used.

2.4.1 Selectivity

Selectivity is defined as the ability of the method to differentiate the analyte and internal standards from components of the matrix or components of the sample. Selectivity should be analysed in 6 different individual sources of the blank matrix. In the case of plasma, usually, some serum albumin can be used.

The absence of interference can be declared if the response is smaller than 20% of the lower limit of quantification of the analyte and smaller than 5% of the lower limit of quantification for the internal standard.

If a drug assay is studied, the possible interference of its metabolites, degradation products and other metabolites must be verified. Also, the potential interference by the co-medication should be considered.

2.4.2 Carry-over

Carry-over is defined as the occurrence of the analyte in the blank sample due to the short elution time.

Carry-over should be addressed when the method is in the development stage. During the validation of the carry-over, the blank samples should be injected right after injection and analysis of the sample with a high concentration of sample or calibration standard at the upper limit of quantification. Carry-over is considered present if the blank sample following the highly concentrated standard has a response higher than 20% of the lower limit of quantification and 5% for the use of the internal standard.

2.4.3 Lower limit of quantification

The lower limit of quantification is defined as the lowest concentration, where the analysis is reliable according to accuracy and precision. As well as the signal of the analyte has to be at least 5x bigger than a blank sample.

2.4.4 Calibration curve

The calibration curve can be summarised as the set of concentrations of the analyte and the instrument's response. This set of values is then put into the graph.

Calibration standards should be as similar to the samples as possible; this means that the standards should be prepared in the same matrix as the sample to avoid the matrix effect complication.

Before the analysis is run, the concentration range of the analyte should be proposed. Therefore, the calibration curve should fully cover the analyte concentration range. The calibration curve has to have at least six concentration values measured at least three times according to signals with the addition of the blank sample (processed matrix sample without analyte and without internal standard) and the "zero" sample (processed matrix with internal standard). The blank and "zero" samples should not be taken as parameter curve calculation parameters. For all values, measured accuracy values have to be calculated—the regression equation parameters (slope and intercept if the fit is linear).

The back-calculated values for the calibration curve considered usable should be within ± 15% of the original concentration value except for the lower limit, which can fall in ± 20% of the initial concentration. At least six calibration standards have to fulfil these criteria, or 75% of the standards if there are more than 6 of them. If the calibration curve does not meet those criteria, it has unreliable results and should not be used.

2.4.5 Accuracy

Accuracy can be described as the closeness of the values determined by the analysis and the nominal concentrations of the analyte. The accuracy has to be determined for all samples, quality control samples and internal standards.

The accuracy can be divided into within-run accuracy and between-run accuracy. Within-run accuracy should be determined in a single run-on minimum of 5 samples at a minimum of 4 concentrations, which cover the calibration curve range. The mean measured concentration on one value has to be within ± 15% of initial concentrations to

be considered accurate, except for the lower limit of quantification, which should be within ± 20% of the original concentration. Between-run accuracy should be run similarly to within-run accuracy, except that samples should be analysed on at least two days.

2.4.6 Precision

Precision is described as the closeness of repeated individual measures of the analyte. It is expressed in percent as CV (coefficient of variation). It should be run on similar samples as accuracy and also should be run at within-run precision and between-run precision, the same as accuracy.

2.4.7 Dilution integrity

The process of the dilution should not affect the accuracy and precision. The dilution integrity should be shown on a highly concentrated analyte sample, preferably a solution of standard in the matrix in high concentrations (Close to the upper limit of quantifications). Accuracy and precision should be within the criteria described previously.

2.4.8 Matrix effect

The matrix effect should be studied when mass spectrometry is used. It should be done with at least six lots of blank matrices from individual donors; those matrices should not be pooled. This can be applied to plasma or urine matrices.

For each analyte and internal standard matrix, the effect should be calculated. The matrix effect should be calculated as follows: the ratio of peak area in the presence of the matrix and peak area of the peak in the absence of the matrix. The internal standard normalised matrix factor should also be calculated by dividing the matrix effect of the analyte by the matrix effect of the internal standard. The CV of the internal standard normalised calculated from 6 lots of matrices should not be greater than 15%.

2.4.9 Stability

It is essential to study the stability to ensure that any step of sample preparation or sample processing does not affect the sample. Stability should be ensured for every step of the assay and the condition in which the assay is performed. It should be tested for all variables, which can be changed, and it can slightly change the results. If there are any

reference data in the literature, those can be treated as a model situation, thus, cannot be used instead of performing the analysis.

Stability of the analyte should be performed on quality control samples (=blank matrix spiked with analyte), preferably to cover most of the calibration curve range. The quality control samples as to be analysed against the calibration curve and are then compared against the initial concentrations. The mean of the measured values should be ±15% of the initial concentration for a sample to be considered unchanged by the variable.

An essential part of the stability experiments proposed by the EMA norm:

- Stability of freeze and thaw of analyte in the matrix.
- Stability of the stock and working solutions of analytes and internal standards
- Short-term stability of analyte at room temperature (sample processing temperature)
- Long-term stability of the analyte in matrix stored in the freezer (-20°C or -80°C depending on storing conditions)
- On-instrument or autosampler stability (if applicable)
- Stability of the dry extract or stability of the storage conditions

(European Medicines Agency 1922)

3. Experimental part

3.1 Materials

3.1.1 Chemicals

- Water (Honeywell and VWR)
- Methanol (VWR)
- Acetonitrile (Honeywell)
- Isopropyl alcohol (Fluka)
- Formic acid (Superlod)
- Chloroform (Sigma Aldrich)
- Hexan (Mach chemikálie)
- Dithioerythritol (Thermo Scientific)
- Di-tert-butyl-4-methyl-phenol (BHT) (Thermo scientific)
- Plasma (donated - IMTM)
- Standard reference material (SRM 1950, NIST)
- Dried blood spots (DBS) (donated - IMTM)
- Vitamin A (retinol) – standard (Sigma Aldrich)
- Vitamin E (alpha-tocopherol) – standard (Sigma Aldrich)
- Vitamin D3 – standard (Sigma Aldrich)
- Vitamin 25-OHD3 -standard (Sigma Aldrich)
- Vitamin K1 (Phylloquinone) – (Sigma Aldrich)
- Bovine serum Albumin – (Sigma Aldrich)
- NaOH
- Tertbutyl methyl ether (Sigma Aldrich)
- CaCO3

3.1.2 Consumables

- Eppendorf tubes
- Pipette tips
- HPLC vials and caps
- Parafilm
- Gloves
- Glass tubes and caps
- Centrifuge tubes
- Phree phospholipid removal tabbed 1 mL tubes (8B-S133-TAK) (Phenomenex, CA, USA)

3.1.3 Instruments

- pH meter (PO 510, CyberScan)
- Shaker (Wellmix. Lab system)
- Orbitrap Elite (Thermo Fisher Scientific)
- AB Sciex Qtrap 5500 (Sciex, CA, USA)
- Dionex UltiMate 3000 LC system (Thermo Fisher Scientific, MA, USA)
- Kinetex column (1.7 µm C_{18} 100Å, 50 x 2.1 mm, Part No:00B-4475-AN) (Phenomenex, CA USA)

- Rotina 420R Hettich zentrifugen
- TS-100 Thermo Shaker BIOSAN
- IKA Vortex Genius 3
- Raptor column (2.7 µm C_{18}, 100 x 2.1 mm, Cat # 93004A12) (Restek)

3.1.4 Software

- Analyst (Sciex, CA, USA)
- Chromeleon (Dionex chromatography)
- MultiQuant 2.1.1 (Sciex, CA, USA)
- Freestyle (Thermo Fisher Scientific, MA, USA)

3.2 Methods

3.2.1 LC-MS assay

3.2.1.1 Liquid chromatography assay

The column used in the final assay was Kinetex C18 (1.7µm, 2.1 mm x 50 mm ID) due to its separation properties and small particle dimensions. The final assay used gradient elution with two mobile phases (A)Methanol/water (70/30, *v/v*) + 0.2% formic acid and (B) methanol + 0.2% formic acid. The gradient composition can be seen in table 1. The injection volume was set to 2 µl, and flow was set at 0.5 ml/min for the whole analysis.

Table 1: Gradient composition in the final LC-MS assay

Elution time (min)	% of Mobile phase B in the composition of the eluent
0	50
0.3	50
3	100
7.5	100
7.6	50
8.12	50

3.2.1.2 MS Assay

The final MS assay was performed on AB Sciex Qtrap 5500 using specific MRM to identify the fat-soluble vitamins (FSV). All studied FSVs were analysed in positive ion mode. And all used MRM transitions are stated in Table 2.

Table 2: MRM transitions and other parameters for all analysed fat-soluble vitamins

Q1 mass (*m/z*)	Q3 mass (*m/z*)	DP (V)	CE (V)	CXP (V)	Compound ID
287.2	43.2	81	23	16	Vitamin A – Retinol
287.2	57.3	81	37	16	Vitamin A – Retinol
287.2	55.2	81	41	12	Vitamin A – Retinol
401.3	383.2	116	15	36	Vitamin 25-OH-D_3 – calcidiol
401.3	91	116	97	12	Vitamin 25-OH-D_3 – calcidiol
401.3	105	116	71	10	Vitamin 25-OH-D_3 – calcidiol
385.3	90.9	56	83	16	Vitamin D_3 – cholekalciferol
385.3	107.1	56	35	12	Vitamin D_3 – cholekalciferol
385.3	95	56	41	8	Vitamin D_3 – cholekalciferol
473.4	165.3	241	85	26	Vitamin E – alpha-tocopherol acetate
473.4	137.4	241	87	24	Vitamin E – alpha-tocopherol acetate
473.4	69.1	241	61	22	Vitamin E – alpha-tocopherol acetate
451.4	187.1	61	37	24	Vitamin K_1 – Phylloquinone
451.4	128.2	61	101	14	Vitamin K_1 – Phylloquinone
451.4	57	61	63	8	Vitamin K_1 – Phylloquinone
431.29	165.1	61	41	18	Vitamin E – alpha-tocopherol
431.29	137.1	61	59	18	Vitamin E – alpha-tocopherol
431.29	55.1	61	79	8	Vitamin E – alpha-tocopherol
524.35	268.2	61	17	14	Vitamin A – Retinylpalmitate
524.35	267	61	25	26	Vitamin A – Retinylpalmitate
524.35	144.7	61	35	8	Vitamin A – Retinylpalmitate

3.2.2 Extraction

The extraction procedure is based on liquid-liquid extraction. The extraction process was different for plasma samples and DBS.

The extraction procedure from plasma started with adding 200 µl of ice-cold solution of 37.5 $mmol \cdot l^{-1}$ Dithioerythritol (DTE) in methanol to 100 µl of plasma in a 2 ml test tube. The mixture was vortexed for 30 s, then 600 µl of extraction mixture (n-hexane: chloroform, 9:1, *v/v*). The mixture was vortexed for 30 s and secured with parafilm. The

secured tube was then placed on the shaker set for 1400 rpm for 15 min. After that, the parafilm was removed, and the mixture in tubes was centrifuged for 10 min at 22000g and 4°C. After the centrifugation, 500 µl of the upper organic phase was withdrawn and transferred to a clean tube. Extraction with organic extraction mixture was repeated once more. The collected organic phase was then evaporated with a nitrogen stream. The dried extract was then resuspended in a 1 $g \cdot l^{-1}$ Di-tert-butyl-4-methyl-phenol (BHT) solution in methanol. This mixture was put on the shaker for 5 min at 1500 rpm; then, the mixture was centrifuged for 10 minutes at 22000g and 4°C. 80 µl were then transferred to the HPLC vial and used for LC-MS analysis. The scheme of extraction can be seen in Figure 10.

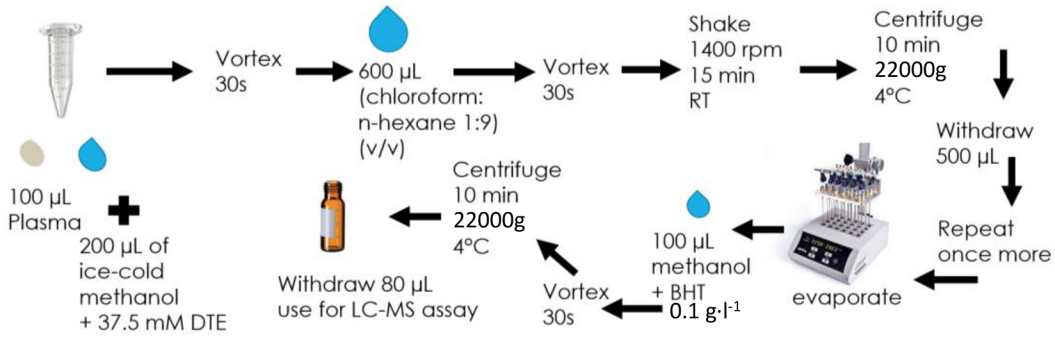

Figure 10: Scheme of final extraction procedure for fat-soluble vitamins from plasma

The extraction procedure from DBS started with adding 200 µl of LC-grade water to one-quarter of DBS in a 2 ml test tube. The tube was secured with parafilm and sonicated for 5 minutes. After the sonication, parafilm was removed, and 200 µl of ice-cold solution of 37.5 $mmol \cdot l^{-1}$ DTE in methanol was added to the mixture. The mixture was vortexed for 30s, then 600 µl of extraction mixture (n-hexane/chloroform, 9/1, v/v). The mixture was vortexed for 30 s and then secured with parafilm. The secured test tube was then placed on the shaker set for 1400 rpm for 15 min. After that, the parafilm was removed, and the mixture in tubes was centrifuged for 10 min at 22000g and 4°C. After the centrifugation, 500 µl of the upper organic phase were withdrawn and transferred to a clean tube. Extraction with organic extraction mixture was repeated once more. The collected organic phase was then evaporated with a nitrogen stream. The dried extract was then resuspended in a 1 g·l-1 BHT solution in methanol. This mixture was put on the shaker for 5 min at 1500 rpm; then, the mixture was centrifuged for 10 minutes at 15000 and 4°C. 80 µl were then transferred to the HPLC vial and used for LC-MS analysis.

4. Results

4.1 Development of extraction assay

After initial research on extraction, the first extraction procedures and mobile phases were proposed. The first version was proposed to be simple as the initial plan was to develop one which would be quick, efficient and preferably monophasic. For the initial experiments, DBS was chosen mainly due to their availability and low impact on the human organism. The extraction started with one-quarter of the DBS cut into smaller pieces, which increased the paper's surface and, therefore, should make the extraction more efficient. As the extraction solvent was used, two mixtures a) methanol/isopropanol (50/50, *v/v*) and b) methanol/acetonitrile/isopropanol (25/25/50, *v/v/v*), each was used in 100 µl. The mixture was vortexed for 30 s and let to be gently shaken for 20 min at 300 rpm at room temperature. After shaking, the mixture was centrifuged for 20 minutes at 15000 rpm and 4°C to ensure all flowing cellulose fibres and other solid particles from DBS were sedimented at the bottom of the test tube. 80 µl of the extract was transferred into HPLC vials and analysed via the LC-MS method. The column used for the LC assay was Raptor C_{18} 2.7µm 2.1mm x 100mm ID column. The results of this extraction method can be seen in Figure 11 - 14. As seen in those graphs, the extraction solvents are too weak, so the extraction procedure has to be modified.

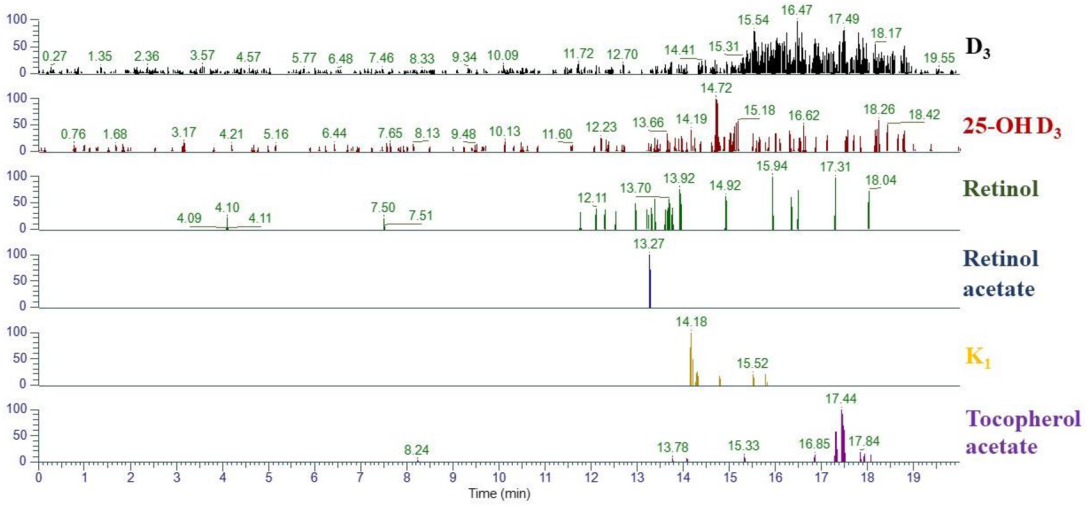

Figure 11: Chromatogram of extract acquired with methanol/isopropanol, Mobile phases: A) methanol/water (50/50, v/v)+ 0.2% formic acid B) methanol/acetonitrile (50/50, *v/v*) + 0.2% formic acid. Suspected vitamins vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet)

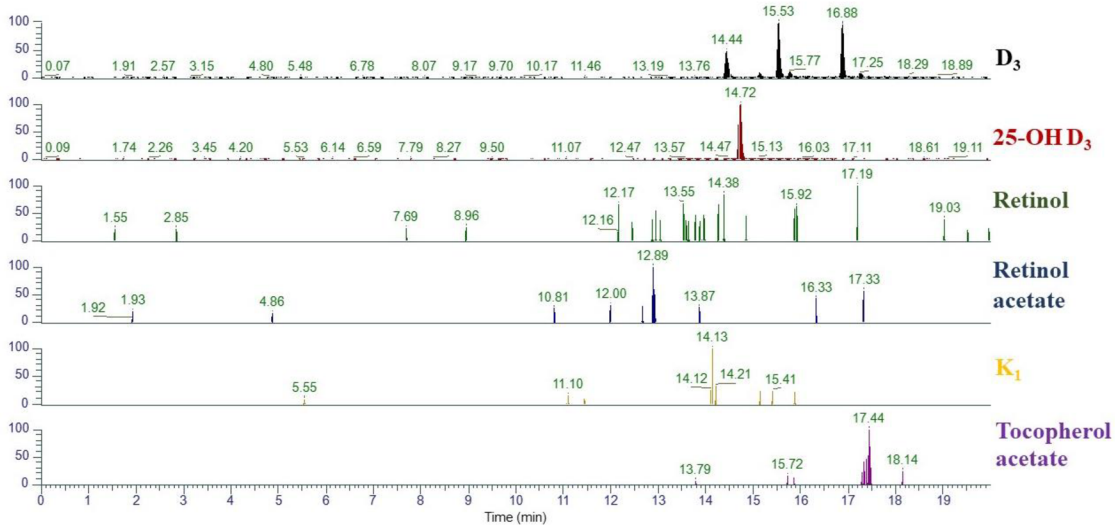

Figure 12: Chromatogram of extract acquired with methanol/isopropanol/acetonitrile, Mobile phases: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet)

