Development of a Derivatization Procedure for the Quantitative Determination of Vitamin D_3 for Clinical Use

Method Development

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PREFACE

This thesis was written as the final part of the Bachelor Chemistry at the University of Applied Science Leiden. The study was performed at the Division of Bioanalyticial Chemistry of the VU University in Amsterdam.

Developing and testing the procedure was extremely time-consuming and labor-intensive. Due to lack of time, the experimental part of this study was partly carried out, under my supervision, by four students of the Bachelor Pharmaceutical Sciences at VU Amsterdam. These students worked on separate systems, explaining the variation in separation conditions. I wish to thank Dennis van der Laan, Marlien van Mever, Tristan Bruning, and Wayne Chang for their work and dedication.

I would like to thank my supervisor, dr. Henk Lingeman, for his guidance, support, and answers to any questions I had. I also wish to thank all my colleagues at the Division of Bioanalytical Chemistry (VU Amsterdam). They have made my internship a pleasant experience and were always there to help me, motivate me, or distract me when necessary. Finally, I wish to thank Coen for being there for me and his support.

Thank you for beginning to read my thesis.

Nikita Boeren
Amsterdam, August 2016

SAMENVATTING

Vitamine D deficiëntie komt voor bij zowel kinderen als volwassenen. Deficiëntie kan leiden tot verschillende ziektes, onder andere misvormingen van het skelet en groeiachterstanden (bij kinderen), osteomalacie en spierverzwakking. Een analysemethode voor vitamine D zonder het gebruik van een massaspectrometer is wenselijk, aangezien niet elk ziekenhuis in bezit is van dit apparaat en het aanzienlijk hoge kosten met zich meebrengt. Aangezien relatief kleine hoeveelheden vitamine D aanwezig zijn in het menselijk lichaam, is detectie met fluorescentiespectrometrie gewenst, vanwege de hoge gevoeligheid. Om de detectie van vitamine D_3 te verbeteren, zal vitamine D_3 moeten worden gederivatiseerd.

Het doel van dit onderzoek is het ontwikkelen van een derivatisatie procedure voor de kwantitatieve bepaling van vitamine D_3 voor klinische doeleinden.

Drie verschillende derivatisatie reagentia en methoden zijn getest. De geteste derivatisatie reagentia zijn naproxenchloride, 3,5-dinitrobenzoylchloride en dansylchloride. Metingen werden uitgevoerd met vloeistofchromatografie in combinatie met ultraviolet detectie (LC-UV), fluorescentie detectie (LC-FL) en massaspectrometrie (LC-MS/MS). Scheiding werd verkregen op een C_{18} kolom.

Methodes met naproxenchloride of 3,5-dinitrobenzoylchloride als reagentia leverden geen derivaat op. Na een langere reactietijd, trad degradatie van vitamine D op. Zowel naproxenchloride als 3,5-dinitrobenzoylchloride werden uitgesloten als bruikbare derivatisatie reagentia.

Een derivatisatie met dansylchloride leverde het gewenste derivaat op, wat een duidelijk signaal gaf tijdens metingen met LC-FL. Het toevoegen van N,N-diisopropylethylamine en 4-dimethylaminopyridine aan het derivatisatie mengsel verbeterde de opbrengst van het derivaat. LC-MS/MS bevestigden de formatie van het derivaat. Het verlengen van de reactietijd resulteerde in een toename van de reactieopbrengst.

Derivatisatie met dansylchloride, met toevoeging van N,N-diisopropylethylamine en 4-dimethylaminopyridine aan de reactie, zou een geschikte derivatisatie procedure voor vitamine D_3 kunnen zijn. De derivatisatie en analyse vereisen verdere optimalisatie. Ook de monstervoorbewerking zal verder moeten worden ontwikkeld.

ABSTRACT

Vitamin D deficiency is common in children, as well as adults. Deficiency can lead to several diseases, such as skeletal deformities and growth retardation (children), osteomalacia and muscle weakness. A new assay for vitamin D deficiency in hospitals, not using mass spectrometry, is desired, since not every hospital is in possession of a mass spectrometer and the considerably high cost of a mass spectrometer. Because relatively small amounts are required and present in the human body, analysis using fluorescence spectrometry is desirable. Vitamin D_3 has to be derivatized in order to improve the detection for the analysis.

The aim of this study is the development of a derivatization procedure for the quantitative determination of vitamin D_3 for clinical use.

Three different derivatization agents and methods are developed and tested, using three different derivatization agents, namely naproxen chloride, 3,5-dinitrobenzoyl chloride and dansyl chloride. Measurements were performed with liquid chromatography in combination with ultraviolet detection (LC-UV), fluorescence detection (LC-FL) or mass spectrometry (LC-MS/MS). A C_{18} column is used for separations.

Derivatization using naproxen chloride or 3,5-dinitrobenzoyl chloride did not yield the preferred derivative. Degradation of vitamin D_2 and D_3 was observed after a longer derivatization time. The derivatization procedures using naproxen chloride or 3,5-dinitrobenzoyl chloride were excluded as viable options.

Derivatization using dansyl chloride yielded the preferred derivative. LC-UV and LC-FL measurements confirmed enhancement of derivatization using N,N-diisopropylethylamine and 4-dimethylaminopyridine. LC-MS/MS measurements confirmed the presence of the preferred derivative. Extending the derivatization time resulted in an increase of formed derivative. Derivatization using dansyl chloride, in the presence of N,N-diisopropylethylamine and 4-dimethylaminopyridine, is shown to be a potentially successful derivatization procedure for vitamin D_3. However, the method requires further optimization. Also, sample preparation has to be developed.

ABBREVIATIONS

ACN	Acetonitrile
D2	Vitamin D_2; ergocalciferol
D3	Vitamin D_3; cholecalciferol
DBP	Vitamin D-binding protein
DIPEA	N,N-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DNBC	3,5-Dinitrobenzoyl chloride
Dns-Cl	Dansyl chloride (5-(Dimethylamino)naftaleen-1-sulfonylchloride)
ESI	Electrospray ionization
FA	Formic acid
FL	Fluorescence detector/spectrometry
HPLC	High performance liquid chromatography
LC	Liquid chromatography
m/z	Mass-to-charge
MeOH	Methanol
MS	Mass spectrometer/spectrometry
MS/MS	Triple quadrupole mass spectrometer
Nap-Cl	Naproxen chloride
S	Singlet quantum state
THF	Tetrahydrofuran
TIC	Total ion chromatogram
UV	Ultra violet detector/spectrometry
XIC	Extracted ion chromatogram

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1. INTRODUCTION

1.1 General background

Vitamin D refers to different forms of the vitamin and is one of the four fat-soluble vitamins (vitamin A, D, E and K). All the forms of vitamin D are fat-soluble secosteroids. Secosteroids are steroids with an open ring structure [1, 2]. Vitamins are essential for human life. Vitamin D differs from other classes of nutrients, because it has a different function. For instance, other classes of nutrients provide energy for the human body, whereas vitamin D is mainly involved in regulation of calcium and phosphate levels in the blood. Vitamin D has various other highly specific functions. Because of the highly specific uses, relatively small amounts are required for the human body to function properly [3].

The two most important forms of vitamin D in the human body are vitamin D_2 and D_3. In figure 2 the chemical structures of both vitamins are shown. Vitamin D_3, or cholecalciferol, can be ingested from regular nutrition and/or supplements, but the major source of vitamin D_3 is the human skin. Vitamin D_3 is produced through ultraviolet irradiation of 7-dehydrocholesterol from lanolin. Vitamin D_2, or ergocalciferol, is the vegetal form of vitamin D. Vitamin D_2 is produced through ultraviolet irradiation of ergosterol from yeast. Vitamin D that is taken in from diet (nutrition and supplements), represents vitamin D_2 or D_3 [2, 4, 5].

Vitamin D_3 can be dermally synthesized through UVB irradiation of 7-dehydrocholesterol (290-315 nm). 7-dehydrocholesterol can be seen as a provitamin. Vitamin D that is taken in from diet is absorbed with the assistance of chylomicra (lipoprotein particles) [3]. After dermal synthesis (D_3) or obtaining from diet (D_2 and D_3), vitamin D circulates in the bloodstream bound to vitamin D-binding protein (DBP), until it reaches the liver. The liver converts vitamin D to 25-hydroxyvitamin D. This is transported to the kidney, where 25-hydroxyvitamin D metabolized to its active form, 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D). The active form of vitamin D acts as a steroid hormone by binding to the vitamin D receptor [2-4, 6]. A schematic representation of vitamin D circulation in the human body is shown in figure 1.

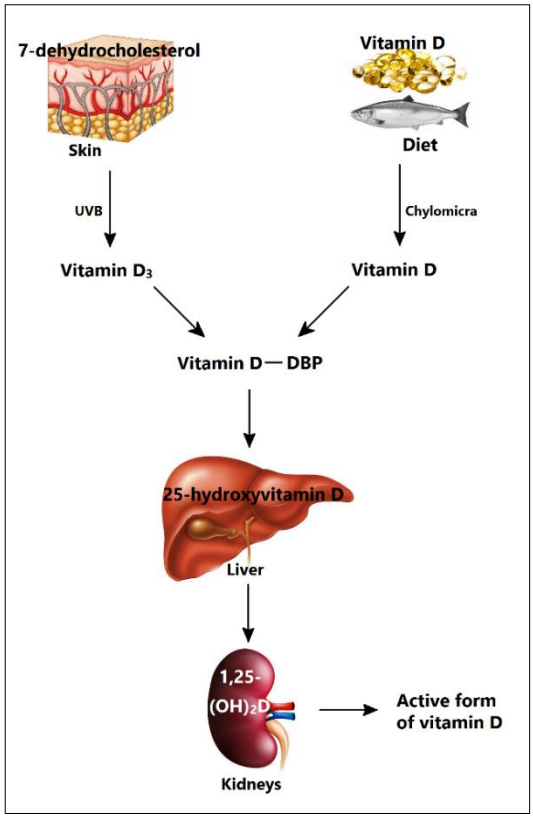

Figure 1. A diagram of vitamin D circulation in the human body.

The active form of vitamin D is responsible for the absorption of the major part of calcium and phosphorus in the human body. It increases the intestinal absorption of calcium for proper mineralization of bones [2, 3, 5].

Vitamin D deficiency or insufficiency is common in children, as well as adults. The number of people worldwide suffering from vitamin D deficiency or insufficiency is estimated around one billion [2, 7]. Possible causes of vitamin D deficiency can be an increased catabolism, reduced synthesis in the skin and reduced absorption of vitamin D in the gastrointestinal tract. Decreased bioavailability, breast-feeding (for infants), decreased synthesis of 25-hydroxyvitamin D or 1,25-dihydroxyvitamin D and acquired or heritable disorders also (have been shown to) play a role in vitamin D deficiency. Examples of patients with an increased risk of vitamin D deficiency are obesity patients, patients with liver failure, nephritic syndrome or chronic kidney disease [2, 5].

Deficiency can lead to several diseases. Deficiency in utero and during childhood can cause skeletal deformities, growth retardation and can increase the risk of hip fracture later in life. In adults it can cause osteomalacia, muscle weakness, increase the risk of fracture and can precipitate or exacerbate osteopenia and osteoporosis [2].

The treatment for vitamin D deficiency is intake of vitamin D supplements and adjusting the diet. People with higher risk factors for vitamin D deficiency are recommended to take supplements as a precaution. Vitamin D can also play a role in decreasing the risk of many illnesses, including common autoimmune diseases, infectious diseases, cardiovascular disease and cancers [2, 4].

Currently, the most commonly used method to measure vitamin D deficiency is the 25-hydroxyvitamin D blood test. 25-hydroxyvitamin D is the converted form of vitamin D in the liver. When the blood level of 25-hydroxyvitamin D falls below 20 ng/ml, it is defined as deficiency by most experts. A blood level between 21 and 29 ng/ml is defined as insufficiency [7].

In hospitals, vitamin D levels are measured by using LC-MS. These measurements are often performed by the pharmaceutical department of the hospital, instead of the clinical department. Hospitals that are not in possession of an LC-MS, often outsource the assays to third party companies or better equipped hospitals, which is costly and time-consuming. Furthermore, current quantitative measurements using MS are prone to non-specific interference (matrix related) and proved to be inaccurate [8-11]. The interference is matrix related and due to analytes with the same polarity and molecular weight. This results in inaccurate quantification. The interferences and inaccuracy leads to a demand for accurate assays, preferably with and without detection with mass spectrometry. However, mass spectrometry can still be used for identification purposes, since only quantitative and not qualitative (identification) measurements are affected by interferences.

The detection of vitamin D_3 in human samples is challenging, mainly because of the high sensitivity required to stay within the range of measurement. The detection of vitamin D_3 can be performed with an ultraviolet (UV) detector, but the sensitivity is not high enough for the preferred scale. Mass spectrometry (MS) can be used for the detection, however a few hospitals

(in the Netherlands) are not in possession of a mass spectrometer and the costs of (using) a mass spectrometer are considerably high. Furthermore, not enough (immune)assays are available and do not have the desired sensitivity. Detection using a fluorescence (FL) detector is desirable, since it has the preferred sensitivity. This gives rise to a new problem, the molecule itself does not have a highly fluorescent group. Therefore, derivatization is necessary to improve detection.

Derivatization is an analytical technique, centered on making a chemical manipulation in a molecule in order to improve detection. Vitamin D_3 will be derivatized with a derivatization agent. The derivatization agent should have a couple of characteristics. Firstly, the derivatization agent must be selective for the reaction with the desired functional group of the analyte. Secondly, it should have a fluorescent group for the fluorescence detection. Furthermore, it is preferred that the derivative is easily fragmented for mass spectrometry. Lastly, it is desirable for the derivative to have a non-polar region for the preferred chromatographic properties [12, 13]. In theoretical chapter 2.1 the technique will be further explained.

1.2 Aim

The aim of this study is the development of a derivatization procedure for the quantitative determination of vitamin D_3 for clinical use.

Current assays in hospitals use mass spectrometry as the detection method, which is a considerably high priced technique. Furthermore, not every hospital is in possession of a mass spectrometer, leading to outsourcing of assays which is costly and time-consuming. These flaws give rise to the demand for a new assay, using a less costly and more available detection method. The low concentrations of vitamin D in samples require a highly sensitive detection method. Fluorescence spectrometry is selected as the most suitable detection technique, due to the low cost, general availability, and considerably high sensitivity. In order to detect vitamin D_3 with a fluorescence detector, vitamin D_3 has to be derivatized.

The aim of this study can be divided in different sub goals. First, a suitable derivatization agent has to be found. Subsequently, the feasibility of separation via reversed-phase liquid chromatography (LC) and detection using fluorescence spectrometry (FL) must be examined. Finally, the derivatives must be identified through triple quadrupole mass spectrometry (MS/MS).

The derivatization conditions and the system settings have to be optimized. After optimization, the sample preparation method will be developed.