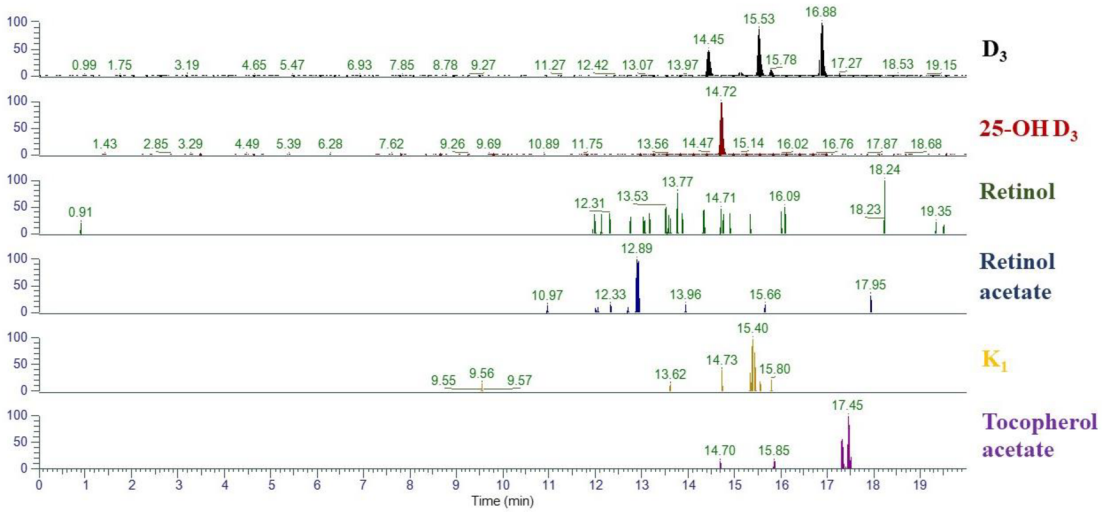

Figure 13: Chromatogram of extract acquired with methanol/isopropanol, Mobile phases: A) methanol/water (50/50, *v/v*)+ 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (Violet)

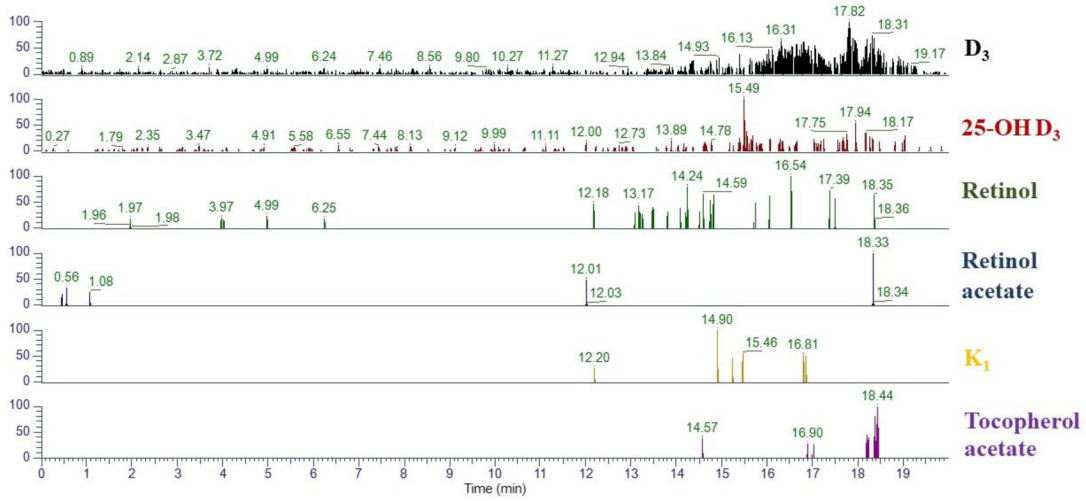

Figure 14: Chromatogram of extract acquired with methanol/isopropanol, Mobile phases: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet)

The extraction solvents applied above did not have enough extraction strength as the intensities of the suspected vitamins were between $1 \cdot 10^3$ to $1 \cdot 10^4$ if anything was extracted. As a result, other extraction mixtures were proposed, but the extraction procedure stayed the same. The proposed extraction mixtures can be found in Table 3. Regarding the mobile phases, it was decided to choose the simpler ones. The resulting chromatograms of those extraction mixtures can be found in Figure 15 – 19.

Table 3: Proposed extraction mixtures and their composition.

Sample	Chemicals	composition
A	Methanol	100%
B	methanol/isopropyl alcohol	50/50 (*v/v*)
C	Isopropyl alcohol/Water	75/25 (*v/v*)
D	Methanol/Water	50/50 (*v/v*)
E	Methanol – extraction, then withdraw 50 µl and dilute with 50 µl of Water	-

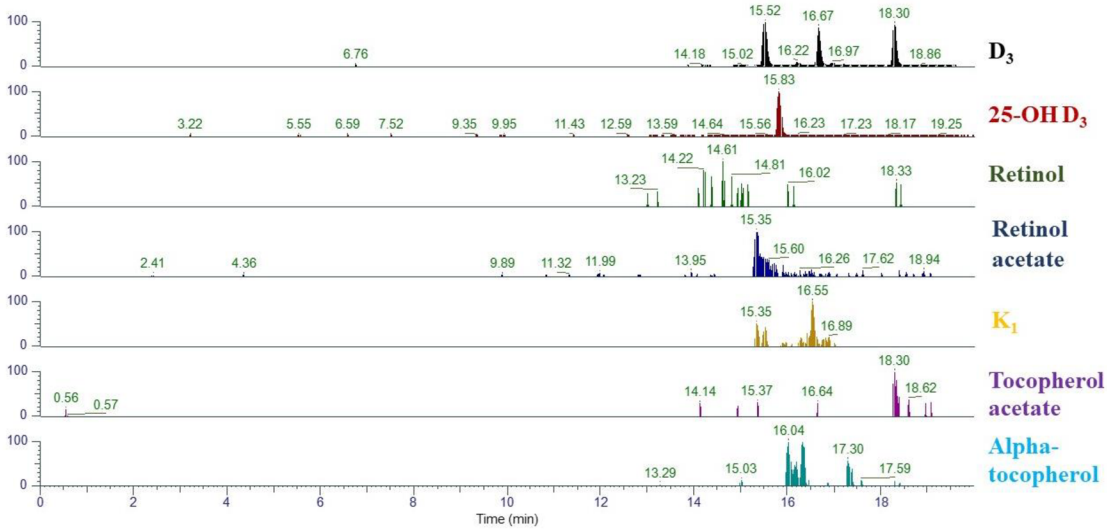

Figure 15: Chromatogram of extract acquired with methanol- extraction mixture A, Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

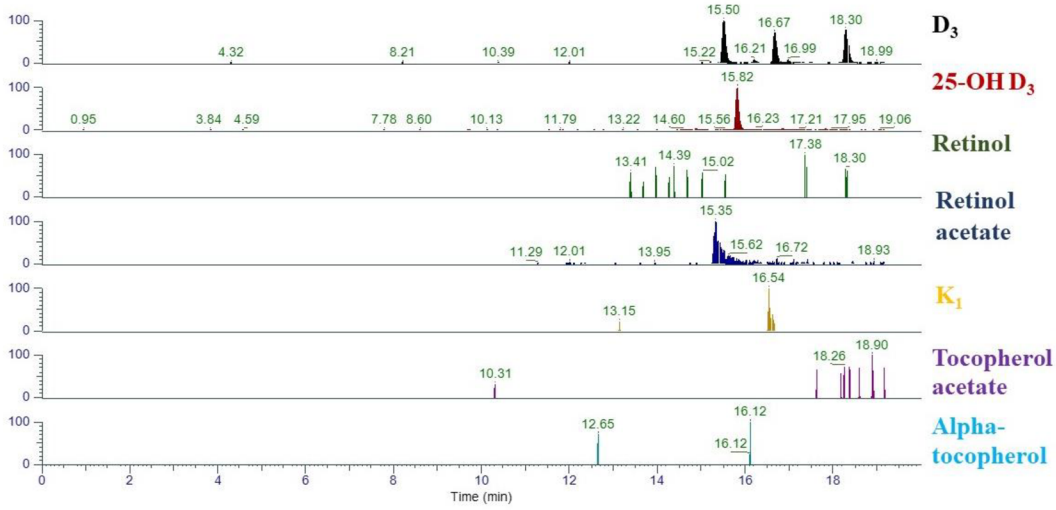

Figure 16: Chromatogram of extract acquired with methanol/isopropyl alcohol (50/50, *v/v*) - extraction mixture B, Mobile phases used: A) methanol/water (50/50, v/v) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

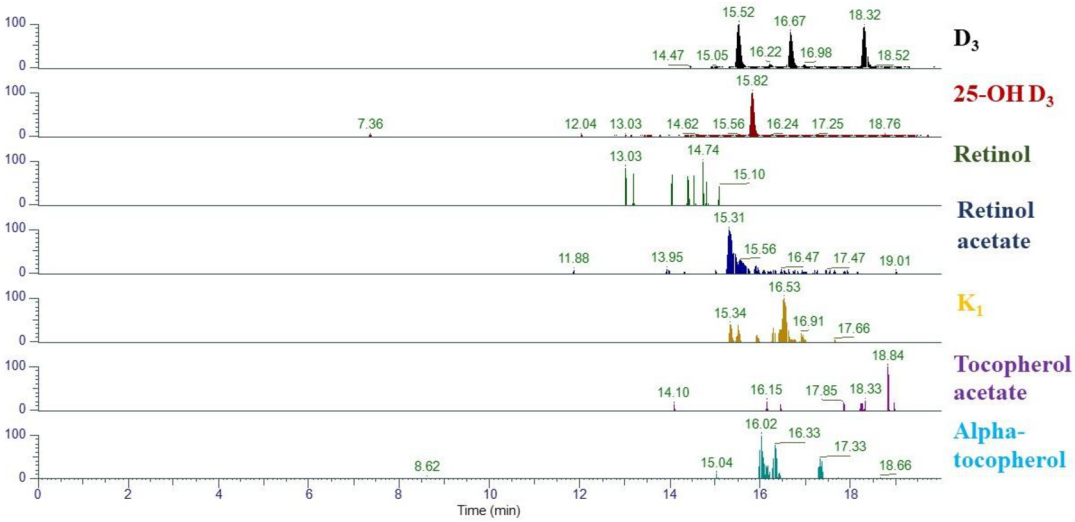

Figure 17: Chromatogram of extract acquired with water/isopropyl alcohol (50/50, *v/v*) - extraction mixture C, Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

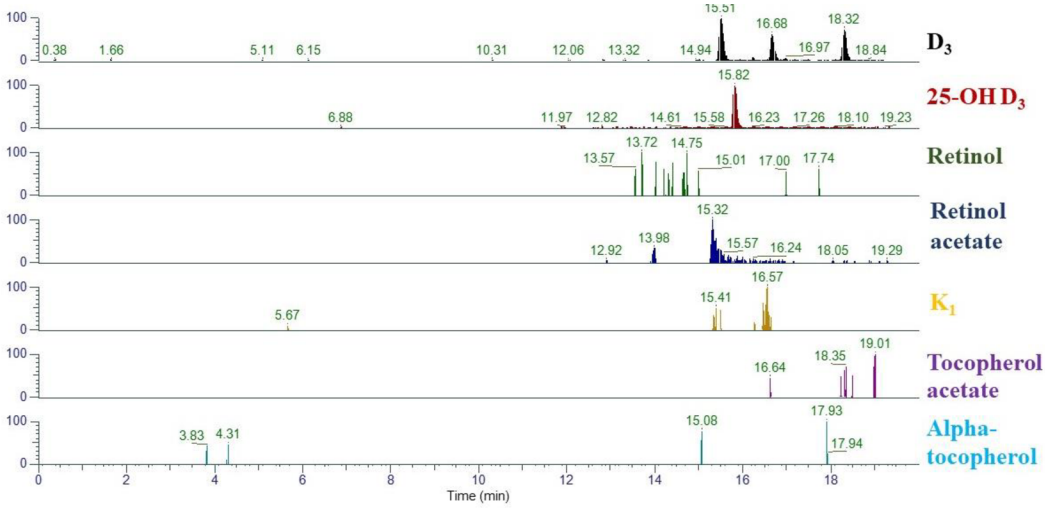

Figure 18: Chromatogram of extract acquired with methanol/water (50/50, *v/v*) - extraction mixture D, Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

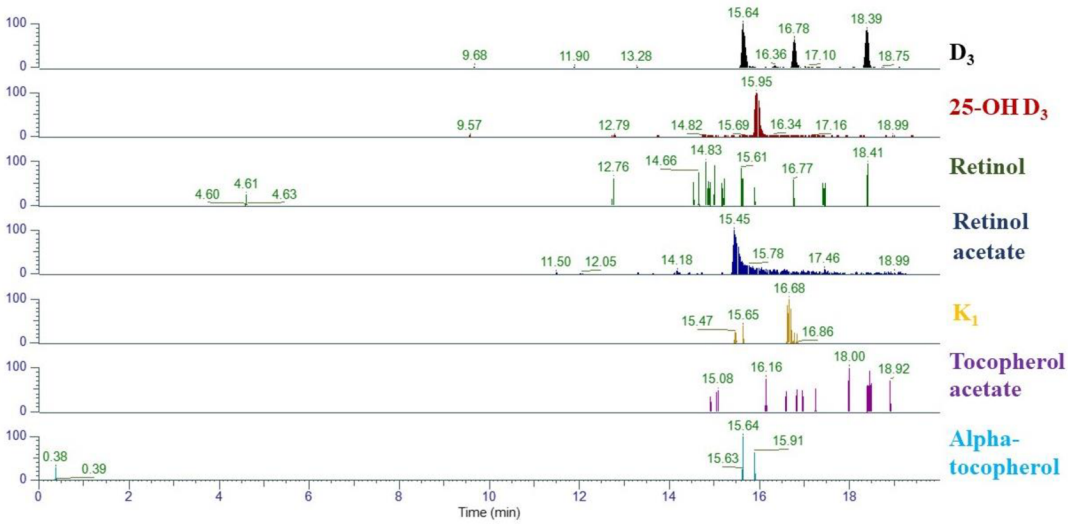

Figure 19: Chromatogram of extract acquired with methanol/water (50/50, *v/v*) - extraction mixture E, Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) Methanol + 0.2% formic acid. Suspected vitamins vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

From the information gained from the chromatograms above, we have decided that the extraction procedure would stay the same and a mixture of water and methanol 50/50, *v/v*, was then used to extract DBS. As this extraction procedure was the best fitted and the peaks of analytes showed the highest intensities $1 \cdot 10^6$ for vitamin D_3, $1 \cdot 10^7$ for 25-OH D_3, $2 \cdot 10^5$ for retinol and retinol acetate, $1 \cdot 10^4$ for K_1 and alpha-tocopherol and $1 \cdot 10^6$ for tocopherol acetate. Samples acquired with this extraction mixture were then used to create a suitable gradient (Chapter 4.2, Development of LC-MS assay). After the optimisation of the gradient, the presence of vitamins was checked with the use of the standard chemicals corresponding to the vitamins. However, the retention times of the standards of vitamin D_3 and 25-OH D_3 did not correspond to the retention times of the analytes, further shown in Figure 24. Above mentioned compounds are predicted to be cholesterol-based and/or cholesterol esters with the same *m/z* ratio, like vitamin D_3 and 25-OH D_3, but have different LogPs and are eluting later than the standards.

Due to the absence of vitamin D_3 and 25-OH D_3, the extraction procedure had to be optimised. The main purpose of the extraction optimisation was to exchange extraction solvents for ones with increased polarity compared to the previous solvents. Four mixtures were proposed, and their composition can be found in Table 4. An important step introduced and incorporated into the extraction procedure was wetting (soaking) of the DBS by adding water to the DBS before the extraction, shaking at 1400 rpm for 15 min and then removing the water and extracting as written previously.

Table 4: Proposed extracting mixtures, their composition and the form of the wetting process

Extraction mixture	Composition of mixture	Wetting
F	100% acetonitrile	water (in the ultrasonic bath)
G	methanol/water (50:50, *v/v*)	0.1% formic acid solution
H	methanol/water (50/50 *v/v*) + 0.1% formic acid	-
I	100% isopropyl alcohol	Water

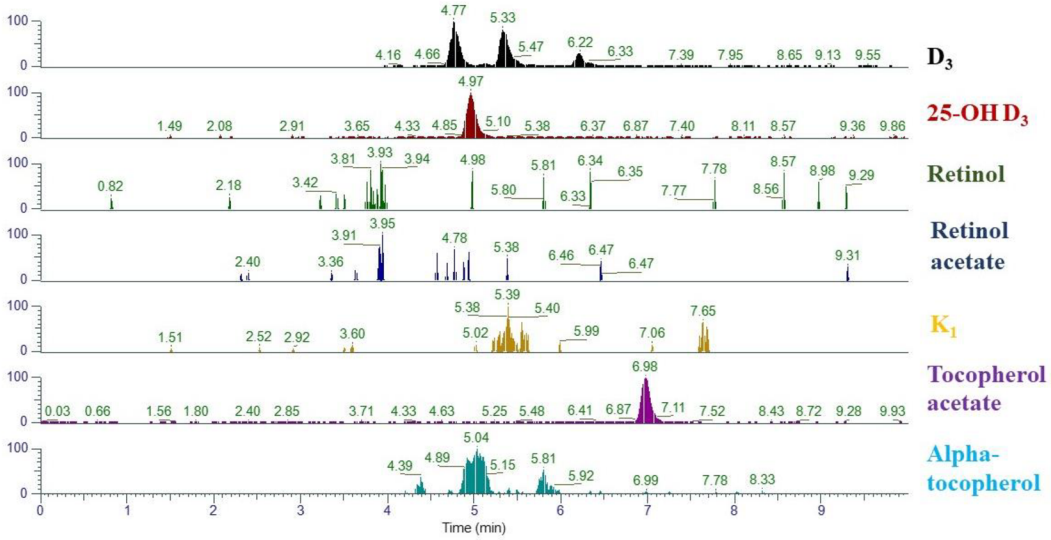

Figure 20: Chromatogram of extract acquired with acetonitrile - extraction mixture F, Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

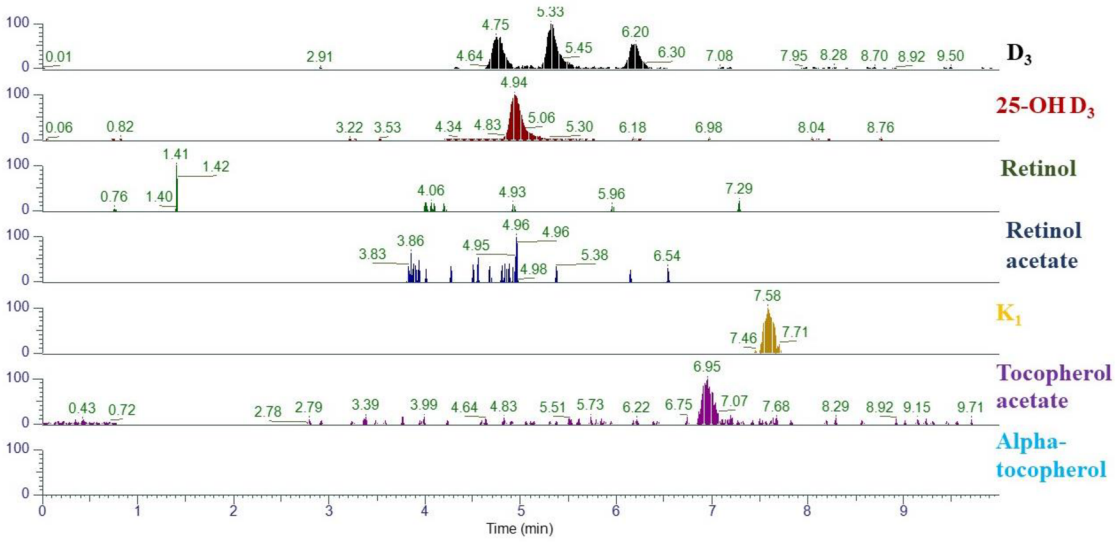

Figure 21: Chromatogram of extract acquired with methanol/water (50/50, *v/v*) extraction mixture G, Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

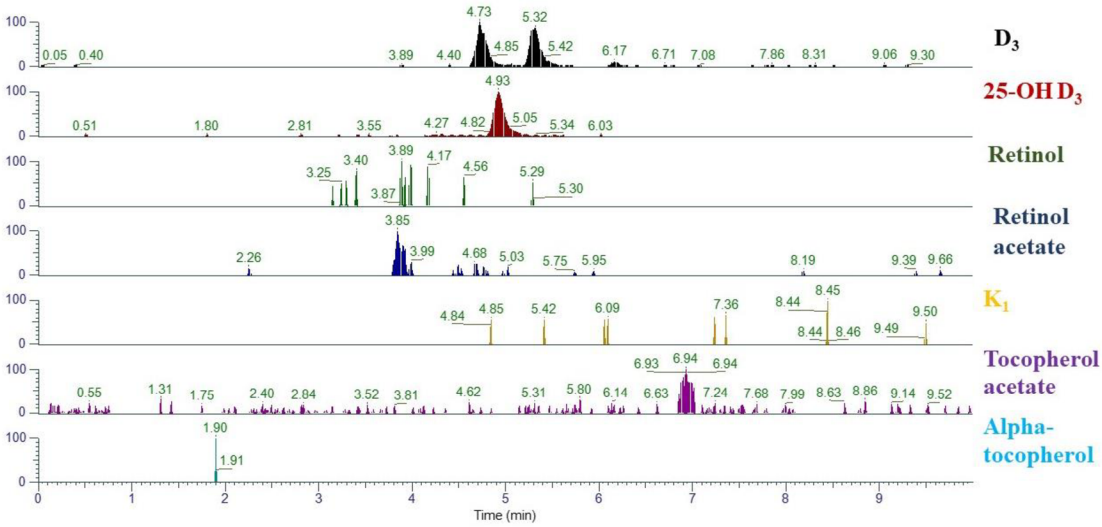

Figure 22: Chromatogram of extract acquired with a mixture of methanol/water (50/50, *v/v*) + 0.1% formic acid- extraction mixture H, Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

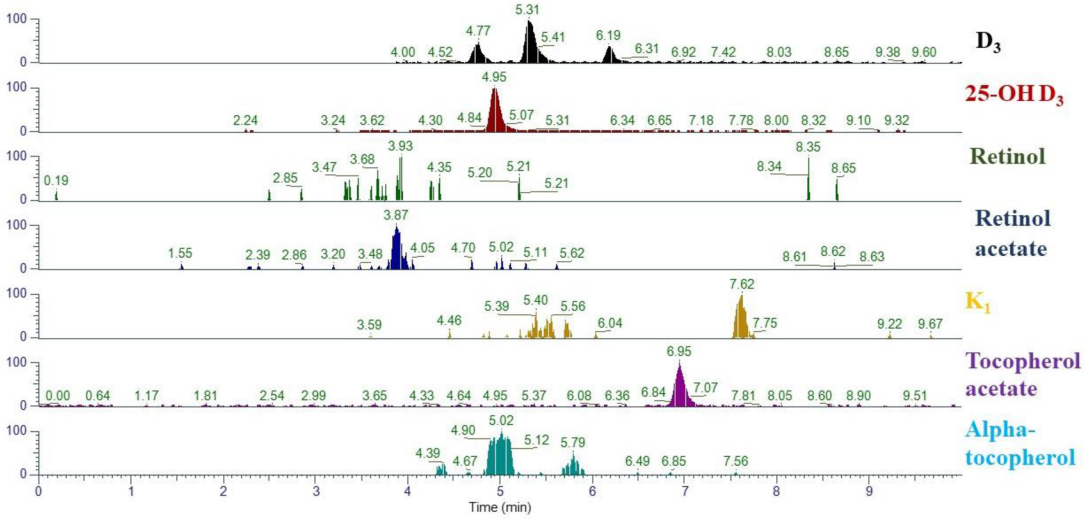

Figure 23: Chromatogram of extract acquired with isopropyl alcohol - extraction mixture I, Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

From the first look at the chromatograms above, Figure 20 - 23, it can be said that mixture F was the most efficient extraction of fat-soluble vitamins. However, one important factor has to be considered, which is the nonspecificity of this analysis, which can be projected into identifying the compounds only by their molecular weight. Therefore, the mixture of analytes was analysed with the same analytical assay to compare the elution time of standards and suspected fat-soluble vitamins. The chromatogram showing the analysis of the standards can be seen in Figure 24.

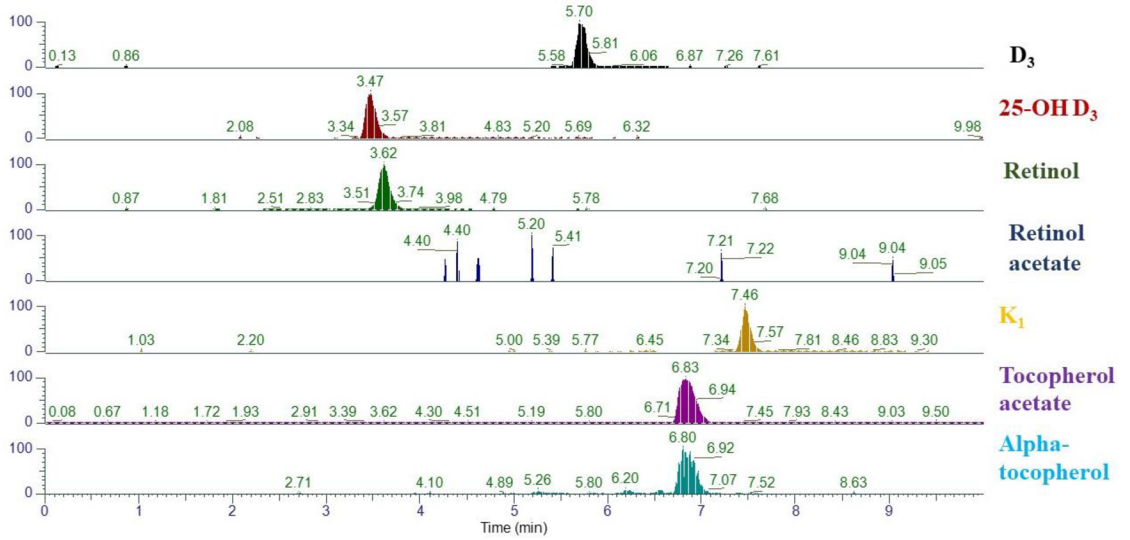

Figure 24: Chromatogram of fat-soluble vitamins standards: Mobile phases used: A) methanol/ water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

As can be deduced, vitamins D_3 and 25 OH-D_3 are absent in any extracted samples; the peak has the same *m/z*. However, the compounds do have different polarities from the vitamins. Also, retinol does not show any significant peaks in any of the previous extracts. Therefore, other changes in the extraction procedure had to be made. By this time, with a lack of alternatives for monophasic and straightforward extractions, we decided to do additional research on liquid-liquid (biphasic, LLE) extractions. We decided to go with liquid-liquid extraction using three extraction mixtures based on the literature and availability of the chemicals. The extraction mixtures were: n-hexane/chloroform (90/10)(*v/v*), pure n-hexane, methyltertbutylether (MTBE)/Methanol 50/50)(*v/v*). Regarding the procedures, we decided to try already published extraction procedures to ensure efficiency but slightly modify them to fit laboratory equipment.

The first procedure (Nimalaratne et al. 2014) used 300 µl of the mixture (n-hexane/chloroform, 90/10, *v/v*, mixture one and n-hexane = mixture two) to extract one-quarter of DBS cut into small pieces. The mixture in tubes was then vortexed for 30 seconds and secured with parafilm to ensure that the volatile mixture did not evaporate from the tube. The tubes were put for 15 minutes into the ultrasonic bath. The mixture was then centrifuged for 10 minutes at 3000 rpm at 4°C. Then 250 µl was withdrawn into a separate clean tube. The extraction was performed again, and the combined extracts were evaporated under a nitrogen stream. The dried extracts were then resolubilised in

100 µl of methanol; the mixture was put on the shaker to ensure maximal solubilisation; after shaking, the mixture was centrifuged for 10 minutes at 3000 rpm and 4°C. 80 µl were transferred into an HPLC vial and analysed via LC-MS assay. The chromatograms of extracts from this extraction procedure are shown in Figures 25 and 26.

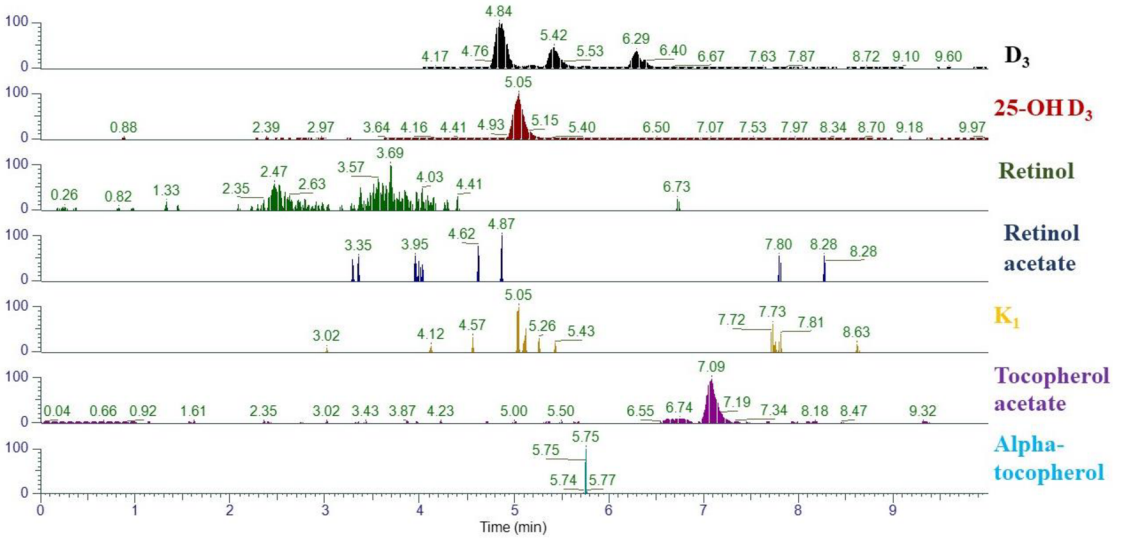

Figure 25: Chromatogram of extract acquired with extraction mixture n-hexane/chloroform, 90/10, *v/v*: Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

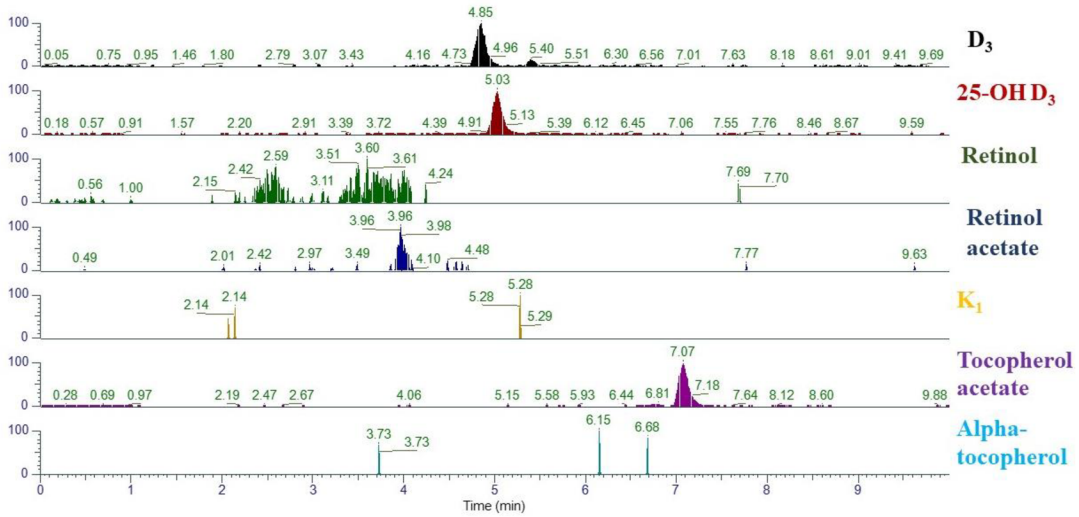

Figure 26: Chromatogram of extract acquired with n-hexane extraction: Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

Based on the comparison of the chromatogram of fat-soluble vitamin standards and chromatograms shown in Figures 25 and 26, it can be said that the method mentioned above is not well-suitable for fat-soluble vitamin extraction because only vitamin that is

extracted in significant amount and form well-shaped peaks is tocopherol acetate, which peaks have intensities around $1 \cdot 10^6$ in both extraction versions.

The second proposed option (Sau et al. 1997) for extraction of fat-soluble vitamins started with a wetting process of one-quarter of DBS using formic acid solution (1% formic acid = sample 4, 0.1% = sample 5). The mixture was vortexed for 30 s and then put on the saker at 300 rpm for 10 minutes. After shaking, the extraction mixture was added (n-hexane/chloroform, 90/10, *v/v*). Then the mixture was centrifuged for 10 minutes and 3000 rpm and 4°C. After centrifugation, the organic layer was withdrawn; in the case of wetting with 1% formic acid solution, the paper in DBS disintegrated, making it difficult to withdraw the organic layer. The organic solvent was then evaporated under a nitrogen stream. The dried extract was then resuspended in 100 µl of methanol. To ensure the complete dissolution of the dried extract, the mixture was put on a shaker at 300 rpm for 5 min. After that mixture was centrifuged for 10 minutes at 3000 rpm and 4°C. 80 µl of the solution was then transferred into an HPLC vial and was used for LC-MS analysis. Chromatograms of those extracts can be seen in Figures 27 and 28.

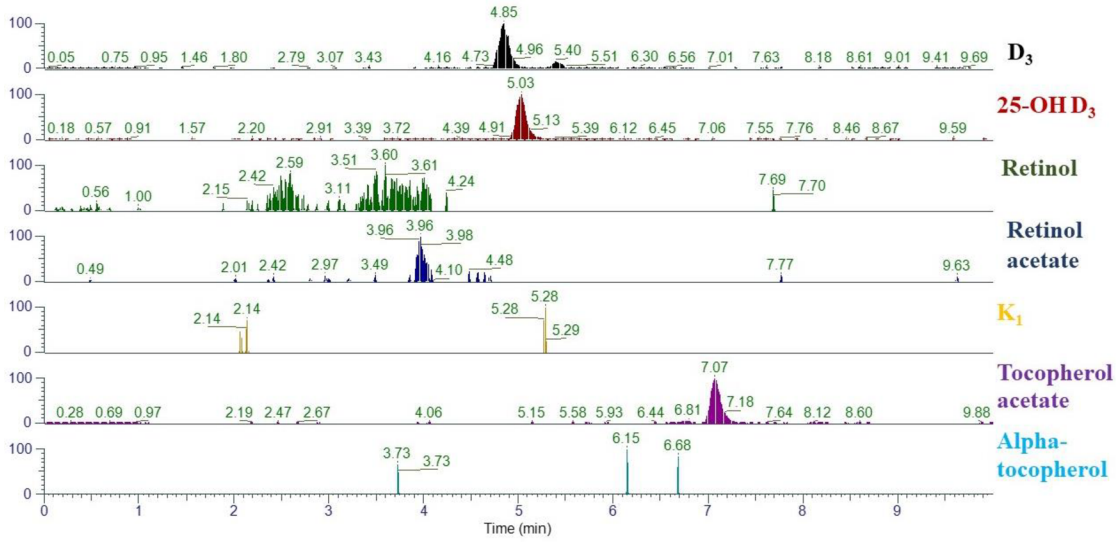

Figure 27: Chromatogram of extract acquired with extraction mixture n-hexane/chloroform, 90/10 *v/v*, wetted with 1% formic acid solution: Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

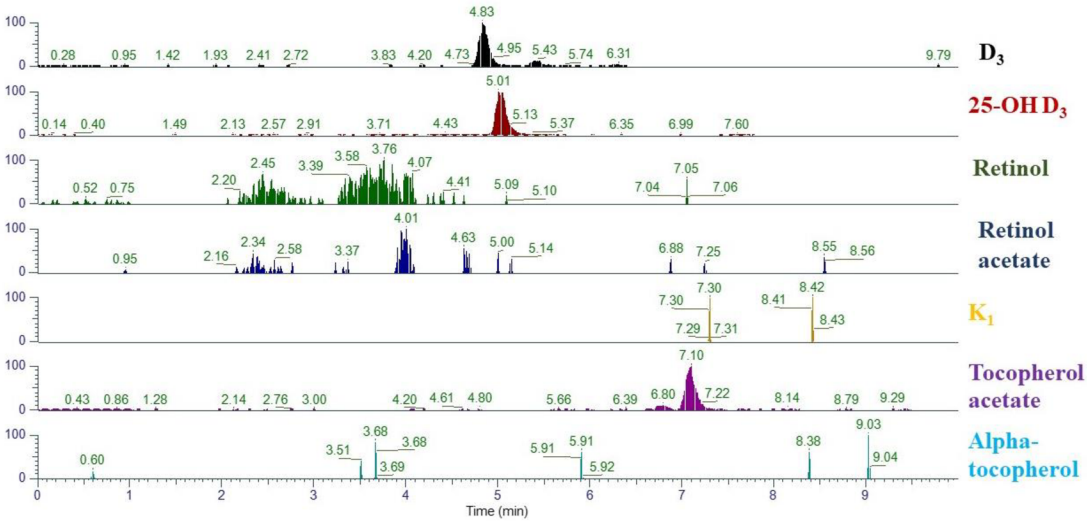

Figure 28: Chromatogram of extract acquired with extraction mixture n-hexane/chloroform, 90/10 *v/v*, wetted with 0.1% formic acid solution, Mobile phases used: A) methanol/water (50/50, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

For Figure 27, the intensities of peaks were as follows: $5 \cdot 10^3$ for retinol, $1 \cdot 10^4$ for retinol acetate and $2 \cdot 10^5$ for tocopherol acetate and for Figure 28 intensity of tocopherol acetate was $5 \cdot 10^4$. Although the results of both versions of the extraction are highly similar to each other, however still do not have high enough extraction strength to extract all of the fat-soluble vitamins.

The third extraction method (Rubió et al. 2020) started with adding one-quarter of DBS and 300 µl of extraction liquid (MTBE: Methanol (50/50, v/v) = sample 1 and n-hexane = sample 2) into the tube. The mixture was vortexed for 30 s and then placed on the shaker for 10 minutes at 300 rpm. Then the mixture was centrifuged for 10 minutes at 300 rpm and 4°C. After that, the organic layer was withdrawn, and extraction was repeated again. The combined organic layers were then dried under a nitrogen stream. The dried extract was then resolubilised in 100 µl of methanol. Then the mixture was put on a shaker at 300 rpm for 5 min. After that mixture was centrifuged for 10 minutes at 3000 rpm and 4°C. 80 µl of the solution was then transferred into an HPLC vial and was used for LC-MS analysis. Chromatograms can be seen in Figures 29 and 30.