1.3 Approach

The aim of this study is focused on vitamin D_3, but for the experimental part of the study not only vitamin D_3 will be derivatized. The experimental part of this study will partly be carried out by four students (BSc. Pharmaceutical Sciences, VU Amsterdam), under supervision of the

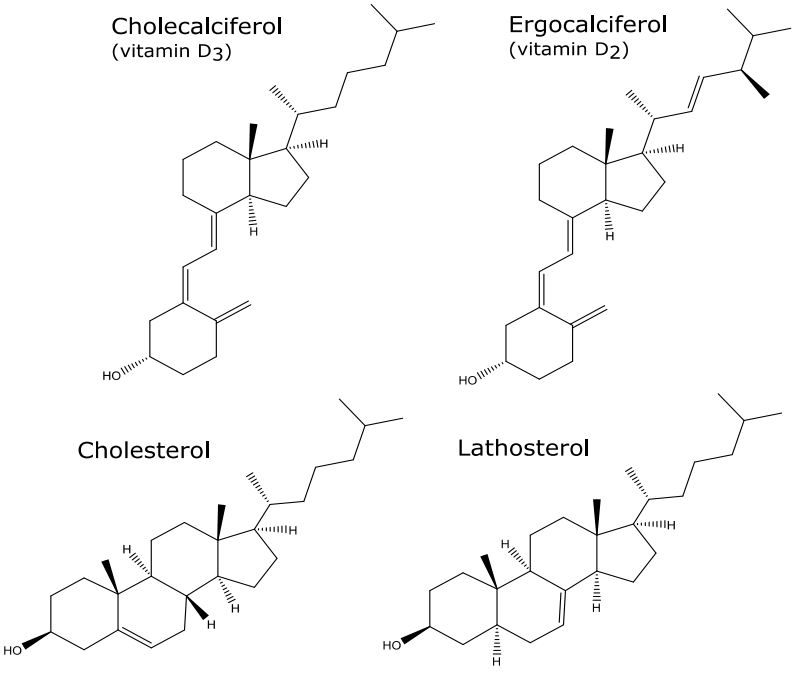

Figure 2. The chemical structures of cholecalciferol, ergocalciferol, cholesterol and lathosterol.

author. Therefore, four different compounds will be used in order to facilitate the practical implementation and to broaden the practical applicability. The different compounds have the same functional hydroxyl group, as vitamin D_3, on which the derivatization will be performed. The compounds that will be used are cholecalciferol (vitamin D_3), ergocalciferol (vitamin D_2), cholesterol and lathosterol. The chemical structures of the compounds are shown in figure 2. Cholesterol and lathosterol are chosen for their chemical similarity to vitamin D, and their clinical background, validating incorporation of both sterols in this study.

The goal for the analysis is that the derivative can be detected via fluorescence and (if possible) mass spectrometry. Fluorescence detection is desirable for the hospitals who are not in possession of a mass spectrometer. A typical fragmentation pattern for mass spectrometry is desirable for the hospitals that possess a mass spectrometer and want to continue the assays using this technique. Therefore, the derivative needs to be fluorescent and must have a typical fragmentation pattern.

Developing a derivatization procedure will be carried out by a number of steps. An overview of the development of a derivatization procedure is presented in figure 3. First, the procedure will be tested and the method will be developed using LC-UV. Students will carry out this part of the study, due to a lack of time and since developing and testing a derivatization procedure is very labor-intensive. Thereafter, the method will be tested with fluorescence detection and further adjusted and developed. Subsequently, the derivatives will be identified using mass spectrometry. The procedure will be evaluated after measurements with mass spectrometry. If a procedure is suitable for further research, and (eventually) assays in hospitals, the procedure has to be optimized further.

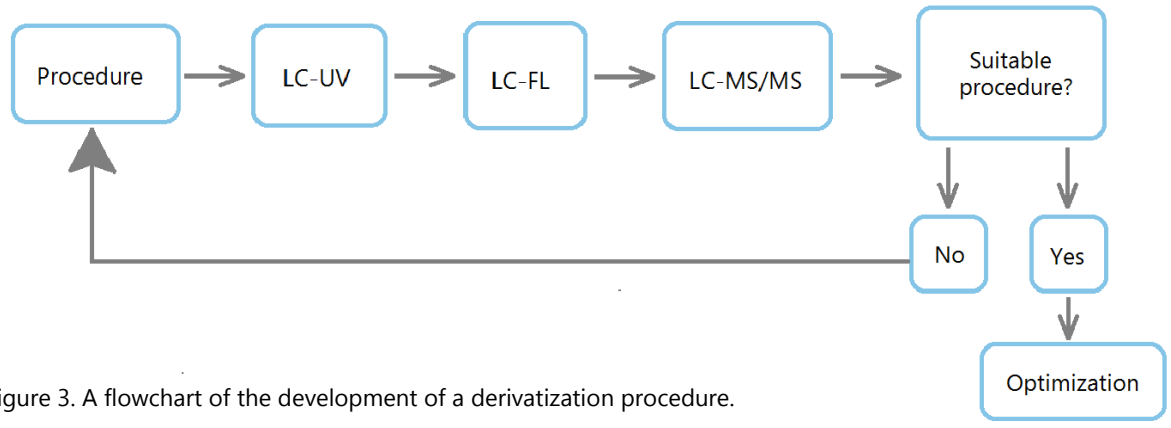

Figure 3. A flowchart of the development of a derivatization procedure.

The development of a derivatization procedure will be started by finding a suitable derivatization agent. The aim is to connect the derivatization agent to the functional part of the compound in a simple (one step) reaction. There has to be examined if the derivatization agent will react with the analyte, in the way that is preferred. The reaction should be descending and specific. The derivatization reactions will be performed prechromatographically. Therefore, optimization of the derivatization is relatively easy, since any solvent can be selected and any by-products of the derivatization reaction can be separated.

Three different derivatization agents will be tested, namely naproxen chloride, 3,5-dinitrobenzoyl chloride and dansyl chloride. These three derivatization agents, and methods, are chosen because of their following characteristics. First, all three derivatization agents have a fluorescent part in the molecule and have a typical fragmentation pattern (facilitating identification steps using MS). Also, two derivatization agents are acyl chlorides and one is a sulfonyl chloride, groups which are both known for reacting rapidly. Lastly, the availability of all three compounds played a role in the selection process.

For the derivatization with naproxen chloride, the method described by *Y.T. Lin et. al., 2007* will be used [14]. This method describes the derivatization of a hydroxyl group with naproxen chloride. After the derivatization, diethylamine will be added to the mixture to neutralize the remaining part of the derivatization agent. The reaction scheme is shown in figure 4 and the mechanism of the reaction is shown in appendix 1.1.

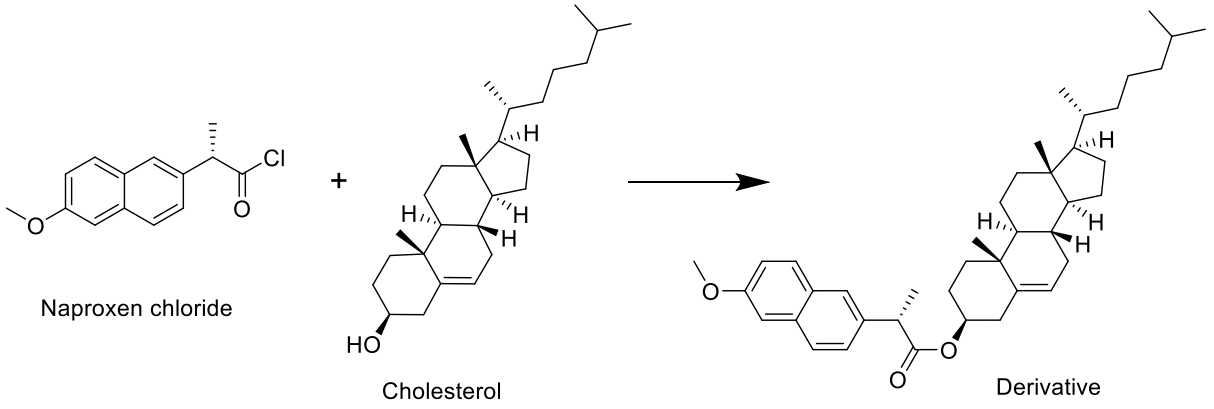

Figure 4. Reaction scheme of the derivatization of cholesterol with naproxen chloride.

3,5-dinitrobenzoyl chloride will be used for the derivatization, based on the method described by *TCI, HPLC labeling agents, 2015* [15]. To aid in the dechlorinating and deprotonating steps of the reaction, pyridine will be added. Figure 5 shows the derivatization, while the reaction mechanism is shown in appendix 1.2.

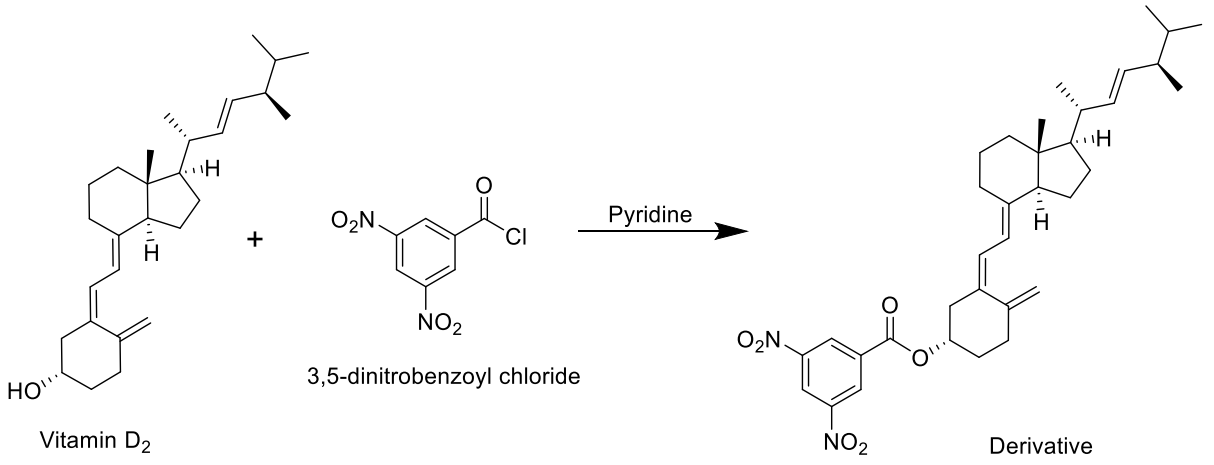

Figure 5. Reaction scheme of the derivatization of vitamin D_2 with 3,5-dinitrobenzoyl chloride.

The procedure described by *Z. Tang and F.P. Guengerich, 2010* will be used for the derivatization with dansyl chloride [16]. Dimethylaminopyridine (DMAP) and N,N-diisopropylethylamine (DIPEA) will be added to the derivatization mixture to enhance yield. The reaction scheme is displayed in figure 6 and the mechanism shown in appendix 1.3.

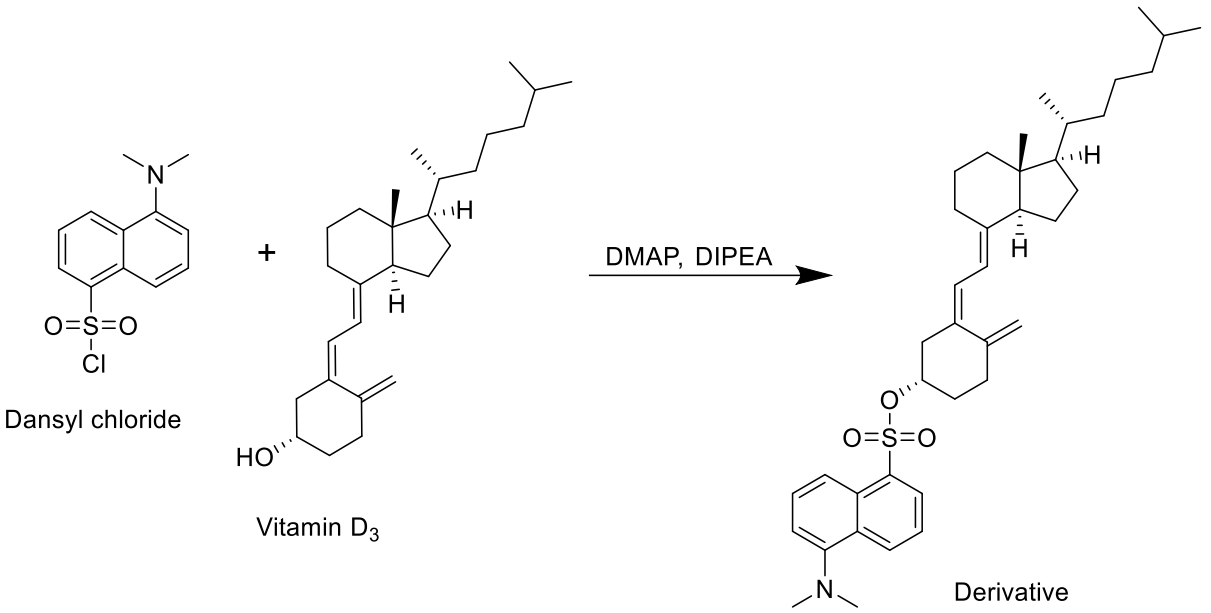

Figure 6. Reaction scheme of the derivatization of vitamin D_3 with dansyl chloride.

The most suitable derivatization procedure will be chosen based on yield, reaction conditions and the lack of formation of any by-products. Subsequently, several variables have to be optimized within the derivatization. Since the variables affect each other, optimization might prove to be challenging.

The derivatives will be measured on reversed-phase LC-UV, LC-UV-FL and also on LC-MS/MS. The settings of the systems require optimization.

A C_{18} column will be used for the separation on reversed-phase LC. The detection will be performed with ultraviolet and fluorescence spectrometry. Ultraviolet spectrometry is a type of absorption spectrometry where UV light is absorbed by the molecule. Monochromatic light of a selected wavelength will be directed through the sample, which will lead to excitation of the electrons of the molecule. The unabsorbed light will be detected. When absorption raises the molecule to a single state of higher energy, emission can occur. Fluorescence occurs when a photon is emitted from the molecule on its return to the ground state from the lowest electronically excited singlet state. Fluorescence spectrometry can be described as the detection of the emitted photons [17]. In chapter 2.2 a further explanation of fluorescence spectrometry is given.

Detection with LC-MS/MS will be performed using an electrospray ionization source and a triple quadrupole mass spectrometer. Electrospray ionization is a soft ionization technique, where a homogenously charged sphere of liquid is evaporated, leaving the charge on any non-

evaporated particle. A triple quadrupole mass spectrometer is a mass analyzer consisting of two quadrupole analyzers and one quadrupole collision cell. Both techniques will be further explained in chapter 2.3 and 2.4.

The method for sample preparation has to be further developed. The method should be suitable for serum and/or urine. At the moment, the most widely used assays are performed with blood samples If a method can be developed for urine measurements, it is less of a burden for the patients.

2. THEORETICAL BACKGROUND

This chapter will elaborate on the four analytical techniques that are essential for this study. Fluorescence spectrometry and a mass spectrometry are both of great importance for this study. Therefore, a general overview of these techniques is given. In order for detection, the analytes must first be derivatized. Derivatization will be discussed first. Subsequently, information about fluorescence and mass spectrometry (ionization and triple quadrupole mass spectrometry) is given.

2.1 Derivatization

The low amount of vitamin D_3 present in the human body makes detection challenging, therefore a detection method with high sensitivity is required. Fluorescence detection has the required sensitivity (and the preferred low costs), however vitamin D_3 is non-fluorescent. Therefore, derivatization is necessary to make detection using fluorescence possible. Also, mass spectrometry can be used as detection method, however sensitivity must be increased in order to improve detection, which can be achieved through derivatization of the compound.

Derivatization is an analytical technique, centered on chemically manipulating a molecule in order to improve detection. Derivatization is widely used in combination with gas chromatography, liquid chromatography and electrophoresis. However, only derivatization in combination with liquid chromatography is of interest for this study. Several derivatization purposes can be distinguished, but this study will only include fluorescence derivatization and derivatization for mass spectrometry. Derivatization can be performed at two points in an assay, prechromatographically or postchromatographically. Both methods have clear advantageous and disadvantageous features.

Prechromatographic, derivatization is usually performed off-line, outside the LC system. One advantage is that there is no need for the solvent, in which the derivatization takes place, to be compatible with the system. Furthermore, chromatographic properties can be optimized and reaction conditions can be varied freely in order to optimize the reaction time and yield. Excess of derivatization agent and side or degradation products that may be formed during derivatization, can be separated from the derivative on the system or with a clean-up step. In short, both derivatization and chromatographic procedures can be optimized separately. However, formation of side products may interfere with the analysis or the reproducibility of the reaction. Furthermore, prechromatographic derivatization is time consuming, since each sample needs to be individually handled. An internal standard is required for prechromatographic derivatization, since potential loss of vitamin D_3 during derivatization must be taken into account [18].

Postchromatographic derivatization, on the other hand, is (mostly) performed on-line. The derivatization takes place in a part between the column and detector, also referred to as reactor. Postchromatographic derivatization has its advantages compared to

prechromatographic. Underivatized compounds are separated and eluted from the column. Therefore, other nondestructable detection methods can be applied before derivatization. Postchromatographic derivatization may also be advantageous when there is a possibility that the derivative will be unstable in the mobile phase and may be (partly) degraded during the measurements or pretreatment. Also, limited sample preparation is necessary. Accuracy and reproducibility are increased by automation of the derivatization procedure. However, online derivatization requires automation of the procedure and may result in difficult instrumental modifications [18].

In this study, prechromatographic derivatization will be performed, since separate optimization of the derivatization procedure and chromatographic procedure is required. Also, prechromatographic derivatization can be accompanied by liquid-liquid extraction or other sample pretreatment steps.

The necessity of removing excess reagent or the solvent prior to derivatization, is common. Evaporation of the mixture under a nitrogen stream (at room temperature or under heating) is a simple and convenient way of removing the solvents. Also, this is a concentration step. If removing of the reagent is necessary, and the reagent is nonvolatile, another compound can be added to the mixture, to inactivate the reagent [18].

An ideal derivatization agent would be selective for a single functional group. Furthermore, the label would introduce a high degree of sensitivity and is nontoxic. The ideal derivatization procedure would be rapid, quantitative under mild conditions and with a minimum of manipulations. The derivatization procedure should yield maximum product and should produce a single derivative. Also, a derivatization reagent that improves the separation from matrix components in sample pretreatment is desirable in most cases [18].

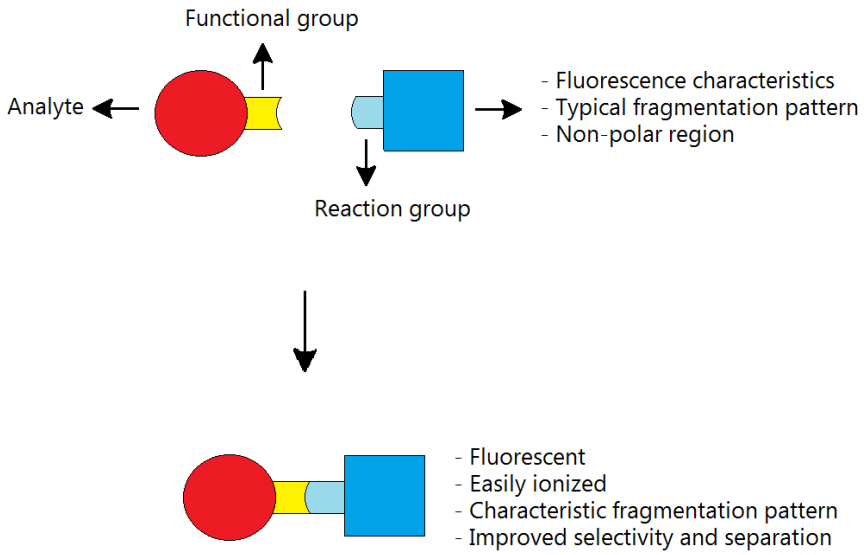

Figure 7. A visual representation of a general reaction mechanism of derivatization with advantages and characteristics.

The requirements for the derivatization agent also depend on the techniques (preferred) to be used and the preferred applications. This study requires a derivatization agent suitable for analysis with fluorescence spectrometry and mass spectrometry. In figure 7 a visual representation of a general reaction mechanism of derivatization is shown with advantages of derivatization.

Fluorescence spectrometry requires the derivative to be fluorescent in order for detection. The presence of rigid planar aromatic ring(s) in a molecule often exhibits fluorescence (UV or visible region) and it is preferred to be present in a derivatization agent [13]. A fluorophore (a fluorescent compound) is preferred to have a longer excitation wavelength than 300 nm. This way, measurements with an excitation wavelength above 300 nm can exclude possible fragments in the detection [18]. The derivatization agent should be non-fluorescent in its native form or should be separable from the desired derivative.

A derivatization procedure for mass spectrometry requires the derivatization agent to be easily ionized and to have a typical fragmentation pattern [18]. However, fragment patterns of the derivatives will also depend on the structures of the formed derivatives. Fragmentation patterns of the derivatives in tandem mass spectrometry should give information. Not only selected ion monitoring, but also the analysis of neutral loss and/or precursor-ions can provide additional data for the analysis [13, 19].

For this study, the derivatization reaction should yield maximum product and should be selective for the functional group (hydroxyl) of vitamin D_3. Measurements with fluorescence spectrometry require the desired derivative to be fluorescent. The reagent should be non-fluorescent in its native form or separable from the desired derivative. Facilitating ionization and typical fragmentation patterns are desirable features for analysis with mass spectrometry. For chromatographic separations, it is desired to have non-polar region in the derivatization reagent.

2.2 Fluorescence

A highly sensitive detection method is required, since vitamin D_3 is present in low amounts in the human body. This makes detection using fluorescence spectroscopy desirable for this study. Also, the significantly low cost of (using) a fluorescence detector makes it a very suitable method.

Fluorescence spectroscopy analyses the fluorescence of a molecule in the ultraviolet or visible light spectrum. In order for fluorescence to occur, a molecule must be excited by a photon. When the molecule falls back to its singlet ground state, a photon is emitted [17].

Excitation occurs when a molecule absorbs energy and raises an electron from its ground state (S_0) to an excited singlet state (S_1). Three excited states can be distinguished, namely electrical, vibrational and rotational states [20, 21]. An in depth explanation of the different states and their role in fluorescence spectroscopy would be out of the scope of this theoretical chapter. Therefore, the different states and their roles will be excluded.

The absorption of the photon may raise the electron to a singlet state of higher energy (S_2) than the lowest excited state (S_1). The electron may reach the lowest excited singlet state by internal conversion. Internal conversion is a radiationless process in which, for example, excess electronic energy is converted to vibrational energy [17, 21].

Fluorescence is the process of emission of a photon from the molecule on its return to the ground state (S_0) from the lowest electronically excited singlet state (S_1). The energy of the photon emitted must be less than the energy of the photon initially absorbed, since the initial excitation energy is partly lost through internal conversion. Figure 8 shows an energy-level for electron absorption and emission. In very specific cases it has been shown that the energy of the photo emitted is equal in energy to the energy absorbed, called resonance fluorescence [20, 21].

Fluorescence is always at a lower energy (or equal energy; resonance fluorescence) than the originally absorbed photon. Thus, the wavelength of the light emitted as fluorescence will be greater that the wavelength of the light absorbed [20].

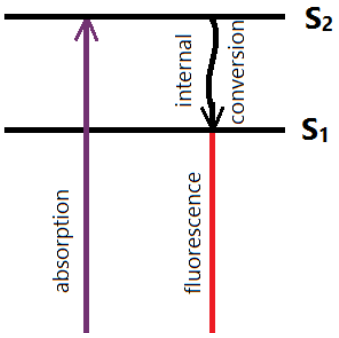

Figure 8. Singlet state energy levels of electron absorption and emission.

A fluorescence spectrometer includes a light source, an excitation monochromator, a sample chamber, an emission monochromator and a highly sensitive detector. A simplified illustration of a fluorescence spectrometer is shown in figure 9.

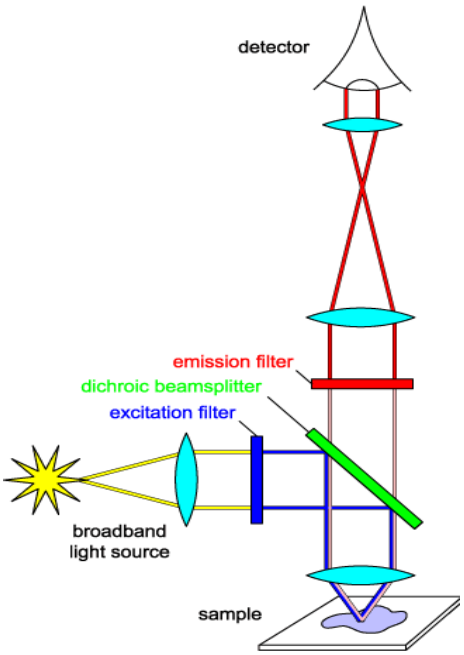

Figure 9. A simplified illustration of the principle of a fluorescence detector [22].

In the figure above, a dichroic mirror is shown in the set-up of a fluorescence detector. A dichroic mirror can reflect light from one angle, while simultaneously transmitting light from another. This property gives it great value for a fluorescence detector. Photons from the light source are being reflected, under a 90-degree angle, and excite molecules in the sample. Any fluorescent light coming off the sample then passes through the dichroic mirror and reaches the detector. This 90-degree angle setup gives a fluorescence detector its advantage over most other types of detectors. Since the only photons that reach the detector have to be either reflected of a molecule or emitted from a molecule through fluorescence, the background 'noise' is reduced to a bare minimum. This results in the extremely high sensitivity that a fluorescence detector is known for. It is this quality that makes it a suitable detection method for this study. However, not many compounds are fluorescent and therefore derivatization is required, hence the derivatization steps.

2.3 Electrospray ionization

In this study mass spectrometry will be used for the identification of the formed derivatives. Mass spectrometry requires the analyte to be ionized for detection. Electrospray ioniziaton (ESI) is a soft ionization technique, where a homogenously charged sphere of liquid is evaporated, leaving a charge on any non-evaporated particle. Electrical energy assists the transfer of ions to the gaseous phase.

A sample solution is let through a fine capillary where a strong electric field is applied to its surface. Charge accumulation at the liquid surface at the end of the capillary will appear and a cone shape will be formed. When the surface tension is broken, due to the electrostatic repulsive forces, positively charged droplets will be formed and the spray appears [23-25].

The charged droplets are dissipated into the ionization chamber, which is heated and at near-atmospheric pressure. The droplets are subjected to heated inert gas to evaporate solvent molecules. The solvent in the droplets will evaporate, which causes the droplets to shrink and increase the charge density. Charge density increases until the surface tension causes the droplets to break apart into smaller charged droplets. The small charged droplets continue to lose solvent and the process repeats. The process continues until tension repulsive forces between ions exceed and desorption of ions from the surface occurs. The positively charged ions are left in the gas phase and can be analyzed [23-25]. Figure 10 shows the mechanism of electrospray ionization.

Negative ions may also be formed in ESI. The formation of negative ions occurs when loss of protons from the sample to basic species in solutions takes place [25].

Not only mono charged ions will be formed. ESI of large molecules often produces multiply charged ions. However, small molecules (<1 kDa) will produce mainly mono charged ions [24].

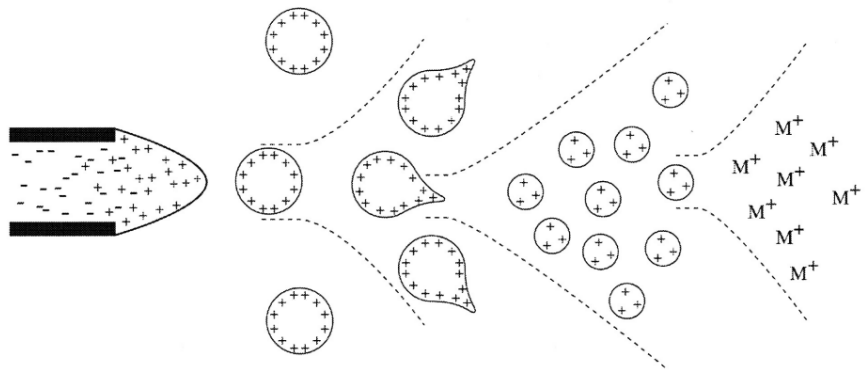

Figure 10. Mechanism of electrospray ionization [23].

ESI is a suitable ionization technique for this study, since it can be expected that mainly mono charged, intact analyte ions will be produced. Therefore, the expected mass of a preferred derivative can be selected for detection. ESI is widely used in combination with a triple quadruple mass spectrometer, since fragmentation prior to the collision cell is redundant.

2.4 Triple quadrupole mass spectrometry

Mass spectrometry will be used to identify any formed (unknown) derivative. A triple quadrupole mass spectrometer allows the molecular ion to be observed, while simultaneously producing fragments. This results in a typical fragmentation pattern with a corresponding molecular ion, by which an (un)known derivative might/can be identified.

A triple quadrupole mass spectrometer (MS/MS) consists of three successive quadrupoles. The first and third quadrupole are mass filters, while the second quadrupole is a collision cell. In a mass filter, ions are separated according to their mass-to-charge (m/z) ratio. The ions are separated by using the stability of the trajectories in oscillating electric fields [24].

A quadrupole is made up of four parallel metal rods. Opposite paired rods are electrically connected. AC voltage overlapped with a radio frequency (RF) voltage are applied to the pair of rods. Opposite voltage is applied to the pairs of rods. This results in an electrical field, which causes the ions to travel through the rods with an oscillatory motion [23]. The amplitude of oscillation depends on the ion and is related to the m/z ratio. The amplitude can be controlled by changing the voltages. These voltages can be set so that specific ions can oscillate through the rods without hitting the rods. The specific ions can reach the detector, but non-selected ions hit the rods and would not reach the detector [23-25].

In the collision cell, selected ions of the first quadrupole are collided with neutral inert gas. Collision adds kinetic energy to the analyte, which can convert into internal energy. A high internal energy can then result in fragmentation of the ions [24]. Subsequently, the formed fragments in the collision cell are let through the third quadrupole. The quadrupole will scan the entire m/z range (m/z 100 - 700) to provide information about the (size of the) fragments.

In tandem mass spectrometry different scan modes are available. For this study only one mode will be used, namely the product ion scan. The first mass filter is the fixed mass filter and is selected on a chosen m/z ratio and the second filter is the scanning mass spectrometer and determines all the product ions, produced in the collision cell. The ion fragmentation can be used to identify the structure of the original selected ion in the first quadrupole.

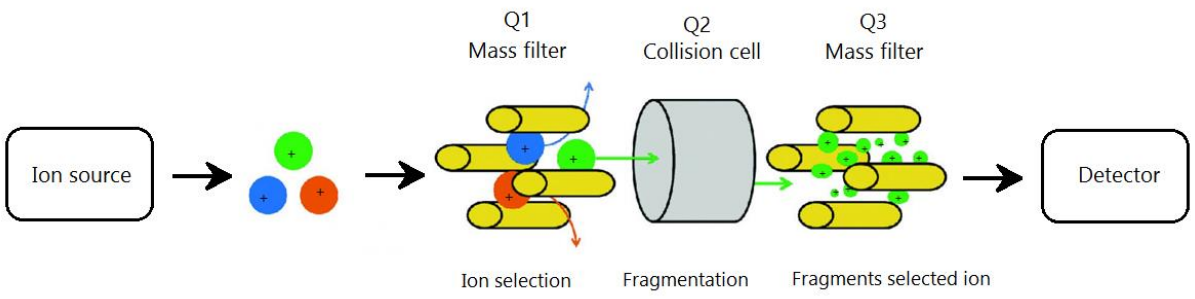

Figure 11. A representation of a triple quadrupole mass spectrometer using product ion scan mode.