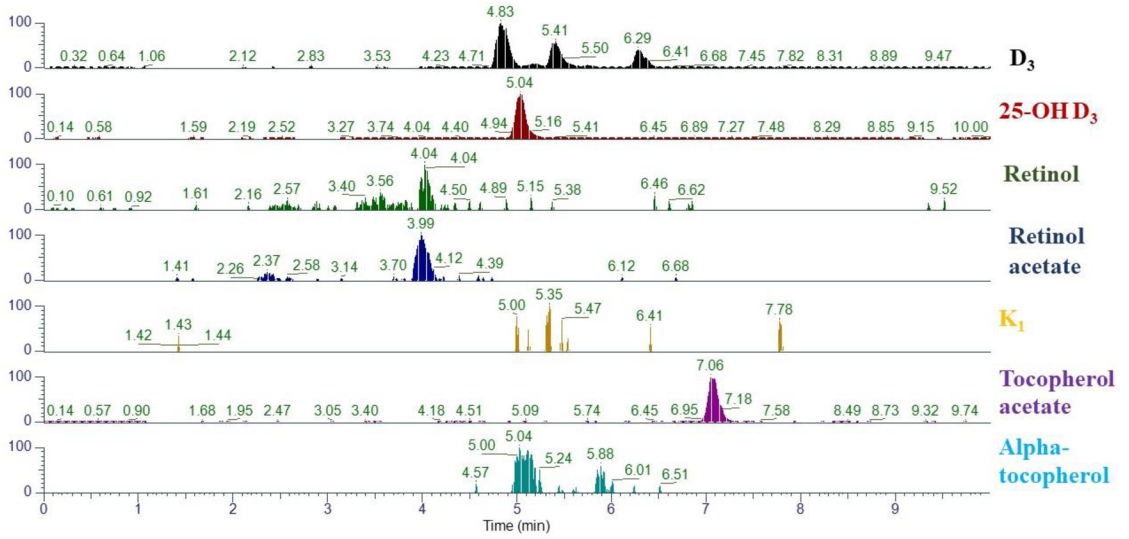

Figure 29: Chromatogram of extract acquired with extraction mixture MTBE/methanol (50/50 *v/v*), Mobile phases used: A) methanol/water (50/50, *v/v*)+ 0.2% formic acid B) methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

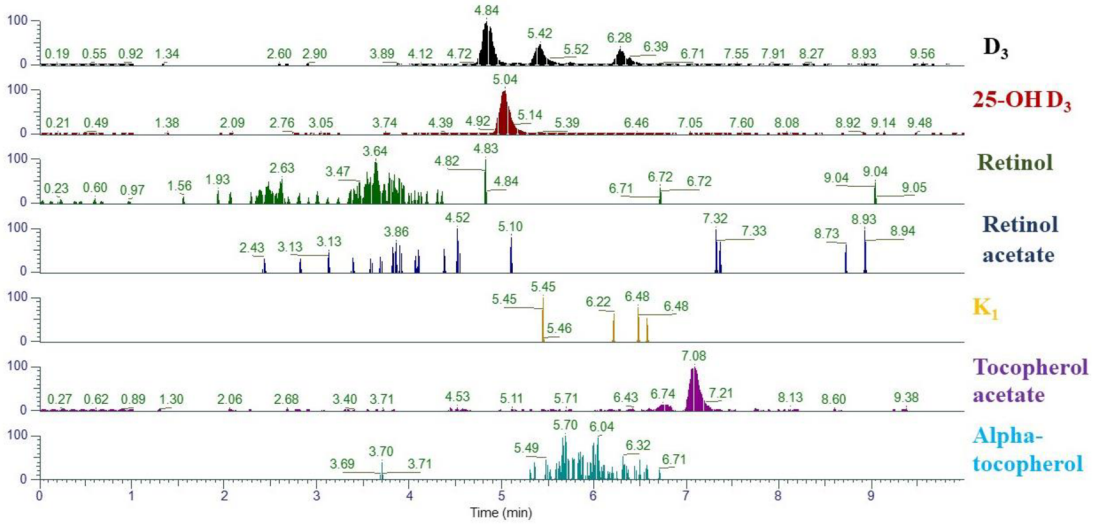

Figure 30: Chromatogram of extract acquired with n-hexane: Mobile phases used: A) Methanol/water (50/50, *v/v*) + 0.2% formic acid B) Methanol + 0.2% formic acid. Suspected vitamins: vitamin D_3 (black), 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), Vitamin K_1 (yellow), tocopherol acetate (violet), alpha-tocopherol (light blue)

This extraction procedure mentioned above using the MTBE/Methanol 50/50 *v/v* had better results than any of the previous ones, primarily due to the ability to extract tocopherol acetate and retinol and retinol acetate, intensities of the peaks were $5 \cdot 10^3$ for retinol acetate and retinol and $1 \cdot 10^5$ for tocopherol acetate. However, there was still no peak containing vitamin D_3 and 25-hydroxyvitamin D_3, as those are important to be determinable via assay. A wide variety of solvents was tried. However, any of the solvents

didn't extract all the fat-soluble vitamins, and none of the solvents extracted vitamin D_3 and 25-hydroxyvitamin D_3. Therefore, a hypothesis was proposed that the concentrations of most of the vitamins are lower than the lower limit of detection in orbitrap MS. As a solution change of the MS instrument was proposed. The mass spectrometer triple quadrupole was used, mainly due to its ability to detect lower concentrations. Multiple reaction monitoring was introduced into the mass spectrometry assay to ensure the correct identification.

The extraction procedures mentioned above were combined into one extraction process. It started with a wetting procedure of one-quarter of DBS using 0.1% formic acid solution. The mixture was vortexed for 30 s and then put on the saker at 300 rpm for 10 minutes. Then the wetted DBS was extracted with 600 µl of the extraction mixture (n-hexane/chloroform, 90/10, *v/v*). The mixture in tubes was then vortexed for 30 seconds and secured with parafilm to ensure that the volatile mixture did not evaporate from the tube. The tubes were put for 15 minutes into the ultrasonic bath. The mixture was then centrifuged for 10 minutes at 22000g at 4°C. Then 500 µl were withdrawn into separate clean. To ensure that the second extraction is needed, two samples were extracted once, and two were extracted two times. The extracts were then evaporated under a nitrogen stream. The dried extracts were then resolubilised in 100 µl of methanol; the mixture was put on the shaker to ensure maximal solubilisation; after shaking, the mixture was centrifuged for 10 minutes at 22000g and 4°C. 80 µl were transferred into an HPLC vial and analysed via LC-MS assay. The resulting chromatograms can be seen in Figures 31 and 32.

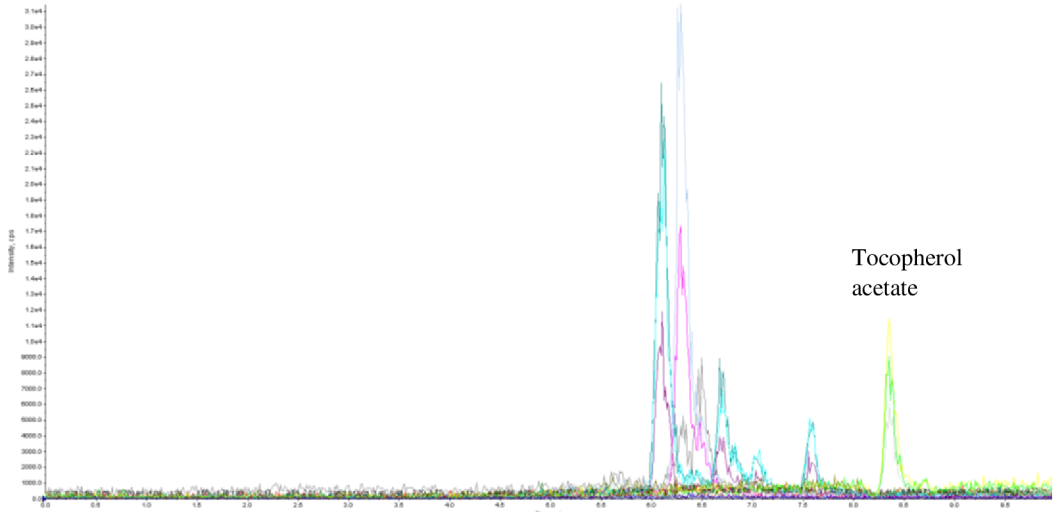

Figure 31: Chromatogram of extract acquired with n-hexane/chloroform 90/10 *v/v*, extracted once. Mobile phases used: A) Methanol/water (70/30, *v/v*)+ 0.2% formic acid B) Methanol + 0.2% formic acid.

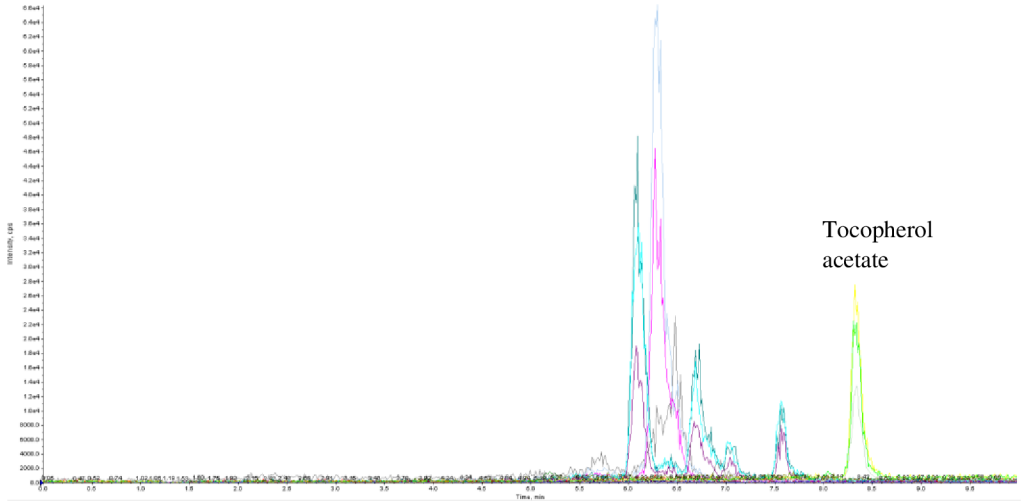

Figure 32: Chromatogram of extract acquired with n-hexane: chloroform 90/10 v/v, extracted twice, Mobile phases used: A) Methanol: water (70/30, v/v)+ 0.2% formic acid B) Methanol + 0.2% formic acid.

From the comparison of Figures 31 and 32, Figure 32 shows that twice extracted sample has higher intensities; therefore, extracting the sample twice gives higher efficiency to the extraction process. However, this extraction procedure can be marked as inadequate, as only tocopherol acetate was extracted.

Above mentioned extraction procedure was then adjusted considering other resources, mainly (Midttun et al. 2016) and the final extraction assay was developed. At this time, plasma was also introduced as a sample. The results of this extraction assay from DBS can be seen in Figures 33 and 34 and from plasma in Figures 35 and 36.

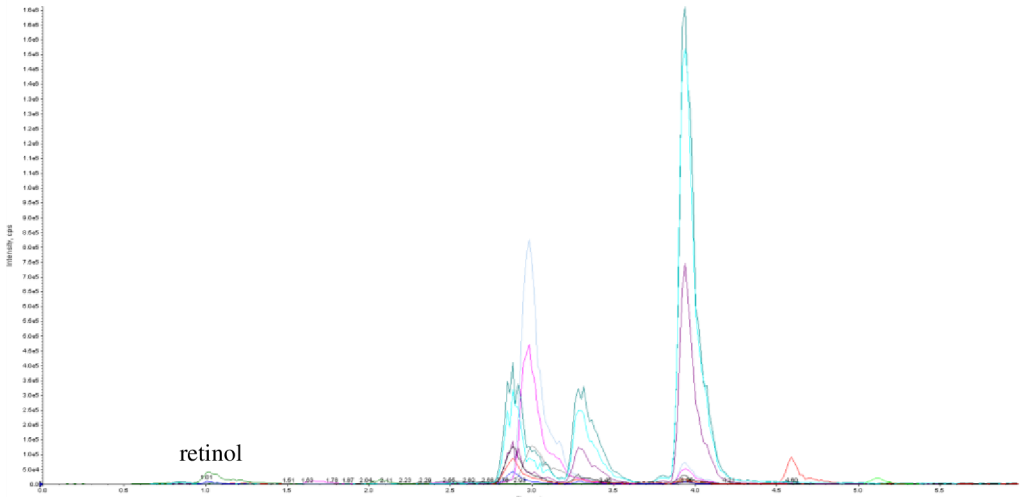

Figure 33: Chromatogram of extract acquired with n-hexane: chloroform 90/10 *v/v*, extracted twice, Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid.

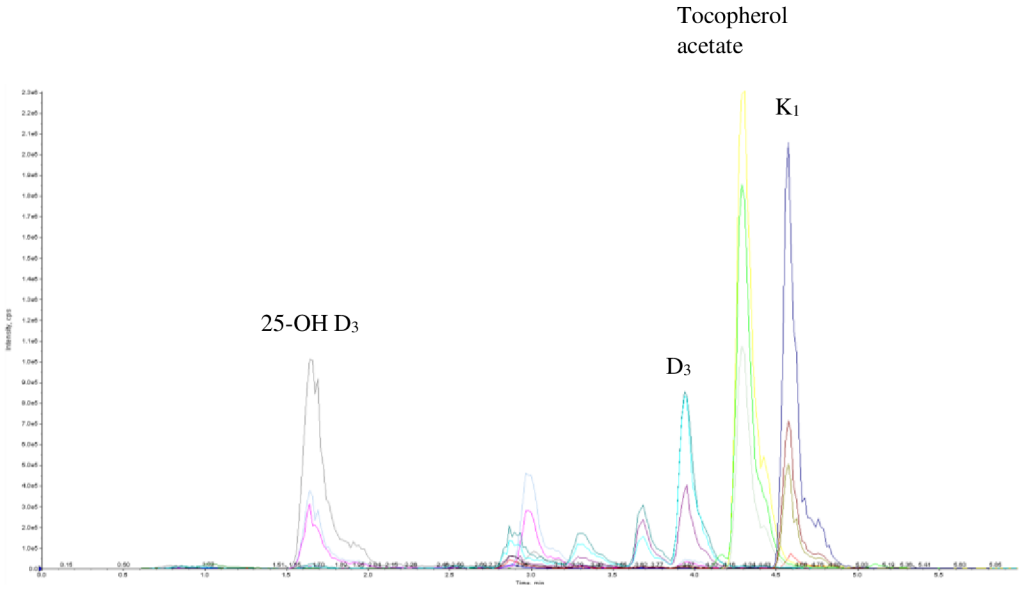

Figure 34: Chromatogram of spiked DBS extract acquired with n-hexane/chloroform 90/10 *v/v*, extracted twice, Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid.

From the comparison of Figure 33 – DBS extract and Figure 34 – spiked DBS extract. It can be said that only tocopherol acetate was efficiently extracted from DBS. However, the extraction of vitamins from spiked DBS was extracted. Therefore the extraction assay is strong enough to extract fat-soluble vitamins.

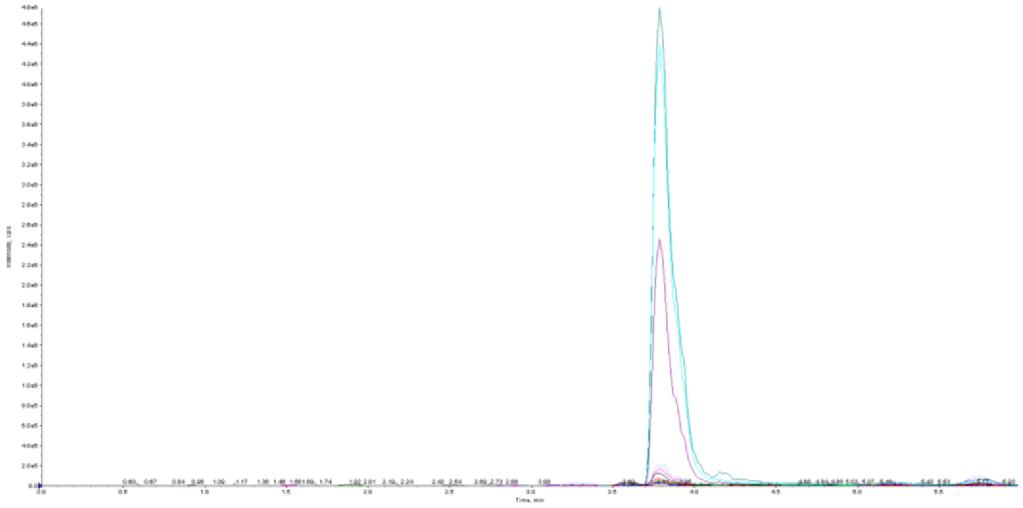

Figure 35: Chromatogram of plasma extract acquired with n-hexane/chloroform 90/10 *v/v*, extracted twice, Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid.

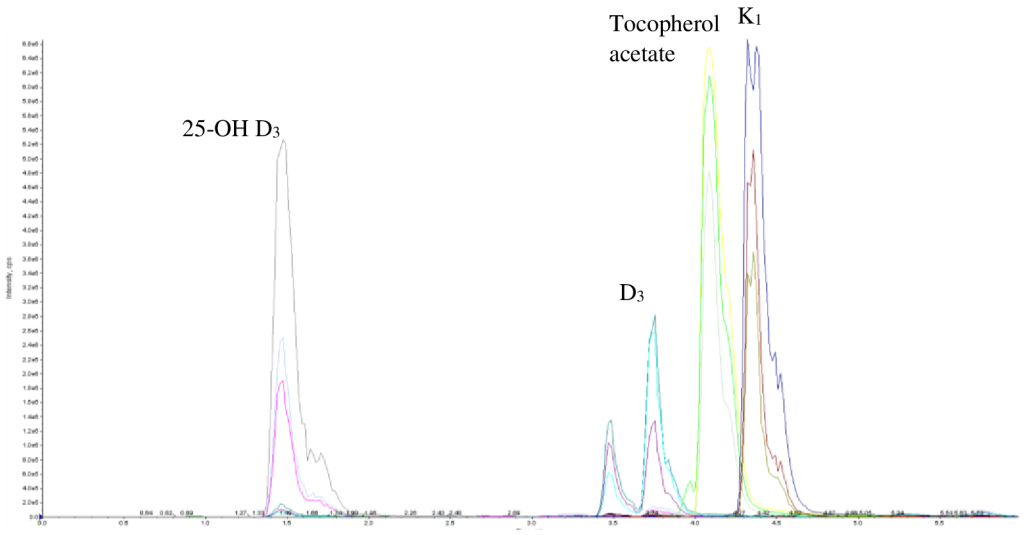

Figure 36: Chromatogram of spiked plasma extract acquired with n-hexane: chloroform 90/10 v/v, extracted twice, Mobile phases used: A) Methanol: water (70/30, v/v)+ 0.2% formic acid B) Methanol + 0.2% formic acid.

The results of plasma extracts are comparable to the results of DBS extracts. Both extracts show the main peak of tocopherol acetate. However, the chromatogram of the spiked plasma sample shows a peak of higher interest, even though the same amount of vitamins were added to the sample. The conclusion can be that it is easier to extract fat-soluble vitamins from plasma than from DBS. The hypothesis was proposed that if vitamins D_3 and 25-OH D_3 are very hard to extract, they can be precipitated together with proteins. To test if vitamin D_3 and 25-OH D_3 are not precipitated with plasma proteins, a solution of bovine serum albumin (BSA)(70 $g \cdot l^{-1}$) was spiked with 50 µl of stock solution of each vitamin (concentration 100 $\mu mol \cdot l^{-1}$). 100 µl of spiked BSA solution was used for

extraction, using the final extraction procedure for plasma. The results of the experiment can be seen in Figure 37.

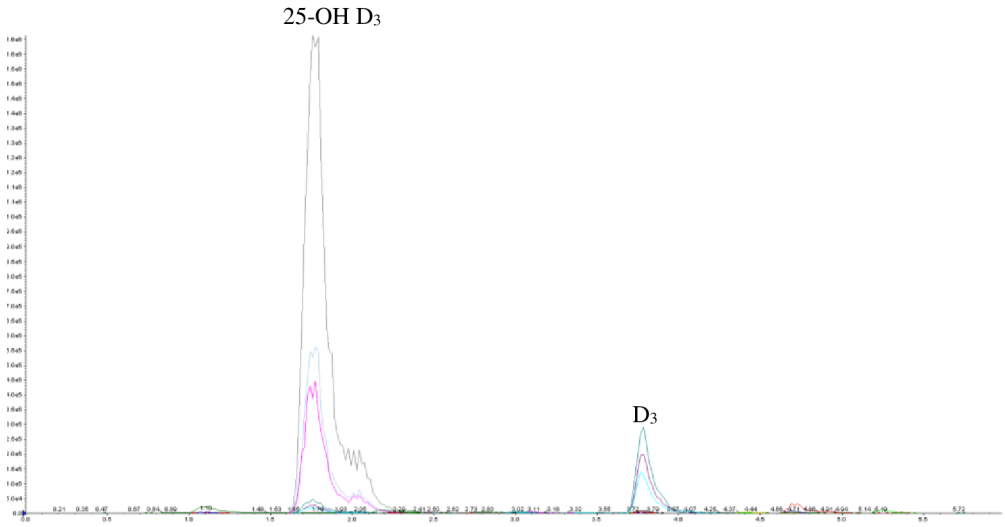

Figure 37: Chromatogram of spiked BSA extract acquired with n-hexane/chloroform 90/10 *v/v*, extracted twice, Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid.

If Figure 37 is compared to previous chromatograms, the intensity is comparable to spiked samples. Also, intensities are similar to the intensities of standards. As a result, the proposed hypothesis was rejected. But all fat-soluble vitamins were still not extracted. Two main hypotheses were proposed. The first one was based on the low polarity of the fat-soluble vitamins, which may then adhere to the surface of the plastic test tubes. This problem may be easily solved by switching plastic test tubes to glass test tubes. The second hypothesis was based on low concentrations of fat-soluble vitamins, which then can be undetectable. To avoid both of those potential problems, it was decided to run the extraction process with 0.5 ml of plasma and in glass bottles. The resulting chromatogram can be seen in Figure 38.

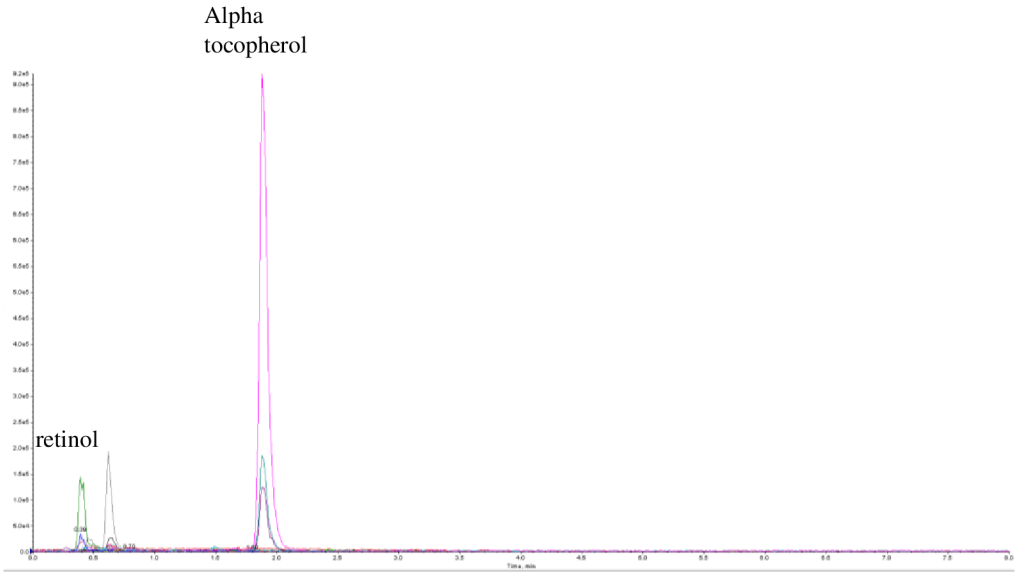

Figure 39: Chromatogram of plasma extract acquired with n-hexane/chloroform 90/10 *v/v*, extracted twice from 0.5 ml of plasma. Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid.

The extraction yielded to obtain retinol in significant amounts. However, there are still no significant peaks containing vitamin D_3, 25-hydroxyvitamin D_3, or vitamin K_1. To eliminate any possible problem caused by LC-MS equipment, it was allowed to run our experiments in the Department of Medical Chemistry and Biochemistry (UP), where a similar instrument is. Those experiments showed the same results as those in previously used lab and equipment.

Consequently, even more complicated and time-consuming methods had to be introduced. One of those methods was saponification. But firstly, phospholipid removing Phree columns by Phenomenex company were used. The primary justification was phospholipids' possible suppressive matrix effect and that phospholipid-removing columns are frequently utilised in extractions for fat-soluble vitamins or other fat-soluble metabolites.

The extraction procedure started with adding 100 µl of plasma and 300 µl of acetonitrile with 1% formic acid. The Phree columns were centrifuged at 1000 rpm, 4°C for 15 minutes. The resulting eluent was extracted with 600 µl of extraction mixture (n-hexane/chloroform 90/10 *v/v*) in the same way as in the final extraction method. The resulting chromatogram can be seen in Figure 39.

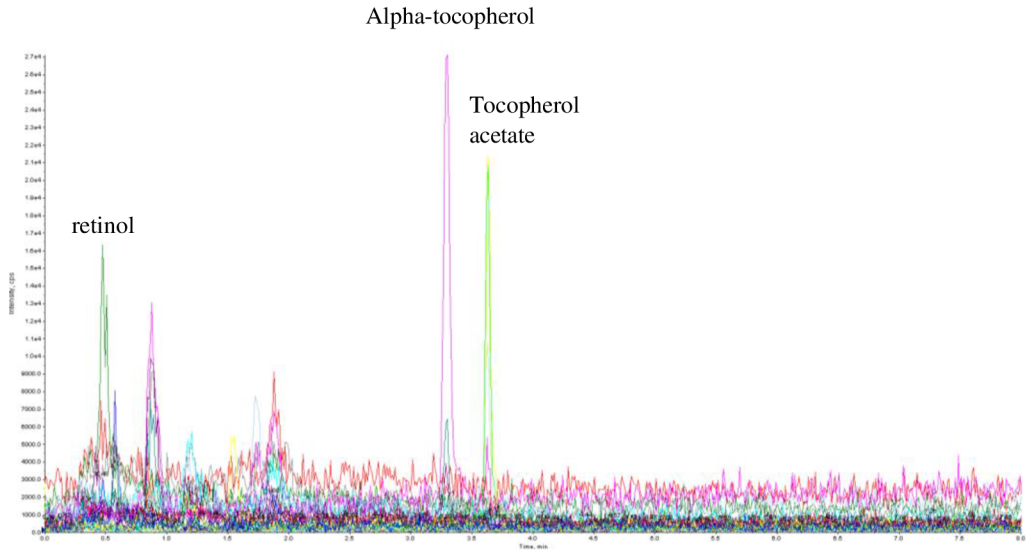

Figure 39: Chromatogram of plasma extract acquired with Phree column and n-hexane/chloroform 90/10 *v/v*, extracted twice from 0.5 mL of plasma. Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) Methanol + 0.2% formic acid.

As seen in the chromatogram, the main problem there is the high noise ratio and relatively low intensity of the peak. This may come from an extraction error. The error may have come from human error, as there wasn't enough acetonitrile or the mixture wasn't acidic enough. The fat-soluble vitamin may also be captured on the Phree column with phospholipids or precipitated together with protein. Still, the result is rather negative due to the low intensities of peaks and high noise.

Then saponification procedure started with adding 100 µl plasma (or one-quarter of DBS) to 2.5 ml of saponification liquid (10% BHT, 10 $mmol \cdot l^{-1}$ KOH in methanol). The mixture was let to shake overnight. Then 70 µl of formic acid was added. The mixture was then divided into three aliquots, as Phree columns fit only 1 ml of liquid. Those aliquots were then transferred into Phree columns and centrifuged for 15 min at 1000 rpm and 4°C. The aliquots were then extracted in the same way as in the final extraction assay. The resulting chromatograms can be seen in Figures 40 and 41.

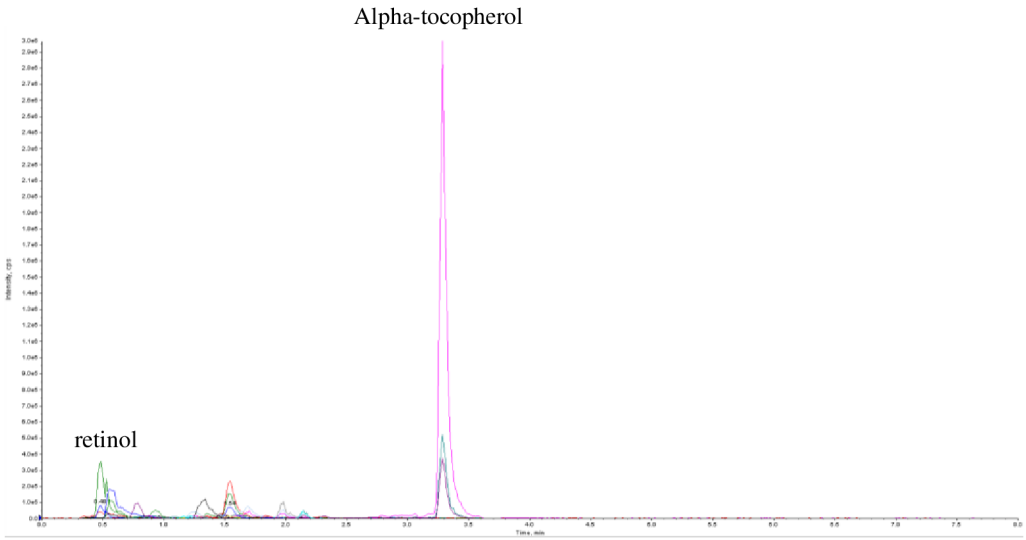

Figure 40: Chromatogram of plasma extract acquired with saponification, Phree column and n-hexane: chloroform 90/10 *v/v*, extracted twice from 0.5 ml of plasma, Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid.

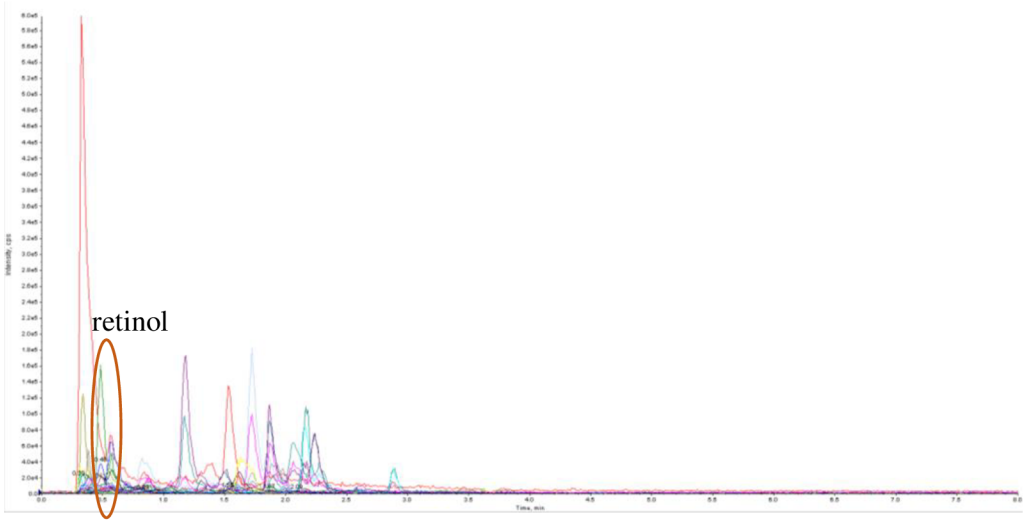

Figure 41: Chromatogram of DBS extract acquired with saponification, Phree column and n-hexane: chloroform 90/10 v/v, extracted twice from 0.5 mL of plasma, Mobile phases used: A) methanol/water (70/30, v/v) + 0.2% formic acid B) methanol + 0.2% formic acid.

The saponified plasma extraction had the best results so far, as it did extract retinol and alpha-tocopherol in significant amounts. However, the extraction is very time-consuming and laborious. It is tough to regulate external conditions in saponification as temperature and light intensity, which can substantially influence the extraction efficiency and the concentration of vitamins, as they can degrade under certain conditions.

Due to failures with the extraction of vitamins D_3 and 25-hydroxyvitamin D_3, we decided to turn to already published and standardised materials (Huq and Safan n.d.) It started with adding 300 µl of acetonitrile/methanol (85/15, *v/v*) to Phree columns. Then

to Phree columns was added 100 µl of plasma. The mixture was left to precipitate for 30 minutes. After precipitation mixture in Phree columns was centrifuged for 15 minutes at 2000 rpm and 4°C. The resulting eluent was collected into the clean test tube. 200 µl of a mixture of acetonitrile/methanol (85/15 *v/v*) was added into the previously used Phree column and vortexed for 30 s. Then the mixture in Phree columns was centrifuged for 15 minutes at 2000 rpm and 4°C. The eluent was collected, and 80 µl of eluent was used for analysis. The rest of the eluent was evaporated under a nitrogen stream, resuspended in 100 µl of methanol with 1 $g \cdot l^{-1}$ BHT, vortexed and 80 µl was used for analysis. The results of this analysis can be seen in Figures 42 and 43.

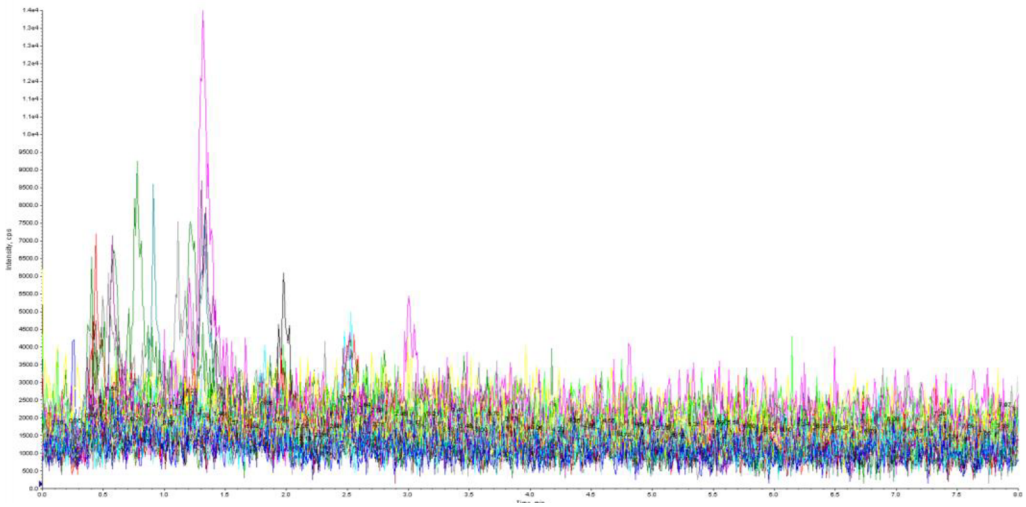

Figure 42: Chromatogram of Plasma extract acquired by Phenomenex procedure. Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid

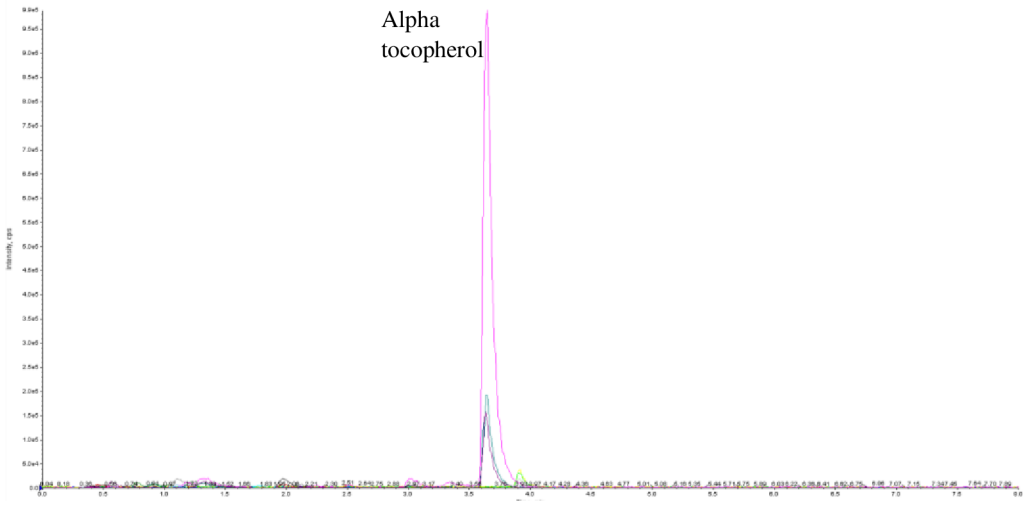

Figure 43: Chromatogram of Plasma extract acquired by Phenomenex procedure, concentrated. Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid

Figures 42 and 43 show that the chromatogram of non-concentrated extract does not have significant peaks. The concentrated extract showed only a peak of alpha-tocopherol.

As one last haulm of hope extraction using pH change was used. (Gonzalez and Choquette n.d.) The extraction started with adding a solution of 0.1 $mol \cdot l^{-1}$ Na_2CO_3 solution to the plasma until pH reached the value of 9.8. Then the solution was left to equilibrate on ice for 30 minutes. Then the mixture was extracted as in the final version of the extraction assay. The result of this extraction procedure can be seen in Figure 44.