Figure 11 gives a representation of a triple quadrupole mass spectrometer using product ion scan mode. First, ionization takes place and ions travel to the first quadrupole. The selected ion will be let through to the collision cell, where it will be fragmented. The fragments are scanned with the third quadrupole and reach the detector.

A total ion chromatogram (TIC) is typically obtained from any measurement. This is a chromatogram created by summing up intensities of all signals (peaks) of the same scan. Background noise is also present in the total ion chromatogram. An extracted ion chromatogram (XIC) is the chromatogram of a single (selected) mass. In this study, the selected masses will be of the preferred derivative, the analyte, and the derivatization agent. A spectrum with the fragmentation pattern of the selected mass can be obtained at a selected time. This spectrum shows all fragments belonging (/observed of) to the selected ion. Figure 12 shows an example of a TIC, XIC and mass spectrum of one measurement.

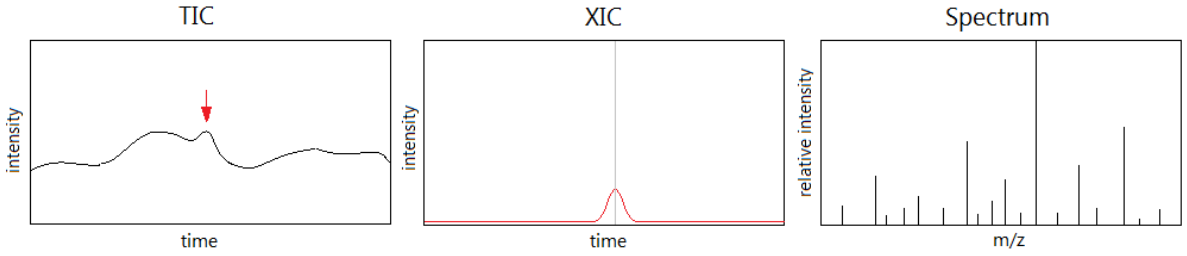

Figure 12. An example of a total ion chromatogram (TIC), an extracted ion chromatogram (XIC) and a mass spectrum of the same measurement.

Current assays for vitamin D deficiency commonly use LC-MS/MS [9, 11]. Analysis with LC-MS/MS is a favorable technique, since it has the capability to overcome most of the problems associated with protein binding assays. Also, the run time is very short, sample derivatization is not required and an internal standard is used [11]. However, studies show an inaccuracy in the assays using LC-MS/MS and development of new LC-MS/MS assays [8, 9, 11, 26]. Most of inaccuracy in the assays are due to interferences, such as matrix related, instrumental and analytical interferences. Also, deterministic errors, both epimerically and isobarically, have been shown to occur [11]. Therefore, quantitative assays using mass spectrometry are not adequate. However, these errors solely affect quantitative measurements, and do not impair value of mass spectrometry in qualitative measurements. For this reason, this study will use mass spectrometry exclusively for identification purposes.

3. MATERIALS

3.1 Instruments

Five different LC systems were used. All the systems consisted of two LC pumps, an auto/manual injector, and a C_{18} column. Four systems comprised a UV detector (systems A), one system comprised a fluorescence spectrometer (system B), as well as a UV spectrometer, to serve as detectors. The specifications of the systems are shown below. Also, an LC-MS/MS system (system C) was used. The specifications of the LC-MS/MS system are also shown below. An electrospray ionization source and a triple quadrupole mass spectrometer were used.

LC-UV (System A.1)
Manual Injector
Shimadzu LC-20AD pumps
Shimadzu UV/VIS SPD-20A detector

LC-UV-Fluorescence (system B)
Shimadzu LC-20AD pumps
Gilson Auto Injector 234
Applied Biosystems 759A Absorbance Detector
Shimadzu RV-10A XL Fluorescence detector

LC-UV (System A.2)
Spark Holland Autosampler
Shimadzu LC-10AD master pump
Shimadzu LC-20AD slave pump
Shimadzu UV/VIS SPD 20A detector

LC-MS/MS (system C)
Agilent 1100 series Auto Sampler
Shimadzu SCL-10A VP System Controller
Shimadzu LC-10AD VP Pumps
Shimadzu DGU-14A VP Degasser
Turbo ionspray SCIEX
PE SCIEX API 3000 LC/MS/MS

LC-UV (System A.3)
Manual Injector
Agilent 1100 series pumps
Shimadzu SPD-20A Prominence UV/VIS detector

Gilson pipettes (P2, P10, P20, P100, P200, P1000) and a balance (XA105 DualRange) from Mettler-Toledo were used.

3.2 Software

The used software was Ariadne, designed by the Vrije Universiteit Amsterdam, Agilent ChemStation Online/Offline and Analyst as software for the mass spectrometer.

3.3 Columns

Phenomenex Luna C18	3 µm, 4.6 x 150 mm	System A.1
Restek Ultra II Aqueous C18	3 µm, 2.1 x 100 mm	System A.2
Water Symmetry Shield RP18	3.5 µm, 4.6 x 100 mm	System A.3
Agilent XDB-C18	1.8 µm, 4.6 x 50 mm	System B
Agilent Eclipse XDB-C18	3.5 µm, 1.0 x 50 mm	System C

3.4 Chemicals

The following chemicals were used. Cholecalciferol (≥98%); ergocalciferol (≥97%); tetrahydrofuran (≥99%) (THF); 3,5-dinitrobenzoyl chloride (≥98%); diethylamine (≥99.5%); (S)-(+)-naproxen chloride (≥97%); lathosterol (≥98%); dansyl chloride (≥99%); N,N-diisopropylethylamine (≥99%) (DIPEA); 4-dimethylaminopyridine (≥99%) (DMAP); cholesterol (≥99%); formic acid (≥98%); pyridine (99.8%). All chemicals were purchased at Sigma-Aldrich (Zwijndrecht, the Netherlands). Methanol (MeOH), acetonitrile (ACN), toluene and chloroform were purchased at Biosolve (Valkenswaard, the Netherlands). The specifications of all the chemicals are shown in appendix 2.

4. EXPERIMENTAL

The first part of the study was the development of a derivatization procedure and testing the procedure on an LC-UV system. After development with LC-UV, an LC-UV-FL system was used for the measurements and further testing and developing. Subsequently, the derivatives were identified with LC-MS/MS.

Four students (BSc. Pharmaceutical Sciences, VU Amsterdam) performed the first part of the selection/optimization process of the experimental part of this study, under supervision of the author. This comprised the development of the derivatization procedures and measurements with LC-UV.

Three different derivatization procedures, using three different derivatization agents, were tested. Besides vitamin D_3, three different compounds were tested in order to facilitate the practical implementation and to broaden the practical applicability. The tested compounds were cholesterol, lathosterol, vitamin D_2, and vitamin D_3.

The first procedure tested was (derived from) a procedure described by *Y.T. Lin et. al., 2007*, which involved the derivatization of cholesterol using naproxen chloride [14]. In the second procedure vitamin D_2 and D_3 were derivatized using 3,5-dinitrobenzoyl chloride [15]. Subsequently, a third procedure involving derivatization of lathosterol using dansyl chloride was performed [16].

All separations were performed using reversed-phase liquid chromatography on a C_{18} column, since it is the most widely used column, significantly low in cost and widely available. Detection was done with an ultraviolet detector (UV), a fluorescence detector (FL) and a triple quadrupole mass spectrometer (MS/MS).

4.1 Derivatization procedures

Three different derivatization procedures were tested. One student tested the derivatization procedure of cholesterol using naproxen chloride. Two students tested the derivatization procedure of vitamin D_2 and D_3 using 3,5-dinitrobenzoyl chloride and the fourth student tested the derivatization of lathosterol using dansyl chloride. For every derivatization procedure, different variables were tested. The derivatization temperature was varied between 50 and 80 ˚C. Derivatization time was varied between 0 and 48 hours and the concentrations of the reactants were varied greatly. The derivatization procedures below, were the final procedures.

4.1.1 Derivatization with naproxen chloride

A solution of cholesterol in toluene (10 mM, 200 µl) and naproxen chloride in toluene (50 mM, 200 µl) was heated (in a water bath) at 90 °C for 1.5 h. After derivatization, the solution was cooled down to room temperature. Thereafter, 100 µl of diethylamine in toluene (60 %, v/v) was added and the solution was heated for 5 minutes at 30 °C.

After cooling to room temperature, 100 µl of the solution was pipetted in an Eppendorf cup. The mixture was dried under a nitrogen stream. The residue was dissolved in 200 µl acetonitrile (ACN).

In figure 13 an overview of the derivatization procedure is presented.

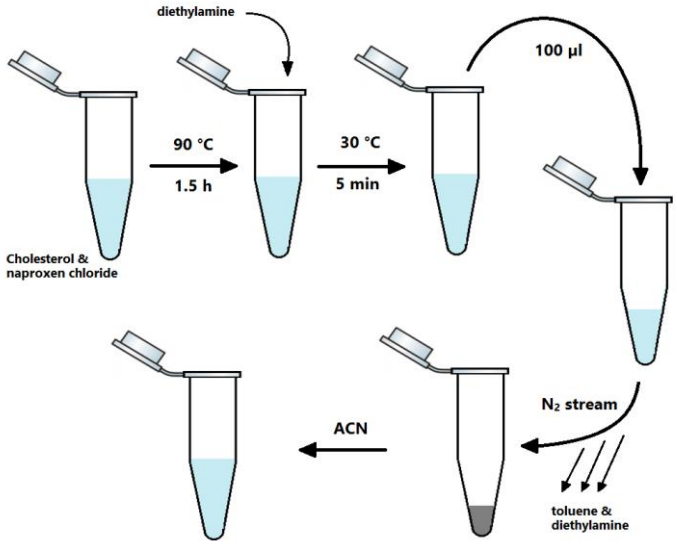

Figure 13. An overview of the derivatization procedure of cholesterol with naproxen chloride.

4.1.2 Derivatization with 3,5-dinitrobenzoyl chloride

Vitamin D_2 and vitamin D_3 were used for the derivatization with 3,5-dinitrobenzoyl chloride. Solutions of both vitamins were made by dissolving the vitamin in a 0.2% v/v solution of chloroform in tetrahydofuran (THF). End concentrations were 0.95 mM for vitamin D_2 and 0.69 mM for vitamin D_3.

900 µl of 3,5-dinitrobenzoyl chloride in THF (7.52 mM), 100 µl of the above described vitamin solution and 50 µl pyridine were mixed using a vortex mixer for 30 s. The mixture was incubated (in a water bath) at 60 °C for 3 h. After incubation, the mixture was cooled down to room temperature.

In figure 14 an overview of the derivatization procedure is presented.

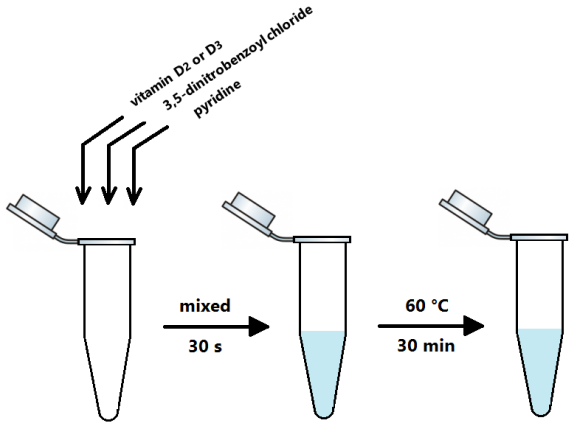

Figure 14. An overview of the derivatization procedure of vitamin D_2 or D_3 with 3,5-dinitrobenzoyl chloride.

4.1.3 Derivatization with dansyl chloride

A solution of 250 µl dansyl chloride in toluene (3.7 mM) and 250 µl lathosterol in toluene (18 µM) was made. 50 µl of N,N-diisopropylethylamine (DIPEA) in toluene (2% v/v) and 50 µl of 4-dimethylaminopyridine (DMAP) in toluene (10 mg/ml) were added. The solution was mixed using a vortex mixer for 30 s. Thereafter, the solution was incubated (in a water bath) at 65 °C for 1 h.

After incubation, the solution was cooled down to room temperature and centrifuged for 10 minutes, followed by drying the solution under a nitrogen stream. The residue was dissolved in 500 µl ACN.

An overview of the derivatization procedure is shown in figure 15.

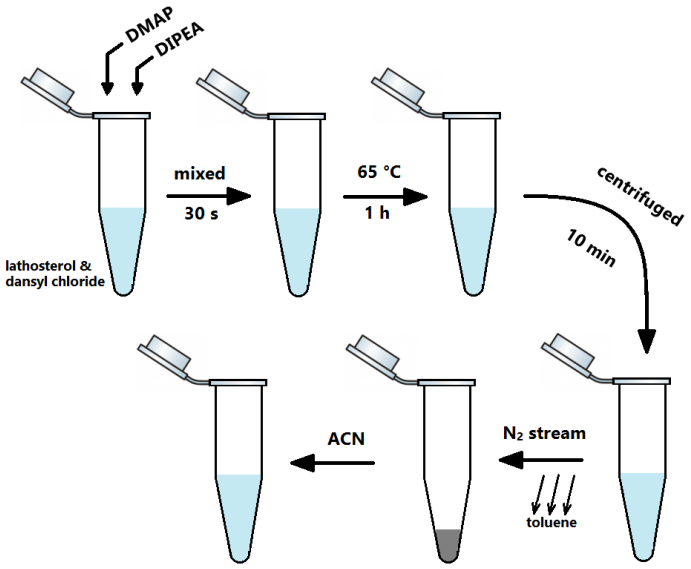

Figure 15. An overview of the derivatization procedure of lathosterol with dansyl chloride.

4.2 Measurements with LC-UV and LC-UV-Fluorescence

The derivatives were measured using different liquid chromatography (LC) systems. All the systems used a UV detector and one system also comprised a fluorescence (FL) detector. The measurements during the development of the derivatization procedures were first performed with a UV detector, carried out by students under supervision of the author. After the development with LC-UV, the method was applied to the LC-UV-FL system. If the method was not fit for the LC-UV-FL system, the method was adjusted. If this is the case, any adjustments made will be indicated as such and described. The derivatives were diluted for the measurements with a fluorescence detector, because of the high sensitivity.

All measurements were performed with gradient elution using two different mobile phases. The mobile phases for the LC-UV and LC-UV-FL system were identical. Mobile phase A consisted of 0.1% formic acid (FA), 1% methanol (MeOH) and 98.9% water. Mobile phase B

consisted of 0.1 % FA, 1% water and 98.9% MeOH. The acquisition rate was 1 data point per second.

The first measurement of every procedure started with a gradient of 10% B to 90% B in 10 minutes. The concentration of B was kept at 90% for 40 minutes. The concentration was lowered to 10% B in 10 minutes and the column was reconditioned for several minutes. Subsequently, the gradient was adjusted to achieve optimal separation. Step gradient elution was used, since relatively polar and nonpolar compounds had to be separated. The final methods are discussed below. Optimization was performed by adjusting gradient, flow rate and wavelength selection for UV and FL.