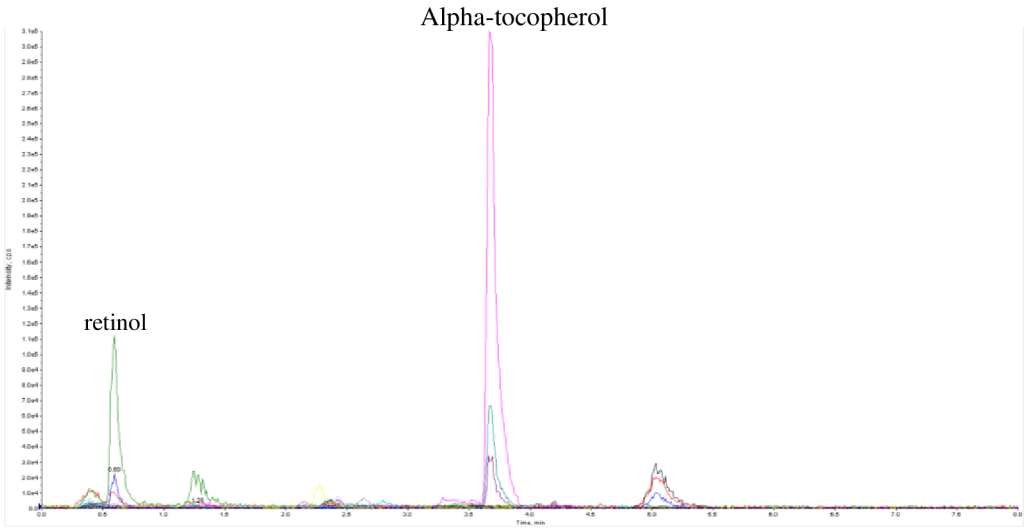

Figure 44*:* Chromatogram of Plasma extract acquired by using carbonate solution, extracted with n-hexane/chloroform 90/10 *v/v*, extracted twice. Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid

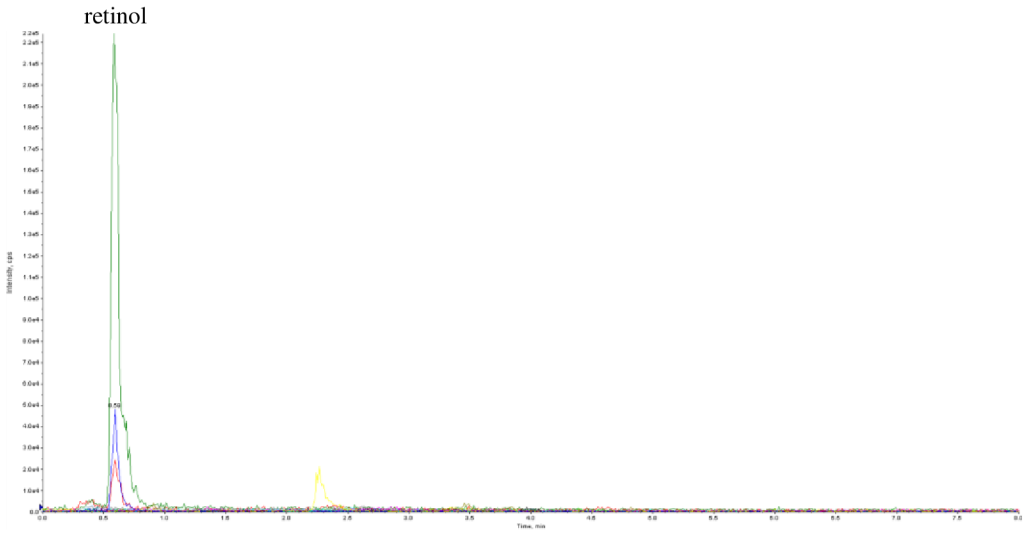

Figure 45: Chromatogram of the blank of Plasma extract acquired by using carbonate solution, extracted with n-hexane/chloroform 90/10 *v/v*, extracted twice. Mobile phases used: A) methanol/water (70/30, *v/v*) + 0.2% formic acid B) methanol + 0.2% formic acid

From the first look at Figure 44, it can be assumed that alpha-tocopherol and retinol are successfully extracted. However, if compared with Figure 45, which shows blank. The blank does have a significant peak containing retinol. Therefore, the retinol peak in Figure 44 comes from carry-over or from contamination of methanol used for resuspension of the dried extract.

4.2 Development of LC-MS assay

The main goals of the LC-MS assay were to create a short assay which would enable the quantification of all fat-soluble vitamins.

The first gradient was designed to be very simple using mobile phases: A) methanol/water 50/50 *v/v* + 0.2% formic acid, B) methanol + 0.2% formic acid with flow 0.3 $mL \cdot min^{-1}$. The composition of the first version of the gradient can be seen in Table 5 - Chromatogram showing the elution of preselected molecular weights, which correspond to molecular weights of fat-soluble vitamins, which can be seen in Figure 46. The chromatograms used the extract acquired from methanol extraction with incorporated wetting using 0.1% formic acid solution.

Table 5: Composition of first gradient elution, Mobile phases: A) methanol/water 50/50 *v/v* + 0.2% Formic acid, B) methanol + 0.2% formic acid

Retention time (min)	% composition of mobile phase B
0	5
2	5
16	100
18	100
18.1	5
20	5

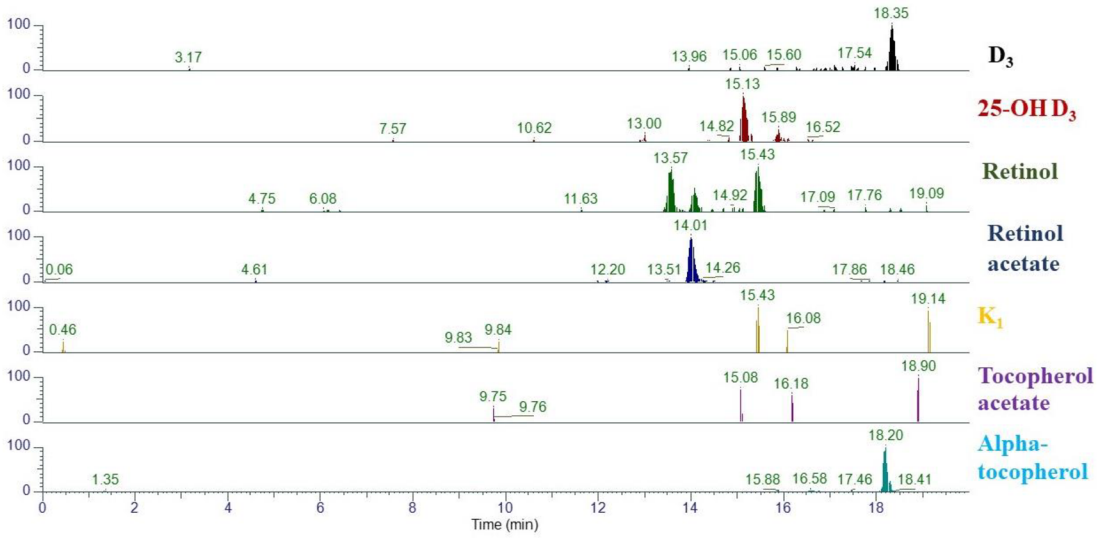

Figure 46: Chromatogram of Plasma extract acquired by the first version of gradient, extracted with methanol, the column used: Raptor C_{18} 2.7µm 2.1 mm x 100mm ID column, showing suspected vitamins: vitamin D_3(black) and 25-hydroxyvitamin D_3(red), Retinol (green), retinol acetate (blue), vitamin K_1 (yellow), tocopherol acetate (purple) and alpha-tocopherol (turquoise) Mobile phases: A) methanol/water 50/50 *v/v* + 0.2% Formic acid, B) methanol + 0.2% formic acid

From the look at Figure 46 can be seen that peaks are packed in the last 8 minutes of the chromatogram. Therefore, the gradient can be steeper and can start on a higher percentage representation of mobile phase B. Also, the time which has 100% of mobile phase B should be longer, as most of the vitamins elute at a very high-volume percentage of mobile phase B. As a result, changes were made. The initial mobile phase composition was increased to 45% of mobile phase B and the time, where the composition was 100 % of mobile phase B, was elongated. The results of the elution change can be seen in Figure 47.

Table 6: Composition of seventh gradient elution, Mobile phases: A) methanol/water 50/50 *v/v* + 0.2% formic acid, B) methanol + 0.2% formic acid

Retention time (min)	% composition of mobile phase B
0	45
1	45
10	100
18	100
18.1	45
20	45

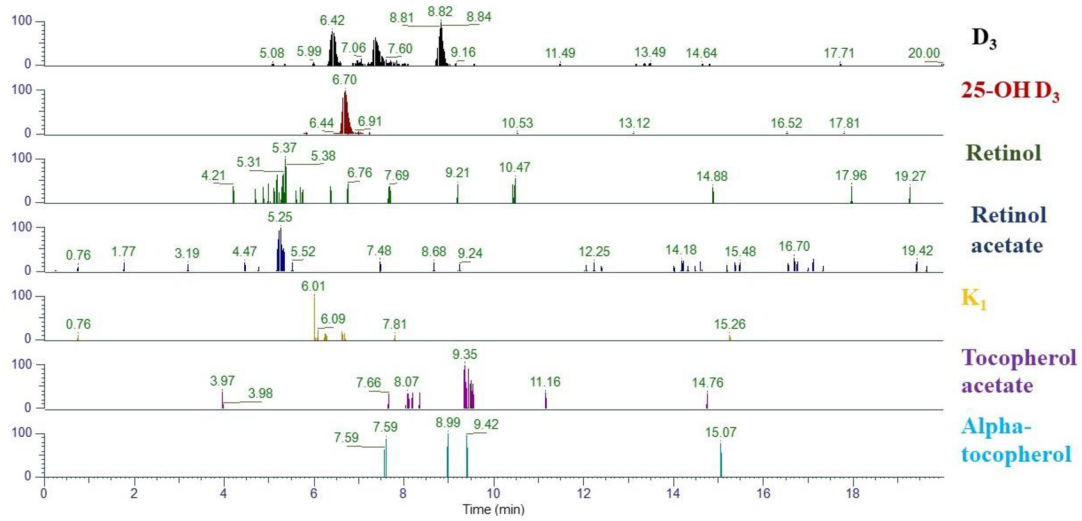

Figure 47: Chromatogram of Plasma extract acquired by the seventh version of gradient, extracted with methanol, the column used: Raptor C_{18} 2.7µm 2.1mm x 100mm ID column, showing suspected vitamins: vitamin D_3 (black) and 25-hydroxyvitamin D_3 (red), Retinol (green), retinol acetate (blue), vitamin K_1 (yellow), tocopherol acetate (purple) and alpha-tocopherol (turquoise) Mobile phases: A) methanol/water 50/50 *v/v* + 0.2% Formic acid, B) methanol + 0.2% formic acid

As shown in Figure 47, most of the peaks shifted into the range of 7.5 to 12.5 minutes. Additionally, the shape of the peaks improved even though tailing can be seen in some of the peaks. However, the assay is now too long. Therefore, it should be shortened; the vitamins may elute earlier, combined with better separation of peaks. A note at the end, the initial % composition is very high. As a result, the mobile phase had to be interchanged to a mobile phase with lower polarity. The initial propose was to interchange the mobile phase B from methanol + 0.2% formic acid to methanol/isopropyl alcohol (50/50 *v/v*) + 0.2% formic acid, mainly to decrease polarity and therefore elute the vitamins earlier. The composition of the gradient elution can be seen in Table 7, and the resulting chromatogram is in Figure 48.

Table 7: Composition of ninth version of gradient elution, Mobile phases: A) methanol/water 50/50 v/v + 0.2% formic acid, B) methanol/isopropyl alcohol 50/50 v/v + 0.2% formic acid

Retention time (min)	% of mobile phase B
0	40
1	40
6	100
18	100
18.1	40
20	40

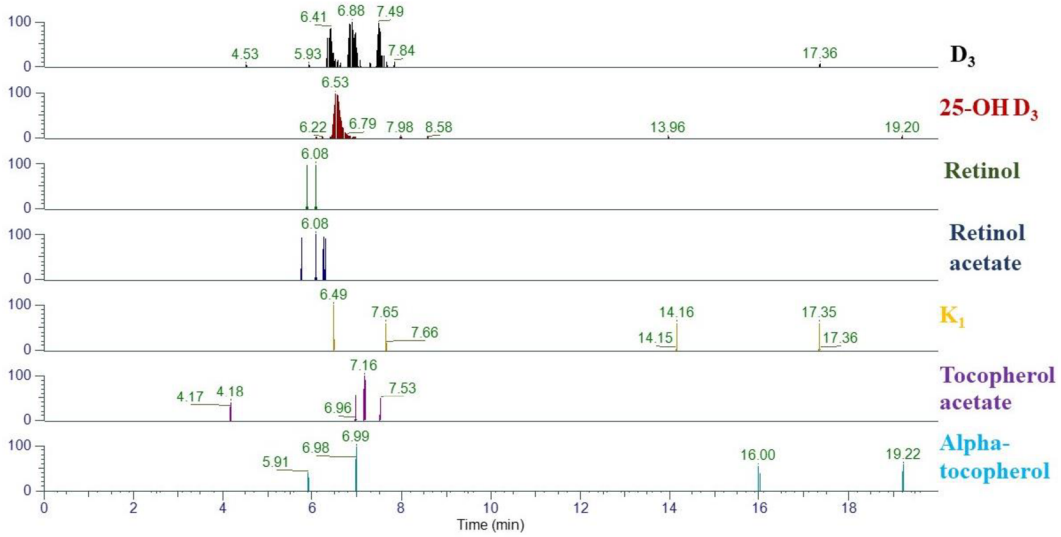

Figure 48: Chromatogram of DBS extract acquired by the ninth version of gradient, extracted with methanol and incorporated wetting process using 0.1% formic acid solution, the column used: Raptor C_{18} 2.7µm 2.1mm x 100mm ID column, showing suspected vitamins: vitamin D_3(black) and 25-hydroxyvitamin D_3(red), Retinol (green), retinol acetate (blue), vitamin K_1 (yellow), tocopherol acetate (purple) and alpha-tocopherol (turquoise) Mobile phases: A) Methanol: water 50/50 v/v + 0.2% Formic acid, B) methanol/isopropyl alcohol 50/50 v/v + 0.2% formic acid

From the look in Figure 48, it can be decided that the composition of mobile phases is not usable for fat-soluble vitamins, mainly due to the absence of most of the vitamin peaks that were previously present. The reason is probably improper mixing of the sample and mobile phase. Therefore, the sample may precipitate, or the mobile phases and sample compatibility were not ideal. Either way, the mobile phase B methanol/isopropyl alcohol 50/50 v/v is not suited for fat-soluble vitamin analysis, at least in combination with mobile phase A methanol/water 50/50 v/v.

As a result, the further progress was to change back the mobile phase B to methanol + 0.2% formic acid. The flow must be increased if the mobile phases have the same elution strength. Thus, the flow was increased to 0.4 $ml \cdot min^{-1}$. The gradient composition can be found in Table 8, and the resulting chromatogram is in Figure 49.

Table 8: Composition of the eleventh version of gradient elution, Mobile phases: A) methanol/water 50/50 v/v + 0.2% formic acid, B) methanol + 0.2% formic acid

Retention time (min)	% of mobile phase B
0	60
1	60
6	100
18	100
18.1	60
20	60

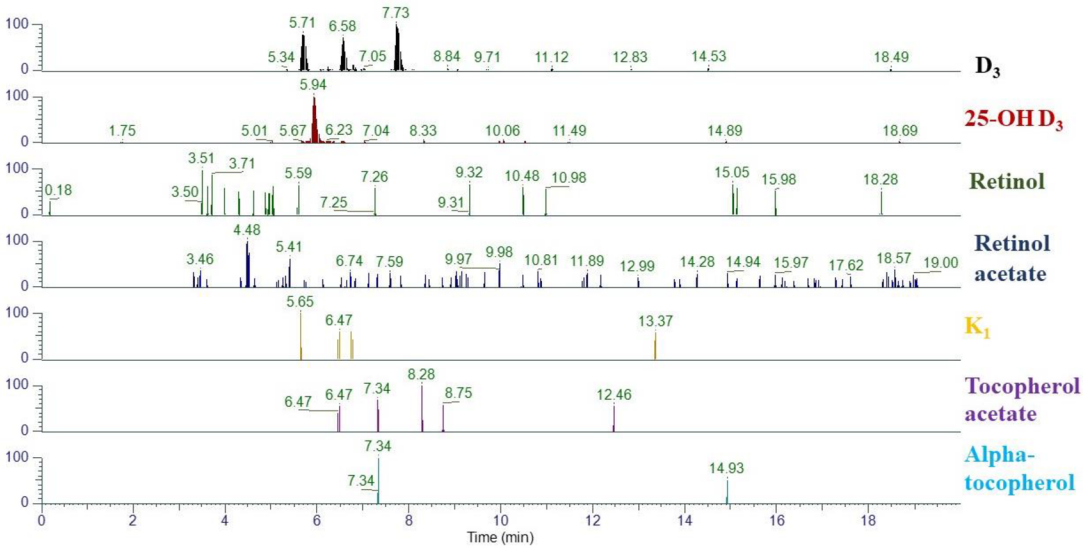

Figure 49: Chromatogram of DBS extract acquired by the eleventh version of gradient, extracted with methanol and incorporated wetting process using 0.1% formic acid solution, the column used: Raptor C_{18} 2.7µm 2.1mm x 100mm ID column, showing suspected vitamins: vitamin D_3(black) and 25-hydroxyvitamin D_3(red), Retinol (green), retinol acetate (blue), vitamin K_1 (yellow), tocopherol acetate (purple) and alpha-tocopherol (turquoise) Mobile phases: A) methanol/water 50/50 *v/v* + 0.2% Formic acid, B) methanol + 0.2% formic acid

As can be seen, the suspected vitamins are eluting early but can elute earlier to shorten the assay further. Hence the flow was increased to 0.5 ml·min^{-1}, and the gradient was shortened to 10 minutes due to the earlier elution of analytes and, consequently, no necessity for a more prolonged analysis. The resulting composition of the gradient can be found in Table 9, and the resulting gradient can be found in Figure 50.

Table 9: Composition of the thirteenth version of gradient elution, flow = 0.5 ml·min^{-1}, Mobile phases: A) methanol/water 50/50 v/v + 0.2% formic acid, B) methanol + 0.2% formic acid

Retention time (min)	% of mobile phase B
0	60
1	60
6	100
8	100
8.1	60
10.2	60

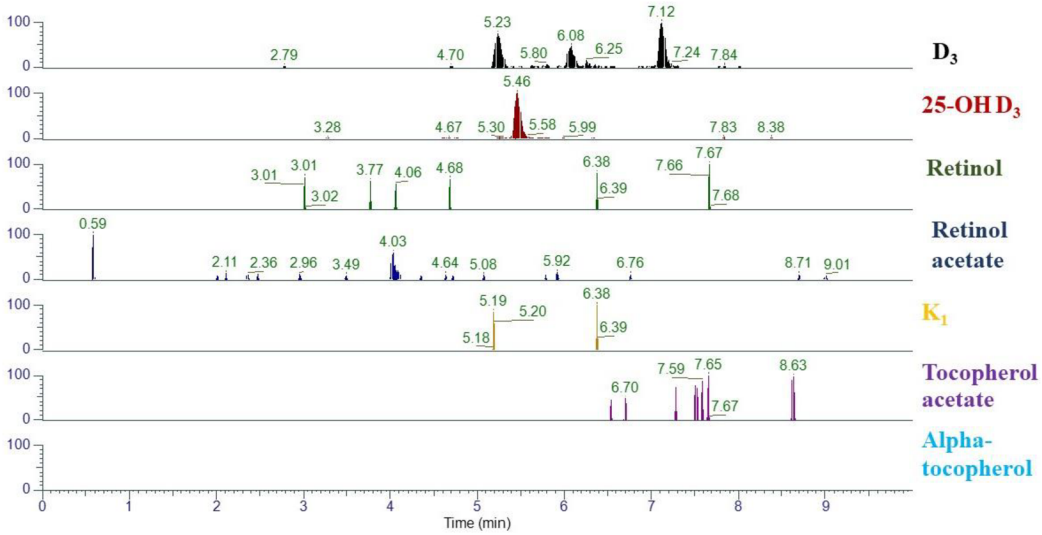

Figure 50: Chromatogram of DBS extract acquired by the thirteenth version of gradient, extracted with methanol and incorporated wetting process using 0.1% formic acid solution, the column used: Raptor C_{18} 2.7µm 2.1mm x 100mm ID column, showing suspected vitamins: vitamin D_3 (black) and 25-hydroxyvitamin D_3 (red), Retinol (green), retinol acetate (blue), vitamin K_1 (yellow), tocopherol acetate (purple) and alpha-tocopherol (turquoise) Mobile phases: A) methanol/water 50/50 *v/v* + 0.2% formic acid, B) methanol + 0.2% formic acid

As can be seen in Figure 50, the length of the assay is more than sufficient. However, the peaks still elute late in gradient, and the peaks still need shape improvement. Also, the pressure values had risen to high values. This resulted in the proposal to increase methanol and water in mobile phase A to methanol/water 70/30 + 0.2% formic acid. A less polar mobile phase allowed the lower initial percentage of mobile phase B. To improve the shapes of the peaks and separation, the column was interchanged for Kinetex column 1.7 µm C_{18} 100Å, 50 x 2.1 mm. As smaller particles result in higher inner pressure, the flow decreased to 0.3 ml·min^{-1}.