4.2.1 Separation and detection of derivatization products with naproxen chloride

The separation was performed using a gradient of 60% B to 80% B in 2 minutes. The concentration was raised to 95% B in 13 minutes and kept at 95% for 10 minutes. Subsequently, the concentration was lowered to 60% B in 1 minute. The column had a reconditioning time of several minutes. The flow rate was 0.15 ml/min.

The wavelength of the UV detector was set to 254 nm, measured with system A.1. The fluorescence detector was set to an excitation wavelength of 230 nm and an emission wavelength of 350 nm, measured with system B.

4.2.2 Separation and detection of derivatization products with 3,5-dinitrobenzoyl chloride

The gradient elution started with 60% of mobile phase B. The concentration was raised to 98% B in 5 minutes. After 25 minutes at 98% B, the concentration was lowered to 60% B in 1 minute. The column had a reconditioning time of several minutes. The flow rate was 0.2 ml/min.

For the detection with UV on system A.2, the used wavelength was 230 nm. Measurements using fluorescence detection were not performed.

4.2.3 Separation and detection of derivatization products with dansyl chloride

Separation was performed with a gradient starting at a mobile phase B concentration of 70%. After 1 minute at 70%, the concentration was raised to 95% B in 4 minutes and kept at 95% for 20 minutes. The concentration was lowered to 70% B in 1 minute and the column was reconditioned of several minutes. The flow rate was 0.24 ml/min.

The used wavelength for the UV detection was 330 nm, measured with system A.3.

The measurements with the fluorescence detector were executed with a different gradient. The gradient elution started at 70% B for 1 minute and were raised to 95% B in 4 minutes. After 15 minutes at 95% B, the gradient was lowered to 70% B in 1 minute. The column had a reconditioning time of several minutes. The flow rate was 0.15 ml/min.

The fluorescence detection was performed with an excitation wavelength of 335 nm and an emission wavelength of 500 nm, measured with system B.

4.3 Measurements with LC-MS/MS

The identification of the derivatives was performed using LC-MS/MS. The measurements were performed after the method development with LC-UV-FL.

The LC-MS/MS system (system C) consisted of a C_{18} column, an ionization source and a triple quadrupole mass spectrometer.

The ionization was performed with electrospray ionization (ESI). The following settings were used for the ESI. The capillary voltage was -3000 V, the fragmentor voltage was 200 V and the skimmer voltage was 70 V. The drying gas temperature was set on 300 °C.

The acquisition rate of the MS/MS was set on 1.0 spectra/s. The selected mass range for the total ion chromatogram (TIC) was 100-700 m/z. The settings for the extracted ion chromatograms (XIC) were varied throughout the derivatives of the different derivatizations.

Separation during the LC-MS/MS measurements was performed using mobile phases A; 0.1% FA in water and B; 0.1% FA in 80% ACN. The used flow rate was 0.25 ml/min. The separations on the C_{18} column were performed using a gradient. Different gradients were applied for every derivatization procedure. The different gradients will be discussed further in the next three chapters.

4.3.1 Separation for identification of derivatization products with naproxen chloride

The separation was performed with a gradient of 60% B to 80% B in 2 minutes. Thereafter the concentration was raised to 95% B in 13 minutes and kept at 95% for 10 minutes. The concentration was lowered to 60% B in 1 minute. The column had a reconditioning time of several minutes.

4.3.2 Separation for identification of derivatization products with 3,5-dinitrobenzoyl chloride

The gradient elution started with 60% of mobile phase B. The concentration was raised to 98% B in 5 minutes. After 25 minutes at 98% B, the concentration was lowered to 60% B in 1 minute. The column had a reconditioning time of several minutes.

4.3.3 Separation for identification of derivatization products with dansyl chloride

The gradient elution started at 70% B for 1 minute, and was raised to 95% B in 4 minutes. After 15 minutes at 95% B, the concentration was lowered 70% B in 1 minute. The column had a reconditioning time of several minutes.

5. RESULTS AND DISCUSSION

The aim of this study was to develop a derivatization procedure for the quantitative determination of vitamin D_3 for clinical use. In this study, three different derivatization procedures were tested.

The three different derivatization procedures were performed with three different derivatization agents. The used derivatization agents were naproxen chloride (Nap-Cl), 3,5-dinitrobenzoyl chloride (DNBC) and dansyl chloride (Dns-Cl). Aside from the main target compound, vitamin D_3, four different compounds were used in order to facilitate the practical implementation and to broaden the practical applicability. The compounds used for derivatization were cholesterol, lathosterol, vitamin D_2 and, naturally, vitamin D_3.

The first tested derivatization procedure used the derivatization agent naproxen chloride. In the second and third method, 3,5-dinitrobenzoyl chloride and dansyl chloride were used respectively. These three derivatization agents, and methods, were selected because of the following characteristics. Firstly, all three derivatization agents have a fluorescent group and have a typical fragmentation pattern (facilitating identification steps using MS). Also, two of the derivatization agents are acyl chlorides and one is a sulfonyl chloride, species which are both known for their high reactivity. Lastly, the availability of all three compounds played a role in the selection process.

The derivatives were separated using reversed-phase liquid chromatography (LC) and detected with an ultraviolet detector (UV) and a fluorescence detector (FL).

After testing a derivatization procedure, a triple quadrupole mass spectrometer was used for analysis of the UV and FL chromatograms. LC-MS/MS was used for the identification of the preferred formed compound/product in the derivative. When the preferred compound was not present after derivatization, a new derivatization procedure, using a different derivatization agent, was developed.

5.1 Derivatization with naproxen chloride

The first method tested involved the derivatization of cholesterol using naproxen chloride (Nap-Cl). A mixture of cholesterol and naproxen chloride was incubated. After a given reaction time, diethylamine was added to the mixture, to inactivate excess naproxen chloride, after which the solution was dried under a nitrogen stream. The formed residue was dissolved in acetonitrile.

5.1.1 LC-UV measurements

First, the derivatives were measured using LC-UV. In figure 16 the chromatogram of the final derivatization method is presented. Various initial concentrations of cholesterol were used for the derivatization.

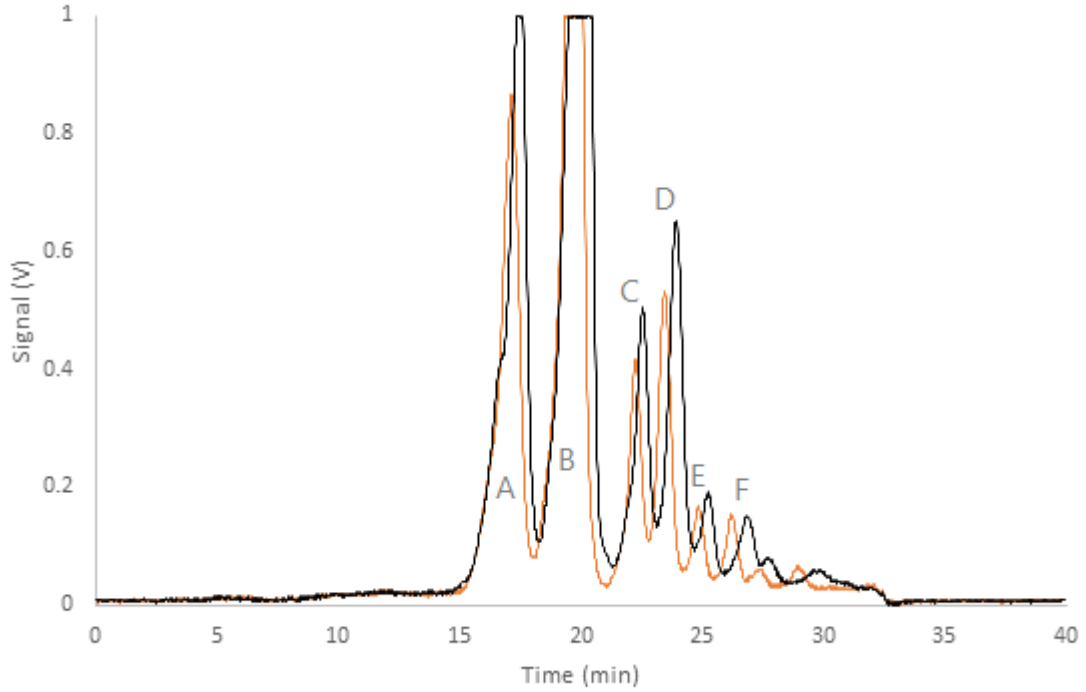

Figure 16. UV chromatograms of the derivatives of cholesterol with naproxen chloride with the signal (V) set out against time (min). — : 10 mM cholesterol; — : 20 mM cholesterol. (measured with system A.1)

In the above presented chromatogram, six peaks can be observed, labeled A through F. A difference in cholesterol concentration did not alter the number of peaks or the peak pattern shown in the UV chromatograms. Observable peaks would be expected of naproxen chloride, cholesterol and possibly a derivative (in that retention order).

The peak area of peak A (17.5 minutes) increased with increasing cholesterol concentrations. However, it seems highly unlikely that this peak was caused by cholesterol, since cholesterol has virtually no absorption at 254 nm. Furthermore, the retention time does not correspond with the retention time expected for a compound as cholesterol.

Throughout the measurements, the initial naproxen chloride concentration was relatively high, and unaltered. However, the cholesterol concentrations varied, resulting in more, or less, excess of naproxen chloride throughout the measurements. The decrease of excess naproxen chloride in measurements with higher concentrations of cholesterol should logically result in a decrease of the area of the peak caused by leftover naproxen chloride. Peak B (at approximately 20 minutes) shows this decrease, indicating this is likely the peak caused by naproxen chloride.

Four peaks were observed with a longer retention than peak B. These different peaks had retention times of approximately 22.5, 23.8, 25.1 and 26.8 minutes. All four peaks also increased in peak area with increasing cholesterol concentration. It might be expected that one of the four peaks represents cholesterol. However, a measurement of a standard solution of cholesterol (in THF; 50 mM) did not show any peaks with a resemblance to any peak in figure

16. This excludes the possibility that one of the unidentified peaks represents cholesterol. The chromatogram of the standard solution of cholesterol is shown in appendix 3.1.

After derivatization, the mixture was dried under a nitrogen stream, so virtually all toluene and remaining diethylamine should be evaporated. Also toluene and diethylamine cannot be detected at this wavelength. So none of the four peaks can confidently be ascribed to any remaining solvent.

Variations in derivatization times (from 0 to 48 h) and temperatures (60 to 80 ˚C) did not make a significant difference in the chromatograms. The measurements were the same and the peak areas did not increase or decrease, nor did peaks occur or disappear.

The four remaining peaks could not be identified based solely on the UV chromatograms of the initial compounds. It is possible that the remaining peaks represent formed compounds of the derivatization.

Final identification of the peaks in figure 16 had to be achieved with the help of LC-FL and LC-MS/MS. Since sterol concentrations in clinical samples fall far below the detection threshold of an average UV detector, development of an LC-FL detection method was already required. Therefore, an LC-FL method was developed prior to an LC-MS/MS method.

5.1.2 LC-Fluorescence measurements

An LC-UV-FL system was used during the developmental process of the LC-FL method, in order to facilitate identification of any peaks in the FL spectrum.

The enhanced sensitivity of the fluorescence detector is demonstrated in the comparison of UV and FL chromatograms of the same measurements. The UV chromatograms of the measurements gave merely a baseline signal, where the FL chromatograms gave two major, off scale peaks. The UV and FL chromatogram of the measurement of the derivatization with 10 mM cholesterol are shown in appendix 3.2.

The fluorescence chromatograms of the measurements of the derivatives of cholesterol (2 mM and 10 mM) with naproxen chloride are shown in figure 17. Changes made in the temperature and the derivatization time did not make a significant difference in the chromatogram.

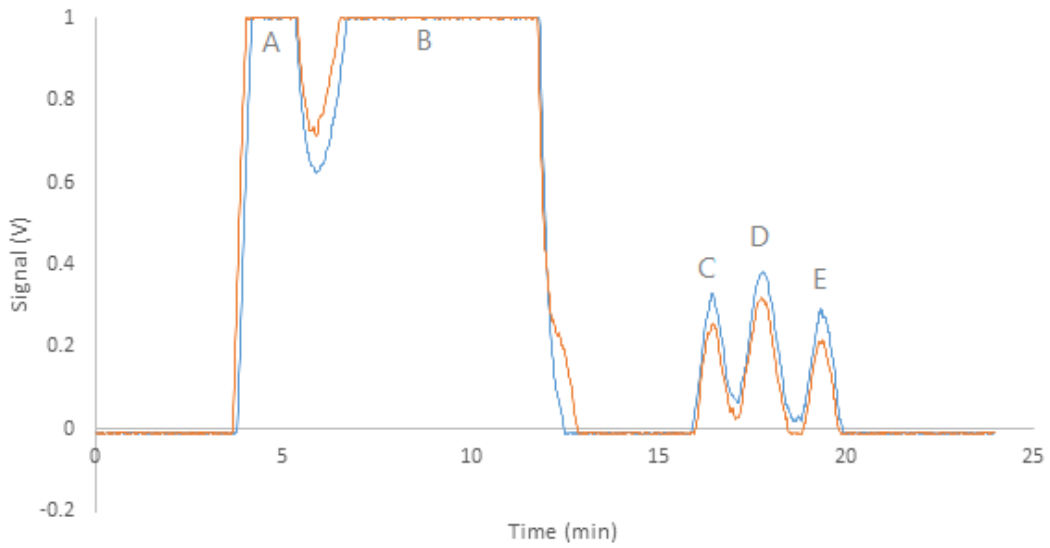

Figure 17. FL chromatograms of the derivatives of cholesterol with naproxen chloride with the signal (V) set out against time (min). — : 2 mM cholesterol; — : 10 mM cholesterol. (measured with system B)

Five peaks can be distinguished in the FL chromatograms, where the UV chromatograms (with the previous LC-UV system) showed an overlapping peak pattern of six peaks. This means that one non-fluorescent compound was observed with LC-UV.

In the fluorescence chromatogram, peak A and B, with estimated retention times of 4.8 and 8.9 minutes respectively, do not display baseline separation and are off scale. Peak B indicates the presence of native naproxen chloride, confirmed by previous UV measurements. The relatively high concentration of naproxen chloride causes the signal of the peak to go off scale. Peak A indicates another compound in similarly high concentration. A possible explanation could be a reaction product of naproxen chloride and diethylamine, which would be expected to have a lower retention time than native naproxen chloride.

Three additional peaks, with retention times of approximately 16.5 (C), 17.9 (D) and 19.5 (E) minutes, can be observed in the FL chromatogram. The presence of multiple peaks in the fluorescence chromatogram indicate the derivatization of a compound, since prior to reaction the only fluorescent compound in the mixture is naproxen chloride. Possibly, a reaction product of Nap-Cl and diethylamine (peak A) is present in the derivative, since diethylamine is added to inactivate Nap-Cl. However, five peaks were observed and, even when assuming a reaction product between Nap-Cl and diethylamine is formed, only two compounds were expected to be fluorescent, excluding the preferred derivative. From these results, it seemed likely that cholesterol was successfully derivatized by Nap-Cl.

The other detected compounds could not be confidently identified based on ultraviolet and fluorescence detection. It might be expected that the unidentified peaks were derivatives of the reaction. Another possible explanation might be found in the high reactivity of Nap-Cl. This high reactivity may have resulted in reaction with any impurity in the mixture, or deactivation by reacting with water. Since the peak area of the three unidentified peaks did not increase as

much as expected with increasing cholesterol concentrations, the latter explanation seemed slightly more probable. Further examination with LC-MS/MS was necessary to identify the formed compounds.

5.1.3 LC-MS/MS measurements

An electrospray triple quadrupole mass spectrometer was used for the identification of the derivatives. The total ion chromatogram (TIC) was recorded for a mass to charge ratio (m/z) range of 100 to 700. The total ion chromatogram of the derivative is shown in appendix 3.3. A TIC is a total sum of intensities of all signals (peaks), including background noise.

The extracted ion chromatograms (XIC) are shown in figure 18. An XIC is a chromatogram of only a single mass selected. The extracted ion chromatograms were obtained for the predicted masses of the cholesterol, Nap-Cl and the preferred derivative, which were m/z 386.7, m/z 248.7, and m/z 598.9 respectively (all with a deviation of m/z ± 0.5).

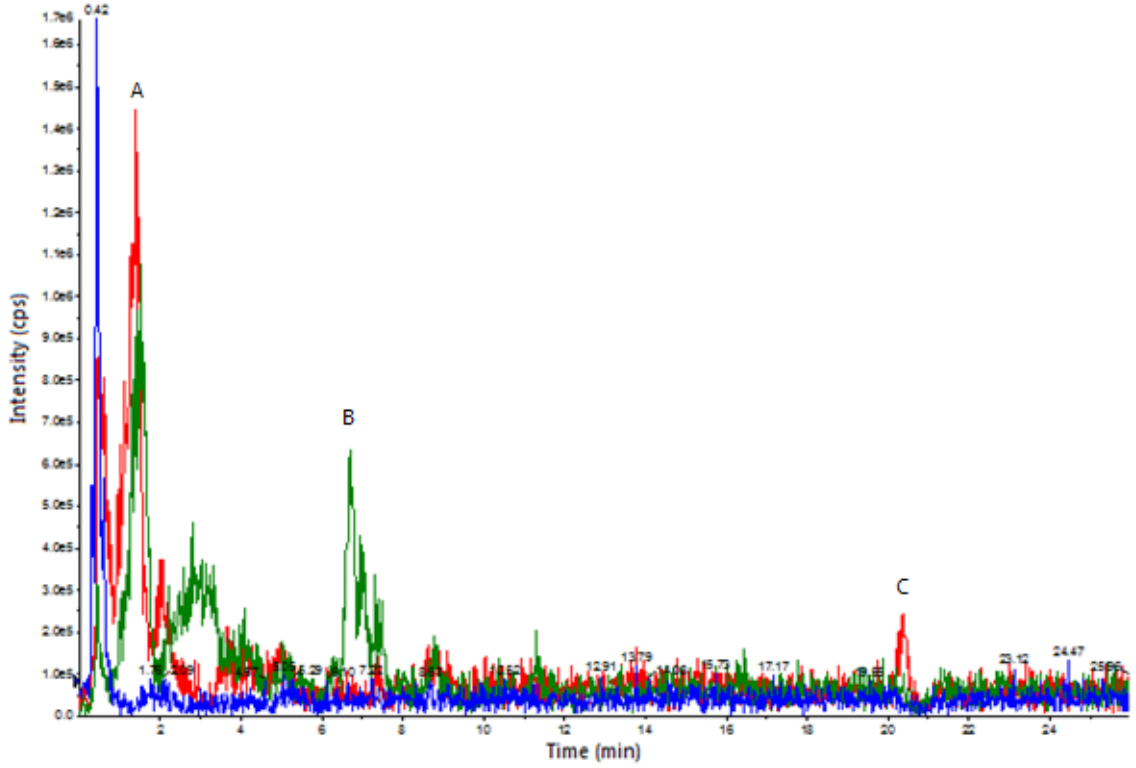

Figure 18. XIC of derivative of 10 mM cholesterol with 50 mM naproxen chloride with intensity (cps) set out against time (min). Three different masses were selected; — : preferred derivative; — : naproxen chloride; — : cholesterol. (measured with system C)

Four signals can be observed in the extracted ion chromatograms of the measurement of the cholesterol derivative (figure 18). The signal at 0.42 minutes (blue trace), was the only signal observed for the m/z of the preferred derivative. This seemed to be the injection peak, since the preferred derivative is not expected to elute so shortly after injection. For the other three peaks that can be observed, at approximately 1.2 (A), 6.7 (B) and 20.3 (C) minutes, the mass spectra were obtained.

Multiple fragments are observed in all three spectra. Changes made in derivatization time and temperature did not make a significant difference in the results of the measurements. Furthermore, variation in the concentration of cholesterol yield significantly different results. The outcome of the interpretation of the spectra is presented in table 1. Not all the fragments could readily be interpreted, all non-interpretable peaks are omitted from table 1.

Table 1. The interpretation of the spectra, obtained from XIC (figure 18), of peaks A, B and C.

Spectrum peak A		**Spectrum peak B**		**Spectrum peak C**	
m/z	**Description**	**m/z**	**Description**	**m/z**	**Description**
153.3	Fragment Nap-Cl	170.0	Fragment Nap-Cl	185.0	Fragment Nap-Cl
170.0	Fragment Nap-Cl	185.0	Fragment Nap-Cl	286.4	Product of Nap-Cl and diethylamine $[M+H]^+$
185.1	Fragment Nap-Cl	286.1	Product of Nap-Cl and diethylamine $[M+H]^+$	369.5	Cholesterol without hydroxyl group $[M-CH_3]^+$
257.4	Rings cholesterol	308.2	Product of Nap-Cl and diethylamine with Na $[M+Na]^+$	385.4	Cholesterol M^+
371.4	Cholesterol without methyl $[M-CH_3]^+$	599.4	Possible derivative of cholesterol and Nap-Cl		
385.4	Cholesterol (minor)				

Some of the observed fragments in the spectra (with m/z 104.0; 132.0; 149.1) are not shown in the table above. These fragments are most likely caused by a contamination of phthalates in the measurement. The contamination of phthalates was observed in every measurement. A list of phthalates and their fragments, including corresponding m/z values, is shown in appendix 6.

The spectrum of peak A is shown in figure 19 and the spectrum of peak C is shown in figure 20. The spectra of A and C show a peak at 385.4, indicating that cholesterol is still present in the sample. Peaks at m/z values of 369.5 and 257.4 indicate the presence of cholesterol lacking a hydroxyl group and an alkyl branch respectively.

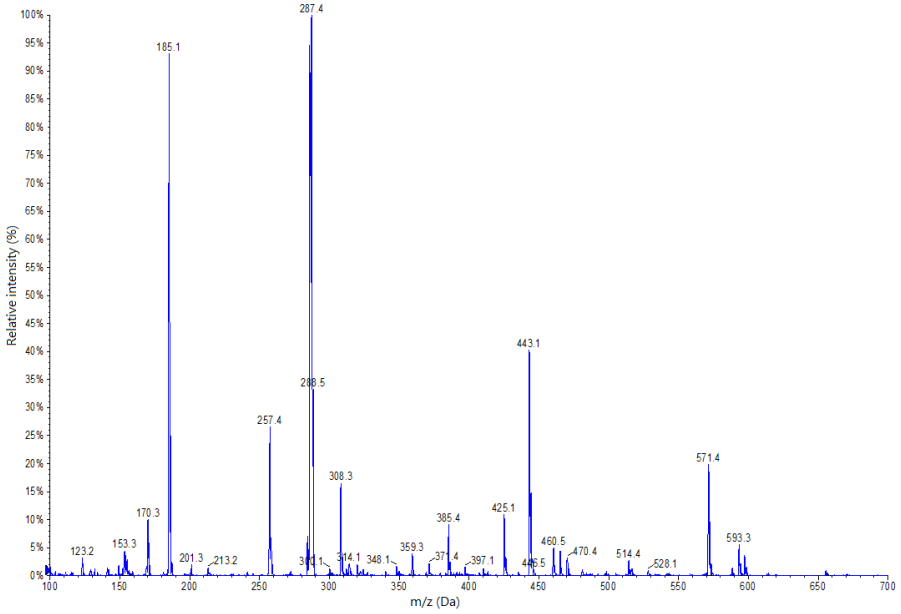

Figure 19. Mass spectrum of peak A of the XIC derivatization of cholesterol with Nap-Cl at 1.2 min, with relative intensity (%) against m/z (Da). (measured with system C)

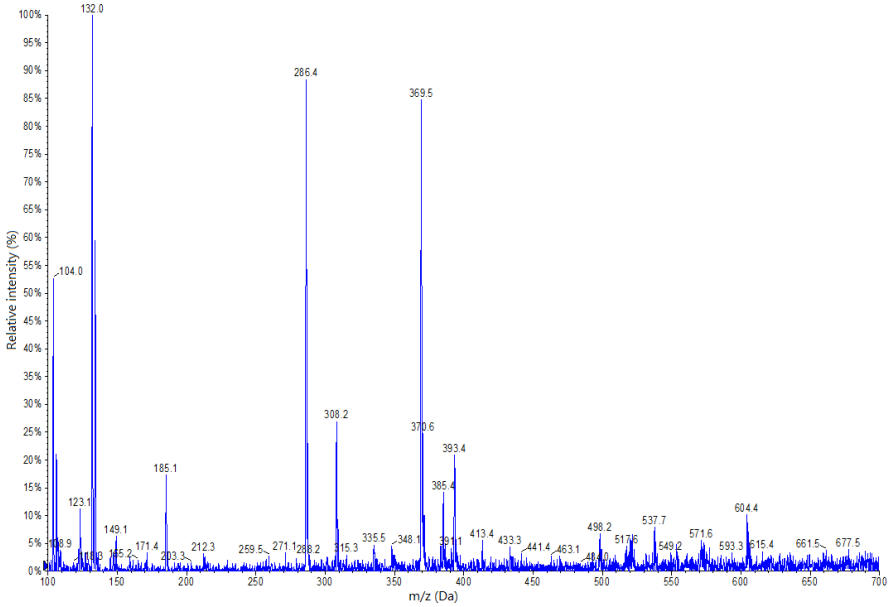

Figure 20. Mass spectrum of peak C of the XIC derivatization of cholesterol with Nap-Cl at 20.3 min, with relative intensity (%) against m/z (Da). (measured with system C)

In both spectra, multiple peaks can be ascribed to fragments of Nap-Cl. However, the spectrum of peak C indicates the presence of a reaction product of Nap-Cl and diethylamine. This renders it impossible to determine whether Nap-Cl or this reaction product cause the aforementioned peaks (A and C). No fragments in both spectra indicated the presence of preferred derivative.

The spectrum of peak B is shown in figure 21. The spectrum of peak B shows a small peak with m/z 599.4, the expected value of the preferred derivative. The peak, however, is very low in intensity and a fragmentation pattern, or additional fragments of the formed derivative, could not be identified. Therefore, the amount of evidence that the preferred derivative was formed can be considered insufficient.

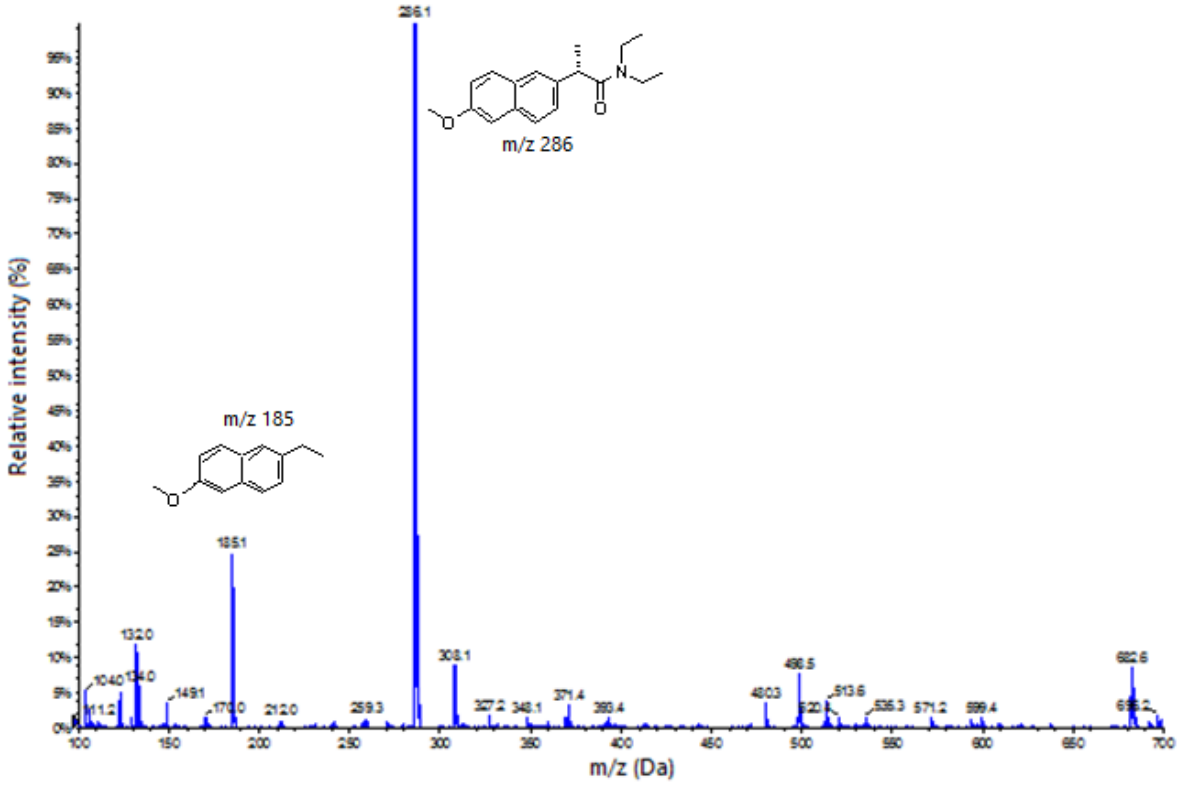

Figure 21. Spectrum of peak B at 6.7 minutes with the relative intensity (%) plotted against m/z (Da). (measured with system C)

Fragments of native Nap-Cl and fragments of a reaction product of Nap-Cl and diethylamine were also observed in the spectrum of peak B. However, it is impossible to determine whether the fragments of native Nap-Cl belong to the reaction product or that native Nap-Cl is still present.

All three spectra indicated the presence of a reaction product of Nap-Cl and diethylamine. The preferred formed compound could not be identified via LC-MS/MS. A small fragment, with the same m/z value expected for the preferred derivative, was observed, yet there was no indication of any additional fragments. A possible explanation for the seemingly unsuccessful derivatization might be found simply in the amount of excess Nap-Cl. It is possible that a higher Nap-Cl : cholesterol ratio would yield better results.

Based on the results presented above, Nap-Cl was eliminated as a viable option for the derivatization of sterols. Further examination of Nap-Cl as a derivatization agent might yield more/different results.

5.2 Derivatization with 3,5-dinitrobenzoyl chloride

The second tested derivatization agent was 3,5-dinitrobenzoyl chloride (DNBC). Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) were derivatized using 3,5-dinitrobenzoyl chloride. Pyridine was added to the mixture to enhance the reaction yield. The solutions of the vitamins contained chloroform and the derivatives were dissolved in tetrahydrofuran (THF).

5.2.1 LC-UV measurements

The first measurements of the development of the derivatization were performed using LC-UV. In figure 22 three UV chromatograms are presented. The difference between the chromatograms was the derivatization time. All derivatizations were performed with vitamin D_3 (D3) using DNBC.