Table 10: Composition of the twentieth version of gradient elution, flow = 0.3 ml·min-^{1}, Mobile phases: A) methanol/water 70/30 v/v + 0.2% formic acid, B) methanol + 0.2% formic acid

Retention time (min)	% of mobile phase B
0	30
1	30
5	100
8	100
8.1	30
10.2	30

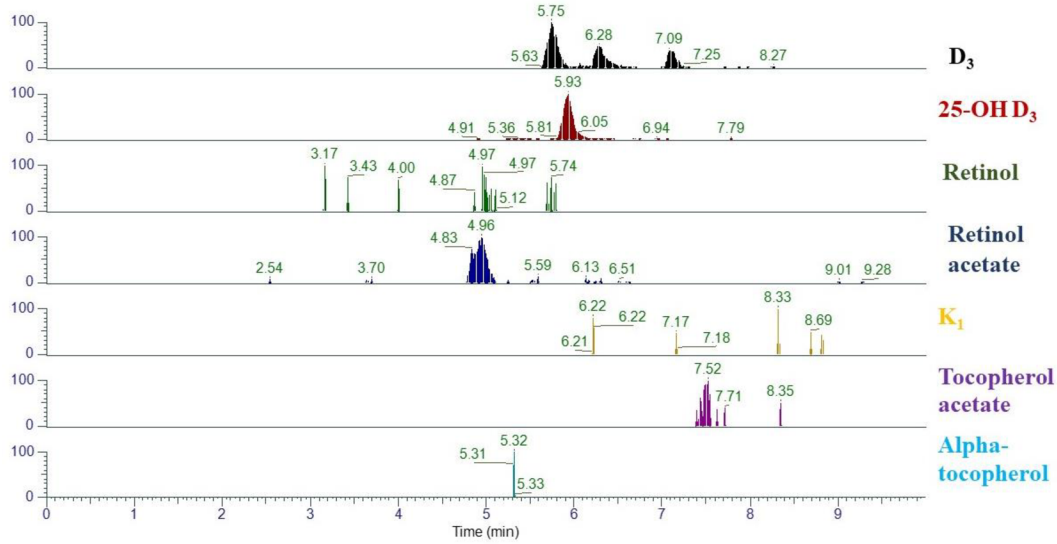

Figure 51: Chromatogram of DBS extract acquired by the twentieth version of gradient, extracted with methanol and incorporated wetting process using 0.1% formic acid solution, the column used: Kinetex column 1.7 µm C_{18} 100Å, 50 x 2.1 mm, showing suspected vitamins: vitamin D_3 (black) and 25-hydroxyvitamin D_3 (red), retinol (green), retinol acetate (blue), vitamin K_1 (yellow), tocopherol acetate (purple) and alpha-tocopherol (turquoise) Mobile phases: A) methanol/water 70/30 *v/v* + 0.2% formic acid, B) methanol + 0.2% formic acid

As can be seen in Figure 51, the column change helped with peak shapes. Also, this results in a more sustainable assay with lower flow and not too high initial mobile phases A and B. However, the peaks can still use better separation and may elute earlier to shorten the assay further.

Table 11: Composition of the twenty-second version of gradient elution, flow = 0.3 $ml \cdot min^{-1}$, Mobile phases: A) methanol/water 70/30 v/v + 0.2% formic acid, B) methanol + 0.2% formic acid

Retention time (min)	% of mobile phase B
0	50
1	50
4	100
8	100
8.1	50
10.2	50

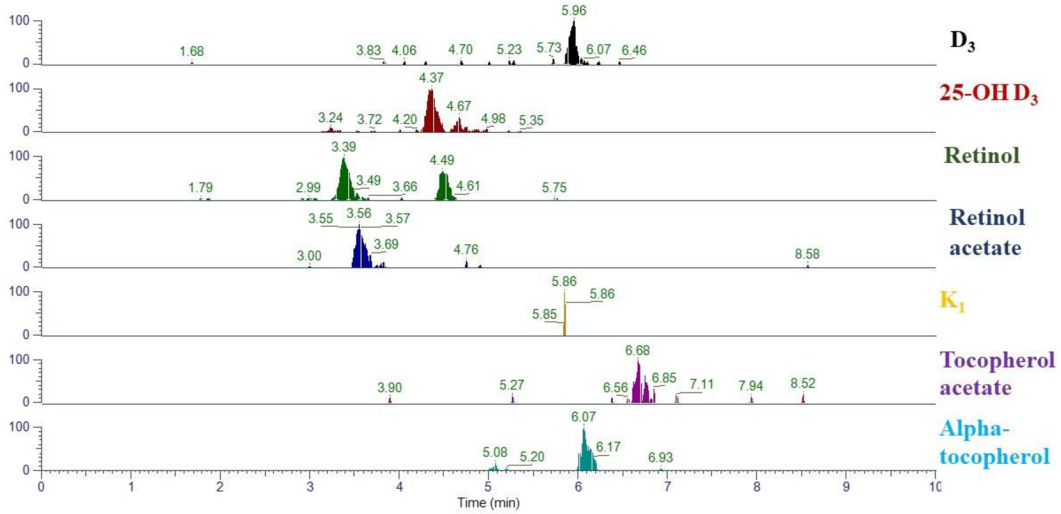

Figure 52: Chromatogram of Plasma extract acquired by the twenty-second version of gradient, extracted with methanol, the column used: Kinetex column 1.7 µm C_{18} 100Å, 50 x 2.1 mm, showing suspected vitamins: vitamin D_3 (black) and 25-hydroxyvitamin D_3 (red), Retinol (green), retinol acetate (blue), vitamin K_1 (yellow), tocopherol acetate (purple) and alpha-tocopherol (turquoise) Mobile phases: A) methanol/water 70/30 *v/v* + 0.2% Formic acid, B) methanol + 0.2% formic acid

The analytes in Figure 52 are now well separated, and the peak shapes improved. The mixture of standards was run on this version of the gradient to ensure that the molecules suspected to be vitamins are what they are.

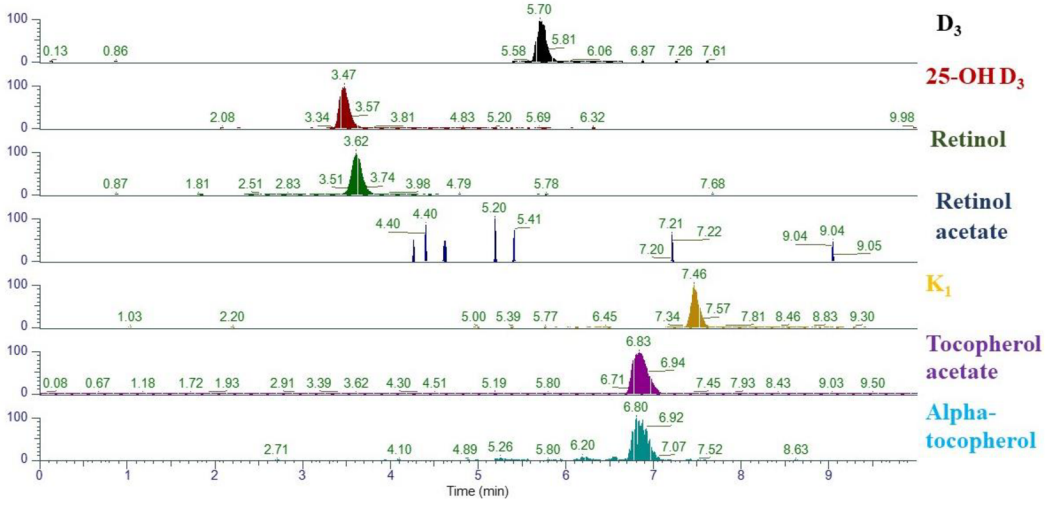

Figure 53: Chromatogram of fat-soluble vitamin standards acquired by the twenty-second version of gradient, the column used: Kinetex column 1.7 µm C_{18} 100Å, 50 x 2.1 mm, showing suspected vitamins: vitamin D_3 (black) and 25-hydroxyvitamin D_3 (red), Retinol (green), retinol acetate (blue), vitamin K_1 (yellow), tocopherol acetate (purple) and alpha-tocopherol (turquoise) Mobile phases: A) methanol/water 70/30 *v/v* + 0.2% formic acid, B) methanol + 0.2% formic acid

From the comparison of Figures 52 and 53, it can be said that only retinol and tocopherol acetate are present. As previously stated, most extraction options were tried or discarded, which may have brought issues to the LC-MS analysis. Therefore it was

proposed to interchange the Orbitrap MS analyser to triple quadrupole MS to improve the selectivity of the MS assay further. The MRM was introduced into the assay. This was done using standards introduced directly into a Mass spectrometer in the solution of methanol/water 60/40 *v/v* with added formic acid resulting in a 1% solution of formic acid. This was done to have a highly similar matrix and obtain the same pseudomolecular ions. The standards were tuned, and the three most intense transitions were used in the MS assay. As the last point, the ion source was changed to APCI, mainly due to APCI being a softer ion source and, therefore, should prevent most in-source fragmentation. To further make the assay more efficient, the assay was shortened. The composition of the resulting LC assay can be seen in Table 12, and the resulting chromatogram in Figure 54.

Table 12: Composition of the sixth version of gradient elution combined with triple quadrupole, flow = 0.3 $ml \cdot min^{-1}$, Mobile phases: A) methanol/water 70/30 v/v + 0.2% Formic acid, B) methanol + 0.2% formic acid

Retention time (min)	% of mobile phase
0	50
0.2	50
2.3	100
4.4	100
4.5	50
6.12	50

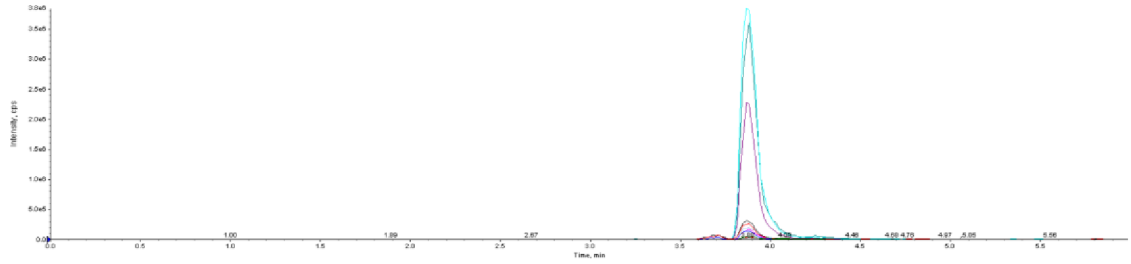

Figure 54: Chromatogram of Plasma extract acquired by the sixth version of gradient analysed with triple quadrupole, extracted with n-hexane: chloroform 90/10 *v/v*, the column used Kinetex column 1.7 µm C_{18} 100Å, 50 x 2.1 mm, Mobile phases: A) methanol/water 50/50 *v/v* + 0.2% formic acid, B) methanol + 0.2% formic acid

As can be seen in Figure 54, most of the peaks elute at a highly similar time, and some of the vitamins are not present at all, therefore can be said that there is a problem with the extraction assay or the lower limit of detection is still too high to detect low concentrations of most of the vitamins that are present. To ensure the error is not in the MRM transition values, MRM transition values from the article (Midttun et al. 2016) were used. The

MRM transitions can be seen in Table 13, and the resulting chromatogram is in Figure 55.

Table 13: MRM Transition ions and parameters

Analyte	Q1 (*m/z*)	Q2 (*m/z*)	CE (V)	CXP (V)	EP (V)	DP (V)
25-hydroxyvitamin D_3	401.4	365.4	30	10	12	50
All-trans retinol	270.1	94.2	30	10	12	30
25-hydroxyvitamin D_2	413.5	355.5	15	10	12	40
Gamma-tocopherol	417.5	151.0	30	14	15	30
Alpha-tocopherol	431.4	137.4	30	10	12	30
Phylloquinone	451.2	187.2	30	15	12	40

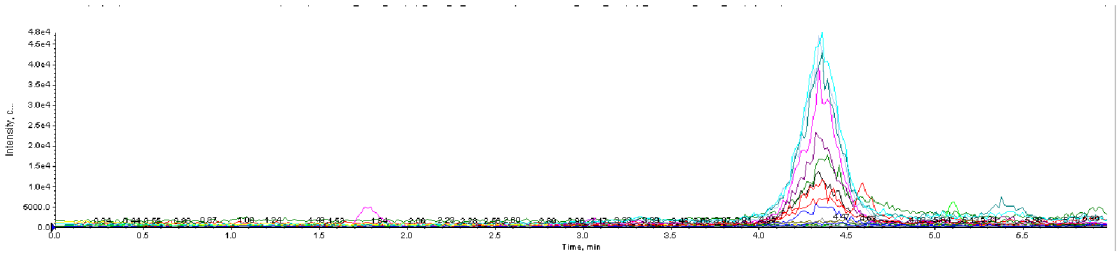

Figure 55: Chromatogram of Plasma extract acquired by the sixth version of gradient analysed with triple quadrupole using MRM transitions from table 13, extracted with n-hexane/chloroform 90/10 *v/v*, the column used Kinetex column 1.7 µm C_{18} 100Å, 50 x 2.1 mm, Mobile phases: A) methanol/water 50/50 v/v + 0.2% formic acid, B) methanol + 0.2% formic acid

As shown in Figure 55, the MRM spectra from Midttun et al. are not adjusted well to the hardware setup existing in the laboratory. Therefore, we decided that the experimentally obtained MRM transitions should be used. As a final step, the ionic source was changed to ESI, as this ionic source was more stable and provided more suitable results for the quantification of vitamins. The composition of the final gradient can be seen in Table 14, and the resulting chromatogram can be seen in Figure 56.

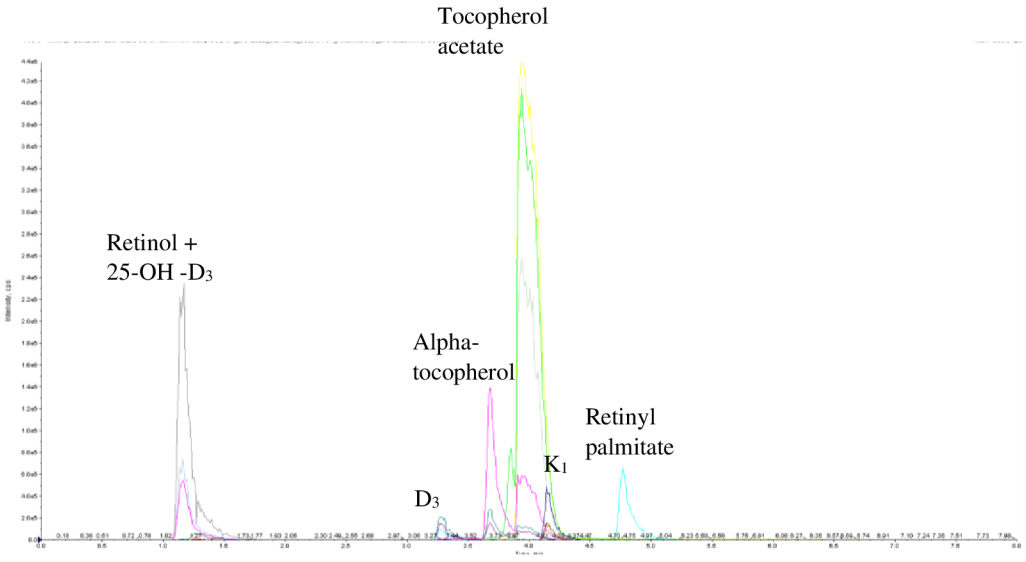

Figure 56: Chromatogram of standards acquired by the ninth version of gradient analysed with triple quadrupole using ESI ionisation, the column used Kinetex column 1.7 µm C_{18} 100Å, 50 x 2.1 mm, Mobile phases: A) methanol/water 50/50 *v/v* + 0.2% Formic acid, B) methanol + 0.2% formic acid

Table 14: Composition of the ninth version of gradient elution combined with triple quadrupole, flow = 0.7 ml·min^{-1}, mobile phases: A) methanol/water 70/30 v/v + 0.2% formic acid, B) methanol + 0.2% formic acid

Retention time (min)	% of mobile phase B
0	50
0.3	50
3	100
7.5	100
7.6	50
8.012	50

From the look of Figure 56 can be assumed that the gradient still lacks the right separation strengths and needs to be improved to separate the peaks further. But the successfully extracted vitamins – retinol and alpha-tocopherol are nicely separated and, therefore, can be quantified without a problem. Also, there is still a tailing of the peaks that is hard to eliminate. Combined with the already very high flow of the mobile phase and power of the gasses in triple quadrupole on edge, it is acceptable.

4.3 Validation of assay

The original aim was to extract all fat-soluble vitamins: retinol, alpha-tocopherol, Phylloquinone, cholecalciferol and calcidiol. However, any of the previously mentioned extraction assays did not successfully extract all the vitamins mentioned above in significant amounts. The only successfully extracted vitamins were retinol and alpha-tocopherol. Therefore, the validation could have been done only on those two vitamins. The selectivity was already used via MRM analysis, which ensured that the analysed

compounds were the analytes. Carry-over was confirmed via analysis of blank matrix–methanol between samples of dilution integrity. No peaks in the blank samples showed the non-existing carry-over in the assay.

4.3.1 Calibration curve

The first plan for both calibration curves was to measure the intensity of the peak in concentrations 100 $\mu mol \cdot l^{-1}$, 20 $\mu mol \cdot l^{-1}$, 2 $\mu mol \cdot l^{-1}$, 200 $nmol \cdot l^{-1}$, 100 $nmol \cdot l^{-1}$ and 20 $nmol \cdot l^{-1}$ and 0 $nmol \cdot l^{-1}$. However, those concentrations were not well suited for both of the vitamins. By measuring the vitamins, it was decided how the calibration curve should look, mainly due to the unproportional response of the detector and the lower and upper limit of detection and quantification. The calibration curves were made using all three fragments used for identification using MRM, and each sample was measured three times to incorporate possible inconsistency in measurement. The calculated mean of each area corresponding to each analyte concentration was used to construct the calibration curve. The calibration curve for retinol can be seen in Figure 58, and one for alpha-tocopherol can be seen in Figure 59.