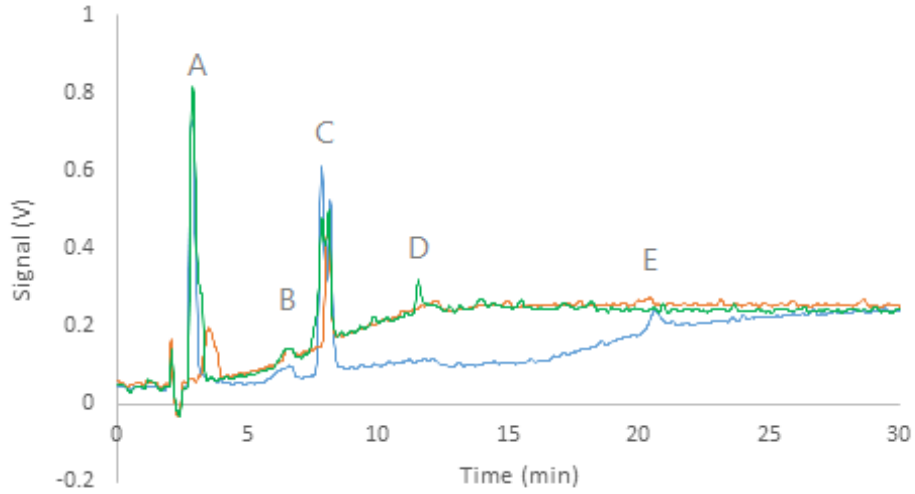

Figure 22. UV chromatograms of the derivatives of vitamin D_3 with DNBC with the signal (V) set out against time (min). Different derivatization times were applied; — : 0 h; — : 2 h; — : 24 h. (measured with system A.2)

Multiple peaks are observed in the chromatograms. Most of the peaks are present in all three chromatograms. The first peak at 2.1 minutes was the injection peak. Peak A and B, at approximately 2.9 and 6.8 minutes, are most likely caused by solvents. A solution of chloroform (5%, v/v) in THF, and standard solutions of vitamin D_3 and DNBC was measured. The chromatograms are presented in figures 46 and 47 in appendix 4.1. Peaks with the same retention time as peak A and B were retrieved in these measurements, eliminating those peaks as possible reaction products.

Peak C, at 8.1 minutes, represents 3,5-dinitrobenzoyl chloride. This can be derived from the measurements of the standard solution of DNBC. The chromatogram of DNBC is shown in figure 46 in appendix 4.1.

A standard solution of vitamin D_3 was also measured and is presented in figure 46 in appendix 4.1. Vitamin D_3 was detected with a retention time of 20.8 minutes and corresponds to peak E in figure 22. Although vitamin D_3 was detected, the signal was very low. A longer derivatization

time (>5 h), resulted in the decrease of area of peak E. After 24 hours peak E was completely reduced in area and peak D, at 11.6 minutes, was observed. Two possible explanations for this observation come to mind. The first option is that vitamin D_3 reacted with the derivatization agent after a longer period of time and a formed compound was detected. Another option is that degradation of the vitamin occurred. Thermal degradation of D3 can occur at higher temperatures, and longer exposure to a high temperature has been shown to stimulate this degradation [27-29].

Different derivatization temperatures were tested, ranging from 50 to 80 °C. There was no difference observed between the chromatograms of the procedures differing only in derivatization temperatures. Adjusting the derivatization time, did however have an effect on the measurements. After a longer period of time the vitamin peak was reduced in area and eventually no longer observed (24 h). In its place, a peak at 11.6 minutes was observed.

To determine whether the new observed peak was a reaction or degradation product, further examination was necessary. Additional measurements had to be performed with mass spectrometry for identification purposes. This was performed prior to the development of an LC-FL method since the LC-UV chromatogram showed only one peak decreasing and one peak increasing, indicating degradation rather than derivatization.

5.2.2 LC-MS/MS measurements

After the development with LC-UV, the derivatives were measured with LC-MS/MS to identify the formed compounds.

A derivatization (3 h) with vitamin D_2 (D2) using DNBC was performed. The total ion chromatogram (TIC) is shown in figure 48 in appendix 4.2. The extracted ion chromatograms (XIC) of this measurement are shown in figure 23. Three masses were selected for the XIC. Firstly, m/z 230.5 was selected for DNBC. For vitamin D_2, m/z 398.5 was selected and m/z 590.7 was selected for the preferred derivative (all had a deviation of m/z ± 0.5).

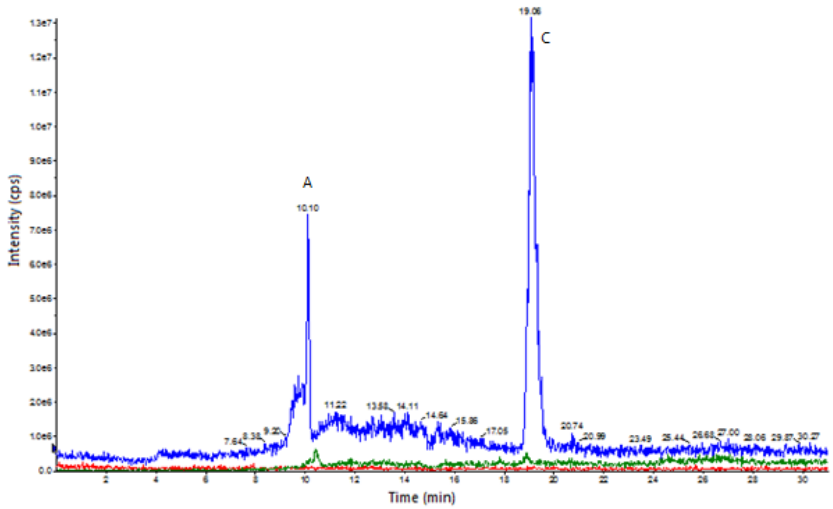

Figure 23. XIC of derivative of vitamin D_2 with DNBC with intensity (cps) set out against time (min). Three different masses were selected; — : vitamin D_2; — : DNBC; — : preferred derivative. (measured with system C)

In the XIC, two peaks at 10.5 and 19.1 minutes (peak A and C) can be observed. In the TIC a major peak at 15.2 minutes (peak B) was observed. Spectra of the three peaks were obtained. The interpretation of the spectra of peak A and C are presented in table 2.

Table 2. The interpretation of the spectra, obtained from XIC (figure 23), of peak A and C.

Spectrum peak A		**Spectrum peak C**	
m/z	**Description**	**m/z**	**Description**
212.0	Degradation product D2	253.2	Fragment D2
286.1	Fragment D2	271.5	Fragment D2 (D2 without alkyl branch)
		286.1	Fragment D2
		309.2	Fragment D2
		327.3	Fragment D2
		379.4	Fragment D2 $[M\text{-}OH]^+$
		397.2	D2 $[M+H]^+$

The spectrum of peak B is shown in figure 49 in appendix 4.2. The interpretation of peak B was not included in table 2 above. The observed fragments (with m/z 104.0; 113.0; 132.0; 149.1; 261.4; 279.0 391.2; 413.3; 429.4) are a result of contamination. These fragments are caused by different sorts of phthalates. Fragments of phthalates were observed in every measurement. A list of phthalates and their fragments, including corresponding m/z values, is shown in appendix 6. Other excluded fragments could not be identified.

The spectrum of peak A is presented in figure 24. Peak A also contained fragments of phthalates, but the base peak (m/z 212) did not represent any phthalate contamination. The retention time of peak A seemed to correspond to peak D of the LC-UV measurements. It was expected to be either derivative or a degradation product. The observed peak with m/z 212.4 corresponds to a degradation product of vitamin D_2, namely Inhoffen-Lythgoe diol, a known degradation product of vitamin D [30, 31].

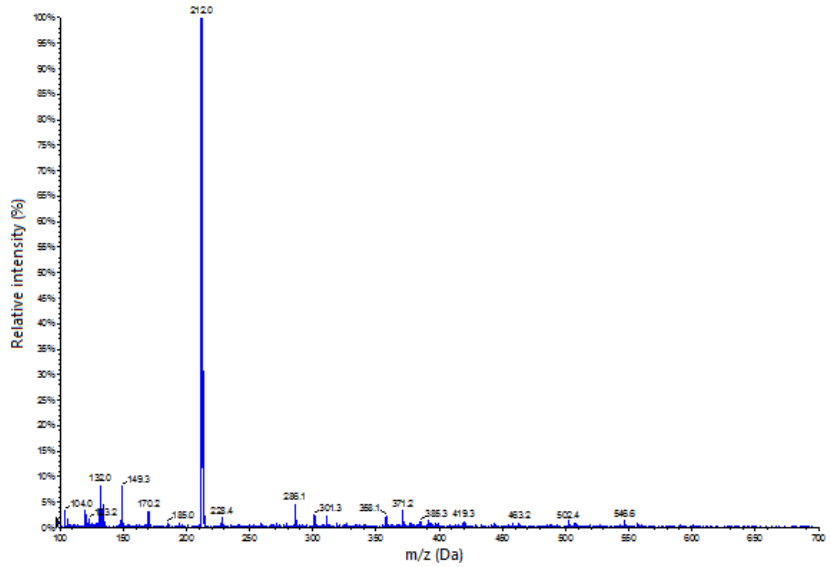

Figure 24. Mass spectrum of peak A of XIC derivatization of D_2 with DNBC at 10.5 min, with relative intensity (%) against m/z (Da). (measured with system C)

Fragments of vitamin D_2 were present in the spectrum of peak C. The spectrum of peak C is shown in figure 25. The molecular ion with hydrogen $[M+H]^+$ was also observed in the spectrum. The fragment of vitamin D_2 without the hydroxyl group (m/z 379.4) was observed and the fragment of vitamin D_2 without the alkyl branch (m/z 271.5). The retention time of vitamin D_2 in the XIC corresponds with the retention time found in the LC-UV measurements.

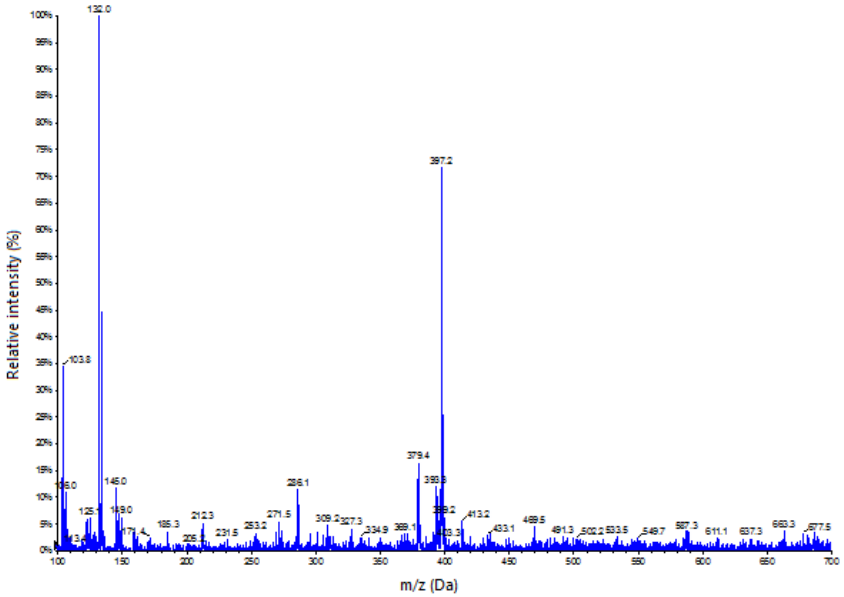

Figure 25. Mass spectrum of peak C of XIC derivatization of D_2 with DNBC at 19.1 min, with relative intensity (%) against m/z (Da). (measured with system C)

In none of the spectra (fragments of) DNBC were observed. One of the expected fragments would be DNBC without chloride $[M-Cl]^+$. However, this fragment and neither of the other (expected) fragments were observed. The lack of observation of DNBC (and fragments) could be due to measuring in the positive-ion mode. The aromatic nitro groups might be troublesome to fragment. Measuring in a negative-ion mode may be an option [13].

The molecular ion of the preferred derivative (m/z 590.7) was not observed. The same goes for typical fragments of the preferred derivative.

Another derivatization was performed, this time using vitamin D_3, and measured with LC-MS/MS. The total ion chromatogram is shown in figure 50 in appendix 4.2 and the extracted ion chromatograms are shown in figure 26. Three masses were selected for the XIC. Firstly, m/z 230.5 was selected for DNBC. For vitamin D_3, m/z 384.6 was selected and m/z 578.7 was selected for the preferred derivative (all with a deviation of m/z ± 0.5).

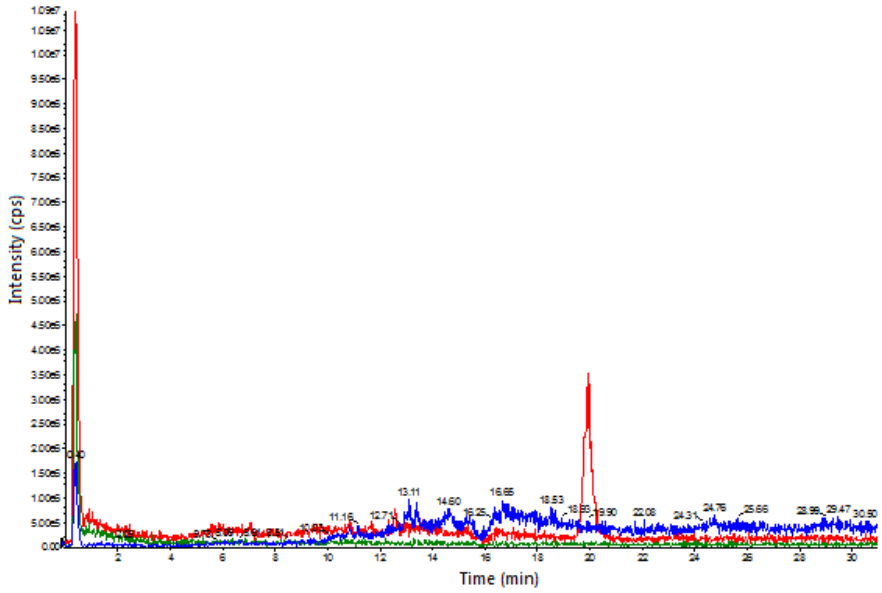

Figure 26. XIC of derivative of vitamin D_3 with DNBC with intensity (cps) set out against time (min). Three different masses were selected; — : DNBC; — : preferred derivative; — : vitamin D_3. (measured with system B)

The XIC of the measurement contained one obvious peak at 19.9 minutes. Four spectra were obtained from the XIC, at 11.2, 15.8, 19.9 and 25.7 minutes. The interpretation of three spectra (at 11.2, 19.9 and 25.7 minutes) is presented in table 3. Not all the fragments could readily be interpreted, all non-interpretable peaks are omitted from both tables.

Table 3. The interpretation of the spectra, obtained from XIC (figure 26), at 11.2, 19.9 and 25.7 minutes.

Spectrum 11.2 minutes		**Spectrum 19.9 minutes**		**Spectrum 25.7 minutes**	
m/z	**Description**	**m/z**	**Description**	**m/z**	**Description**
212.4	Degradation product D3	259.4	Fragment D3	286.1	Fragment D3
286.1	Fragment D3	286.1	Fragment D3	536.1	Unknown
		367.4	Fragment D3		
		385.5	D3 $[M+H]^+$		

As in the previous measurement, a high amount of phthalates fragments was observed. A list of identified fragments of phthalates is shown in appendix 6. The spectrum obtained at 15.8 minutes was excluded from table 3, since only fragments of phthalates were observed in the spectrum. This spectrum is presented in figure 51 in appendix 4.2. The XIC of the measurement also showed an increase in signal observed at 13.1 and 14.6 minutes. However, these spectra only contained phthalates fragments and were deemed of no significant value for this study.