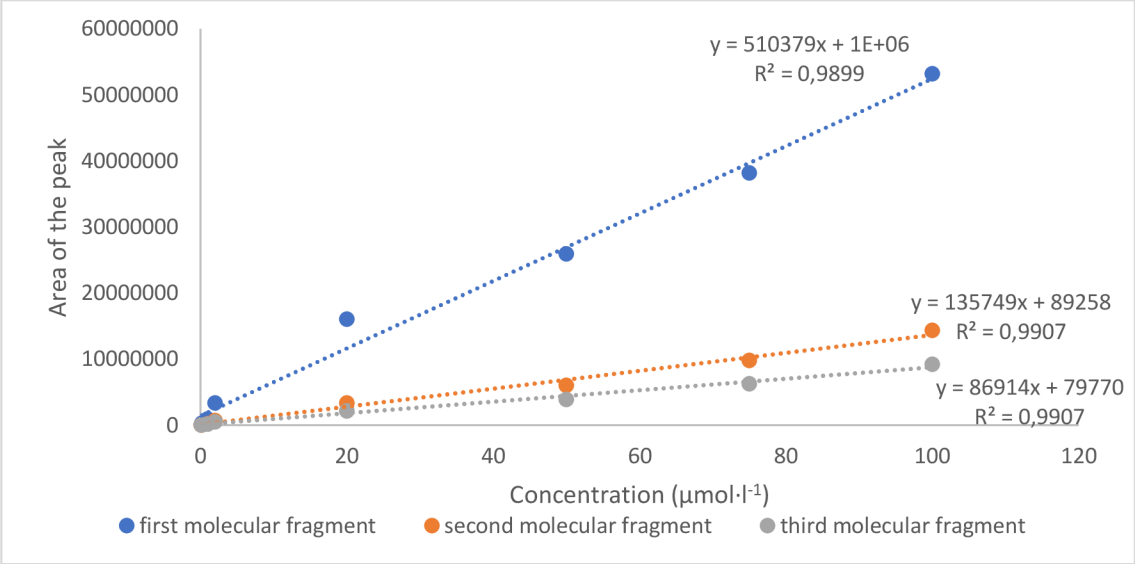

Figure 57: Dependence of the concentration of alpha-tocopherol on the area of the peak area.

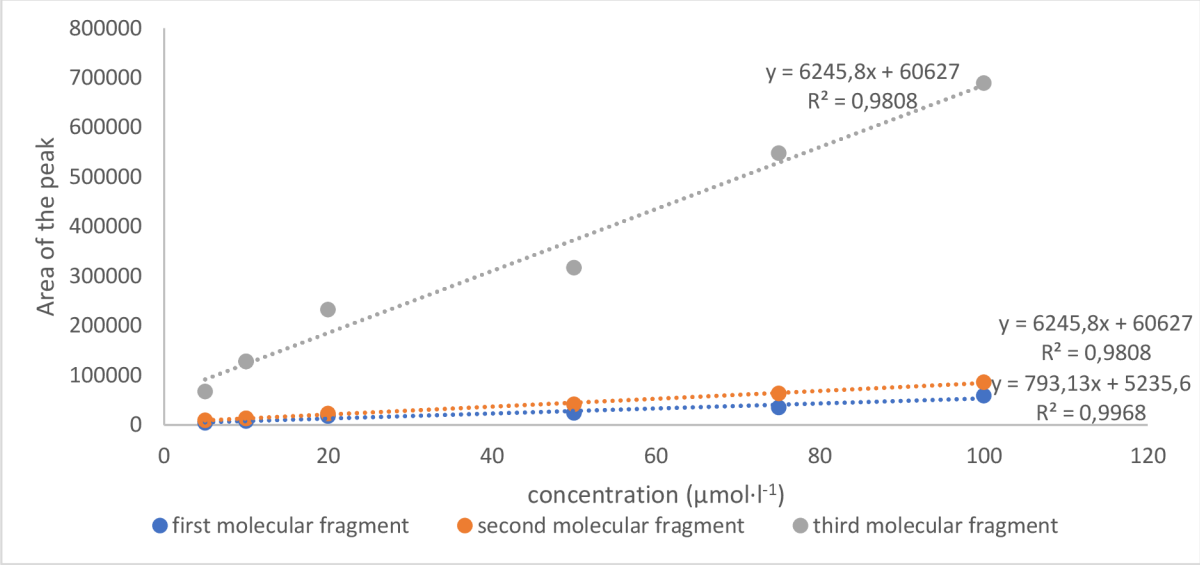

Figure 58: Dependence of the concentration of retinol on the area of the peak area.

The resulting calibration curves for alpha-tocopherol and retinol were made from areas of all three ion fragments for each vitamin. As seen above, those calibration curves have lower correlation coefficients (R^2) than are usually accepted. However, the reason can be seen in the concise calibration range, which can be analysed via triple quadrupole and the fat-soluble vitamin's mediocre stability based on their functions in the organism. Consequently, the calibration curves can be marked as acceptable and used for further validation of the assay.

4.3.2 Precision and Accuracy

The Precision and accuracy of the measurement were measured in a set of 6 values. In the case of retinol, the concentration of the samples was 2.5 µmol·l^{-1}, 7.5 µmol·l^{-1}, 15 µmol·l^{-1}, 30 µmol·l^{-1}, 60 µmol·l^{-1} and 80 µmol·l^{-1}. In the case of alpha-tocopherol, the concentrations were selected as 150 nmol·l^{-1}, 400 nmol·l^{-1}, 1.5 µmol·l^{-1}, 5 µmol·l^{-1}, 30 µmol·l^{-1} and 60 µmol·l^{-1}. Those were constructed to cover the calibration curve range as written in the norm ICH M10. The precision and accuracy were calculated and can be seen in Table 15 for retinol and Table 16 for alpha-tocopherol.

Table 15: Precision and accuracy calculated for retinol samples

Initial concentration [µmol·l^{-1}]	Estimated concentration [µmol·l^{-1}]	Precision (RSD%)	Accuracy (RE%)	Accepted
2.5	3.38	25.34	35.10	No
7.5	18.44	19.47	145.81	No
15	25.44	13.70	69.63	No
30	30.25	17.14	0.82	No
60	50.16	9.57	16.40	No
80	82.43	8.46	3.04	Yes

Table 16: Precision and accuracy calculated for alpha-tocopherol samples

Initial concentration [µmol·l^{-1}]	Calculated concentration [µmol·l^{-1}]	Precision (RSD%)	Accuracy (RE%)	Accepted
0.15	1.01	90.63	572.07	No
0.4	0.58	149.93	44.99	No
1.5	2.09	23.56	39.23	No
5	6.54	4.67	30.71	No
30	61.32	3.79	104.41	No
60	72.13	2.32	20.21	No

From the results, this assay does not show usable precision in low concentrations; the accuracy varies from 0.82 % to 145.8 % for retinol and 20.21 % to 572.07 %. The low accuracy can be caused by the leakage in the mixer, which was detected before the analysis. The low precision and low accuracy can also be caused by high flow in the LC part of the assay; therefore, the gasses must be able to gasify this amount of analyte effectively. Unfortunately, the pipe-source of nitrogen at the IMTM is at insufficient pressure causing problems at the higher LC flow rates with ESI stability and sensitivity.

4.3.3 Dilution integrity

Data for dilution integrity were obtained using samples prepared from the dilution series. Usually, the concentration decreases 10x from one sample to another. The dilution series are supposed to have six concentrations, each measured three times. However, due to the narrow range of concentrations that can be actually measured with this instrument setup, the dilution series were designed as follows: 100 µmol·l^{-1}, 10 µmol·l^{-1} and 1 µmol·l^{-1} for retinol and 100 µmol·l^{-1}, 10 µmol·l^{-1}, 1 µmol·l^{-1} and 100 nmol·l^{-1} for alpha-tocopherol. The precision and accuracy were calculated and can be seen in Table 17 for retinol and Table 18 for alpha-tocopherol.

Table 17: Dilution integrity for retinol samples

Initial concentration [μmol·l^{-1}]	Calculated concentration [μmol·l^{-1}]	Precision (RSD%)	Accuracy (RE%)	Accepted
1	1.91	29.12	90.59	No
10	19.60	20.90	95.98	No
100	108.64	6.15	8.64	Yes

Table 18: Dilution integrity for alpha-tocopherol samples

Initial concentration [μmol·l^{-1}]	Calculated concentration [μmol·l^{-1}]	Precision (RSD%)	Accuracy (RE%)	Accepted
0.1	1.26	8.82	1164.53	No
1	38.01	13.29	3700.78	No
10	11.62	39.13	16.15	No
100	107.18	2.80	7.18	Yes

The dilution integrity results show similar characteristics as precision and accuracy. As the lower concentrations are, the worse the accuracy is. Retinol shows slightly better accuracy than alpha-tocopherol, and alpha-tocopherol shows better precision than retinol. This can also be rooted in the mixer leakage, as the analysis does not show acceptable accuracy. Also, as the lower concentrations have worse accuracy, it can be a pipetting error. The error may come from the user of the pipette, or the calibration of the pipette may have been slightly offset.

4.3.4 lower limit of detection and lower limit of quantification.

Various procedures can determine the lower limit of detection (LLOD), and the procedure from EMA was used there. The lower limit of detection was determined by the ten consecutive measurements of the blank matrix. Therefore in our case, methanol. The LLOD can be calculated from the mean value of noise, which is then multiplied by 3. The lower limit of quantification (LLOQ) can be determined via the multiplication of LLOD by 3. The same matrix was used, and the response of the comparable only one LLOD and LLOQ was calculated. The results can be seen in Table 19.

Table 19: Table showing the LLOD and LLOQ

	Intensity
Mean of the noise signals	80.506
LLOD	241.517
LLOQ	724.551

What must be considered is that each analyte has a different instrument response, which depends on many factors. Experimentally determined LLOD for retinol is 2 $\mu mol \cdot l^{-1}$ and for alpha-tocopherol 100 $nmol \cdot l^{-1}$.

4.3.5 Effect of matrix

The matrix's effect was first studied by preparing the standard solutions in a mixture of acetonitrile/isopropyl alcohol 50/50 *v/v*.

Then the effect of the matrix was studied using spiked urine, plasma and BSA (70 $g \cdot l^{-1}$), which were then extracted with the final version of the extraction assay. The matrices were spiked separately with 5 µl of 1 mmol·l-1 standard of retinol and alpha-tocopherol. The areas of the peaks were then compared, and the possible matrix effect was evaluated. Results can be seen in Table 20 for retinol and 21 for alpha-tocopherol.

Table 20: Matrix effect for retinol samples

Matrix	Initial concentration [$\mu mol \cdot l^{-1}$]	Calculated concentration [$\mu mol \cdot l^{-1}$]	Precision (RSD%)	Accuracy (RE%)	Recovery (%)	Matrix effect
ACN/IPA 50/50 *v/v*	47.62	43.25	9.39	9.18	90.82	none
BSA (70 g/L)	47.62	147.84	35.34	210.46	77.80	Suppressive
Plasma	47.62	165.08	65.01	246.66	346.66	Enhancing
Plasma (analysed 8/11/2022)	12	133.45	31.28	202.423	310.46	Enhancing
Urine	47.62	37.00	22.48	22.11	77.80	Suppressive
DBS (analysed 8/11/2022)	12	9.17	17.43	23.60	76.40	Suppressive

Table 21: Matrix effect for alpha-tocopherol samples

Matrix	Initial concentration [$\mu mol \cdot l^{-1}$]	Calculated concentration [$\mu mol \cdot l^{-1}$]	Precision (RSD%)	Accuracy (RE%)	Recovery (%)	Matrix effect
ACN/IPA 50/50 *v/v*	47.62	35.05	16.86	26.4	73.60	suppressive
BSA (70 g/L)	47.62	319.61	33.67	571.18	671.18	Enhancing
Plasma	47.62	22.65	19.76	52.43	47.57	Suppressive
Plasma (analysed 8/11/2022)	12	8.18	11.19	31.12	68.18	Suppressive
Urine	47.62	182.74	23.26	283.75	383.75	Enhancing
DBS(analysed 8/11/2022)	12	1.95	32.90	83.788	16.21	Suppressive

The mixture of acetonitrile and isopropyl alcohol (50/50,*v/v*) did not have any matrix effect on alpha-tocopherol. However, the mixture had a suppressive effect on retinol. For retinol plasma had enhancing effect, and urine and BSA had a suppressive effect. For alpha-tocopherol, BSA and urine have enhancing matrix effect, and the plasma has a suppressive matrix effect. The knowledge of matrix effects on both vitamins shows the different characteristics. Therefore, it is challenging to find conditions suitable for both of them.

4.3.6 Stability

The stability of the vitamins was studied against temperature and light, which are the main reasons for the degradation of most analytes. The temperature stability was studied at laboratory temperature (20°C), the temperature in autosampler and fridge (4°C) and 45°C. The light sample was placed on the windowsill. All samples were exposed to those conditions for an hour. One control sample was made for comparison purposes. The sample was kept on ice in a dark box. The resulting stability evaluation can be seen in Table 22 for retinol samples and 23 for alpha-tocopherol samples.

Table 22: Stability evaluation for retinol

Stability condition	Initial concentration [µmol·l^{-1}]	Calculated concentration [µmol·l^{-1}]	Precision (RSD%)	Recovery (%)	Stability evaluation
Light	50	15.88	31.86	31.77	unstable
Room temperature (20°C)	50	39.56	13.41	79.12	Partially stable
Temperature (4°C)	50	47.14	13.81	85.93	Partially stable
Temperature (45°C)	50	38.91	6.38	77.82	Partially stable
Control sample	50	37.94	10.13	75.88	Partially stable

Table 23: Stability evaluation for alpha-tocopherol

Stability condition	Initial concentration [µmol·l^{-1}]	Calculated concentration [µmol·l^{-1}]	Precision (RSD%)	Recovery (%)	Stability evaluation
Light	50	57.02	4.29	114.03	stable
Room temperature (20°C)	50	61.02	5.86	122.04	stable
Temperature (4°C)	50	62.18	6.90	124.37	stable
Temperature (45°C)	50	58.77	5.27	117.54	stable
Control sample	50	60.62	6.07	121.23	stable

As can be said, the retinol is most stable at room temperature, kept in the dark, then kept in the dark at 4°C, exactly as it is kept in the autosampler. From this, it is best to keep the retinol in the dark, so using a dark room or dark test tubes would improve the next experiments. Also, the autosampler conditions are suitable for the short-term storage of retinol samples. The worst stability is shown in the sample treated with light, where the recovery reached only 31.77%.

The alpha-tocopherol has shown the best stability at 4°C, similarly to retinol. The stability of the alpha-tocopherol is worst when exposed to direct sunlight, but overall, the stability is substantial for all conditions, and the compound may be labelled as stable.

5. Discussion

Even though the extraction assay successfully extracted spiked samples with standards of fat-soluble vitamins, it is not powerful enough to extract the analytes from the matrix in significant amounts. There are five main predicted hypotheses for the extraction failure, two of which were tried. The first hypothesis is based on the low polarity of the vitamins. Therefore, drying the organic solvent from the extract may have caused adhesion to the non-polar surface of the plastic micro test tubes. This hypothesis was already tested using glass bottles, as those did not affect the result, so this hypothesis was rejected.

The second hypothesis was based on the fact that vitamins are in plasma in very low concentrations, especially fat-soluble vitamins, which are not usually free in the blood, but usually bound to the transport protein or, in some cases, esterified. The first proposed solution was to incorporate the DTE into sample preparation, which should ensure that the analytes would not be precipitated with the proteins. The second proposed solution

was to increase the amount of plasma used for extraction, which was increased from 100 µl to 500 µl. However, this step still didn't ensure the extraction of all the analytes. The next possibility would be to increase the amount of plasma even further. However, that assay would not be applicable for any routine analysis due to the high sample consumption, which is highly undesirable in clinical analyses.

The third hypothesis is strongly linked to the second one, and that is a high lower limit of detection. If this is the case, why are the analytes undetectable, then the solution will be using more sensitive MS analysers and detectors.

The fourth hypothesis is based on an unsuitable extraction process. The main problem may be the stability of the analytes. The first improvement may be the strong regulation of the light. Stability improvement may be achieved by eliminating the natural light and using subdued artificial light in the laboratory. The second improvement may be the cooling of the analytes in every step of the extraction. Even though the extraction is mostly done on ice, some steps still need to be completed. The steps where the analytes' stability may decrease due to an uncontrollable environment are: shaking, evaporating the mobile phase by the nitrogen stream and the ultrasonic bath in the case of DBS. The third option for improvement was using a different vortex shaker, which allows vigorous shaking of the mixture and increases the contact between two phases resulting in more effective extraction.

Lastly, the problem may be rooted in the LC-MS assay. The problem may be a too high flow in the column. This can raise issues in spraying the analytes, as higher volumes are harder to evaporate and require stronger gasses in the ion source. The high flow probably also affects the precision and accuracy in the validation process. The possible solution may be to lower the flow and elongate the assay. This will make the assay more time-consuming and expensive but may solve the issues with the gasification of the analytes. If the solution mentioned above did not successfully spray the analytes, then the derivatisation process may be introduced to the assay.

The poor stability of retinol may be increased with using an antioxidant solution (BHT 0.1 g/ml) from the beginning of the extraction, which was shown effective in Zhang et al. 2019 and add antioxidants in some form to the organic phase before drying under nitrogen stream as the use of antioxidants was found effective in preventing the degradation of the retinol. (Zhang et al. 2019) According to Rubió, antioxidants did not significantly

influence the results. However, each newly developed extraction assay should test the influence on the specific antioxidants. (Rubió et al. 2020)

6. Conclusion

All previously mentioned aims of the work were accomplished. The assay for fat-soluble vitamins analysis was developed. The LC-MS part of the assay is successful in the separation and identification of all fat-soluble vitamins. And the extraction part of the assay successfully extracted retinol – vitamin A and alpha-tocopherol – vitamin E from biological samples. Through the assay's extraction development, monophasic extraction was not satisfactory, and a preconcentration step is necessary to obtain usable results. It was proven through spiked samples that the vitamins could be extracted with the developed extraction assay. The best option for the extraction was a liquid-liquid extraction with a mixture of n-hexane/chloroform (90/10 *v/v*).

The precision and accuracy of analysis of both retinol and alpha-tocopherol are worse with lower concentrations. Therefore, the assay is not suitable for routine analysis yet. The retinol had shown poor stability whilst exposed to light and partial stability in any condition except if kept in the dark at 4°C. The alpha-tocopherol has shown substantial stability in all of the conditions studied. Retinol and alpha-tocopherol have shown polar opposite matrix effects in studied matrices. As a result, precision and accuracy should be improved if the assay is used routinely. The improvement of retinol stability should also be reviewed to obtain robust results.

7. Literature

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8. List of used abbreviations

- APCI – atmospheric pressure chemical ionisation
- BHT - Di-tert-butyl-4-methyl-phenol
- CAD – collision activated dissociation
- CID – collision induced dissociation
- CLD -chronical lung disease
- CV – coefficient of variation
- DAD – diode array detection
- DBS – dried blood spots
- DTE - dithioerithriol
- ECD – electron capture detection
- EIA – enzyme immunoassay
- ELISA - enzyme-linked immunosorbent assay
- EMA – European medicines agency
- ESI – electrospray ionisation
- FID – flame induced detection
- FSVs – fat-soluble vitamins
- GC – gas chromatography
- HMDB – human metabolome database
- HPLC – high performance liquid chromatography
- LC-MS – combination of liquid chromatography with mass spectrometry detection
- LOD – lower limit od detection
- LOQ – lower limit of quantification
- MRM – multiple resonance monitoring
- MS – mass spectrometry
- MTBE - methyltertbutylether
- nano-LC - Nanoscale liquid chromatography
- NARP - Non-aqueous reversed phase
- PFP – pentafluorophenyl
- RIA – radioimmune analysis
- RBP – retinol binding protein
- SPE – solid phase extraction
- UHPLC - ultra-high-performance liquid chromatography
- UV – ultraviolet light
- UVB – medium length ultraviolet light, wavelength 315–280 nm