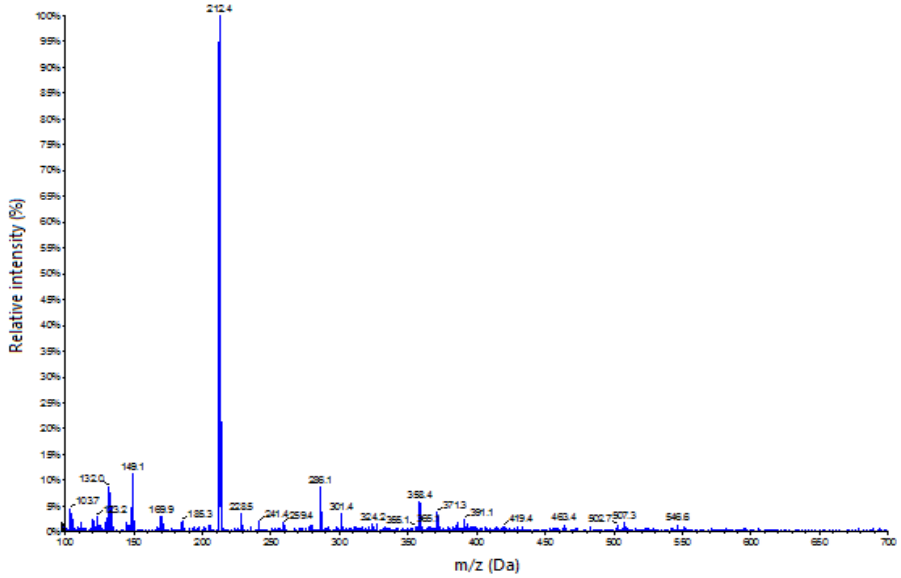

Figure 27. Mass spectrum of XIC derivatization of D_3 with DNBC at 11.2 min, with relative intensity (%) against m/z (Da). (measured with system C)

In the mass spectrum at 11.2 minutes, presented in figure 27, a degradation product of the vitamin (m/z 212.4) was observed, as in the previous measurement with vitamin D_2. Degradation of the vitamins may be due to a longer derivatization time at a higher temperature. Since degradation of vitamin D occurs at high temperatures, a temperature above 80 ˚C was not tested [27-29]. A longer derivatization time did have an increasing effect on the peak area of the degradation product, as seen with LC-UV measurements.

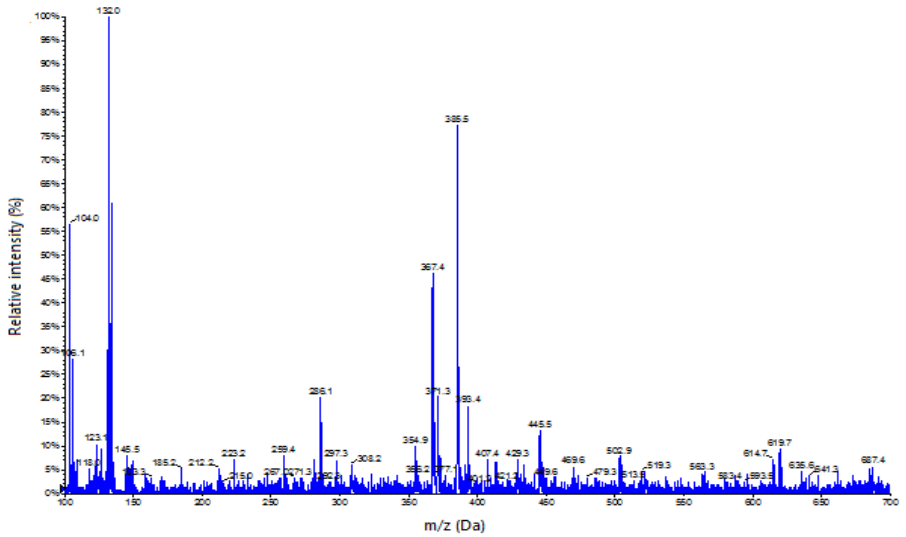

Figure 28. Mass spectrum of XIC derivatization of D_3 with DNBC at 19.9 min, with relative intensity (%) against m/z (Da). (measured with system C)

At 19.9 minutes a peak was observed in the XIC. Fragments of vitamin D_3 were observed in the spectrum, presented in figure 28. The molecular ion of vitamin D_3 with hydrogen $[M+H]^+$ (m/z 385.5) was also present. This confirms that this peak was caused by native vitamin D_3.

At 25.7 minutes in the XIC, a minor increase in signal was observed. The mass spectrum, presented in figure 29, did not yield results. The only interpreted fragments were of vitamin D_3 (m/z 286.1) and phthalates. The base peak in the spectrum was caused by phthalates, but also a peak with m/z 536.1 was observed. The cause of this peak could not be determined. However, m/z value did not correspond to fragments of the preferred derivative or the molecular ion of the preferred derivative (m/z 578.7), so no further research was performed to identify its cause.

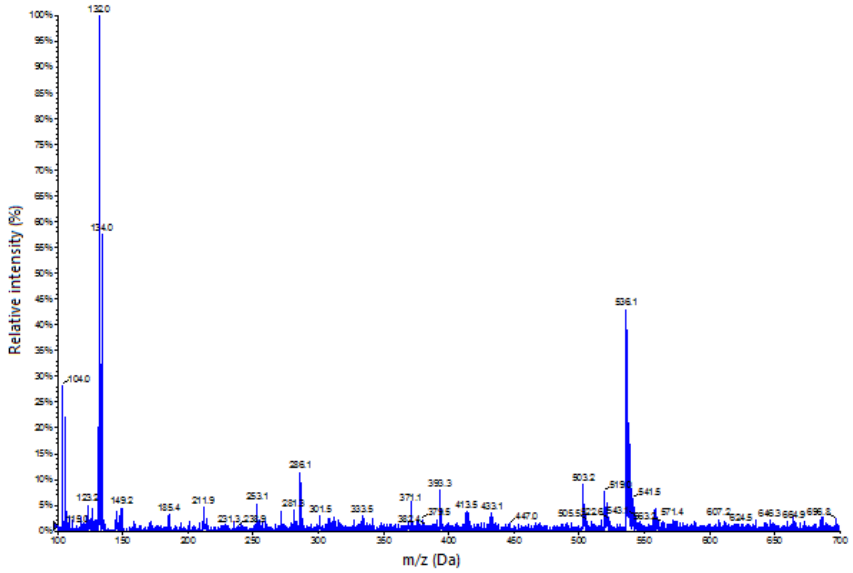

Figure 29. Mass spectrum of XIC derivatization of D_3 with DNBC at 25.7 min, with relative intensity (%) against m/z (Da). (measured with system C)

In the LC-MS/MS measurements no molecular ion or typical fragments of the preferred derivative were present. Only fragments of the vitamins and degradation products of the vitamins were identified. Also, no fragments and/or molecular ion of 3,5-dinitrobenzoyl chloride were observed. This can be caused by measuring in the positive-ion mode. The aromatic nitro groups might be troublesome to fragment. Measuring in a negative-ion mode may be an option [13].

Based on the results of LC-MS/MS, 3,5-dinitrobenzoyl chloride was set aside as derivatization agent, since degradation seemed to occur instead of derivatization. Further research might yield better results. By example, there is a possibility that a higher DNBC : vitamin ratio yield better results.

5.3 Derivatization with dansyl chloride

The final method tested, was a derivatization of lathosterol using dansyl chloride (Dns-Cl). Two compounds were added to enhance the reaction yield. These compounds were 4-dimethylaminopyridine (DMAP) and N,N-diisopropyethylamine (DIPEA). After a given reaction time, the mixture was centrifuged and dried under a nitrogen stream. The residue was dissolved in acetonitrile.

5.3.1 LC-UV measurements

All the measurements were performed at a wavelength of 330 nm, since dansyl chloride has a maximum absorption at this wavelength [32, 33]. In figure 53 in appendix 5.1 two chromatograms are shown of a measurement of a standard solution of lathosterol measured at 254 and 330 nm. Lathosterol was no longer observed at a wavelength of 330 nm.

An additional benefit of using a wavelength of 330 nm is a lower background noise. Moreover, DMAP does not absorb at 330 nm [34]. A measurement of DIPEA in THF was performed (see appendix 5.1 figure 52 and showed no signal other than the baseline at 330 nm.

Figure 30 shows two chromatograms of the derivative. The difference between the measurements was derivatization time and the gradient used during elution.

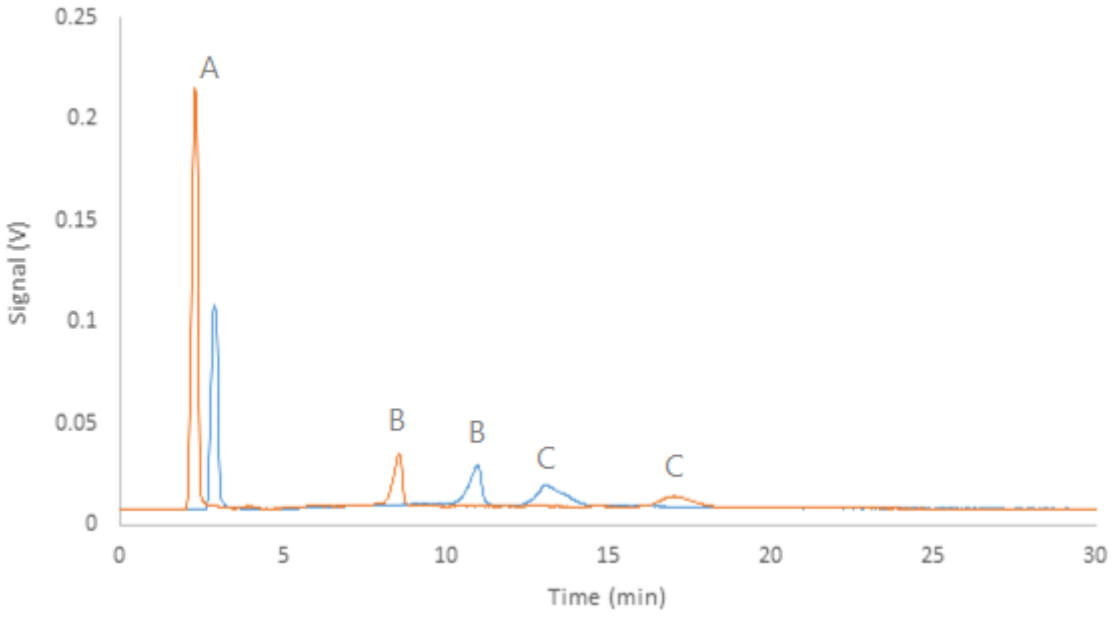

Figure 30. UV chromatograms of the derivatives of lathosterol with dansyl chloride with the signal (V) set out against time (min), with different derivatization times; — : 1 h; — : 3 h. (measured with system A.3)

Peak A was most likely the formed intermediate between DMAP and dansyl chloride. This can be derived from the measurements of the derivative without DMAP. The formed intermediate is an N-acylpyridinium intermediate [16]. Two measurements were performed to give an indication of how essential the use of DIPEA and DMAP was. One measurement was without DIPEA and the other without DMAP. The chromatograms are presented in figure 31. Without

DMAP no N-acylpyridinium intermediate is formed, which can be concluded from the lack of signal at approximately 2.7 minutes.

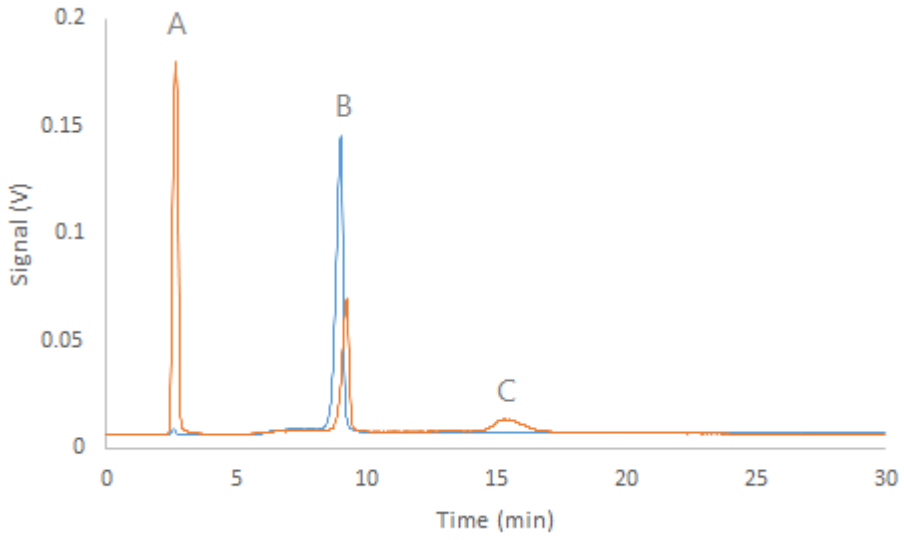

Figure 31. UV chromatograms of two derivatives, one with DMAP and the other with DIPEA, with signal (V) set out against time (min). — : with DIPEA; — : with DMAP. (measured with system A.3)

Peak B (in figures 30 and 31) most likely represents dansyl chloride. In figure 53 in appendix 5.1 a chromatogram of the Dns-Cl standard is shown. This standard shows two peaks, one of which corresponds to peak B. The other observed peak in the Dns-Cl standard was not observed after derivatization. This can be due to the added base(s) for making chloride an efficient leaving group.

Peak C increased in area when the derivatization time was extended. The retention time does not overlap with one of the compounds that was present before derivatization. In figure 31, the measurement without DIPEA or DMAP, peak C is not observed. Also, a derivatization without both DMAP and DIPEA was executed. The chromatogram is shown in figure 54 appendix 5.1. Likewise, peak C was not observed in the chromatogram of the derivatization without DIPEA and DMAP. Derived from these results, it is likely to assume that peak C represent a derivative, which was only formed in the presence of both DIPEA and DMAP.

Different derivatization temperatures (from 60 to 80 ˚C) did not make a significant difference in the chromatograms. A longer derivatization time did, however, make a difference. Extending the derivatization time resulted in an increase in the area of peak C.

To confirm whether or not peak C in the UV chromatogram was a product of successful derivatization, development of an LC-FL method was required. This was performed prior to LC-MS/MS measurements to facilitate the selection process of the peaks of interest in the XIC spectrum.

5.3.2 LC-Fluorescence measurements

In the developmental process of the LC-FL method, an LC-UV-FL system was used in order to facilitate identification of any peaks in the FL spectrum.

First, dansyl chloride was measured separately to determine at what retention time it eluted. In figure 55 in appendix 5.2 the UV and FL chromatogram of the standard solution of dansyl chloride is shown. Because an LC-UV-FL system was used, both UV and FL chromatograms are shown. In comparison to ultraviolet, the fluorescence chromatogram gives a very low signal. Despite the high sensitivity of a fluorescence detector, the UV detector signal gives a higher signal. This can be explained by the fact that dansyl chloride is not fluorescent it its native form. When dansyl chloride reacts with a hydroxyl (or amine) group, the compound becomes fluorescent [33].

A derivatization of lathosterol using dansyl chloride was performed and UV and FL chromatograms were obtained. In figure 56 in appendix 5.2 both chromatograms are shown. A major difference between the two can be observed, mainly displaying the high sensitivity of the FL spectrometer and the necessity to dilute. Different derivatization temperatures (60 to 80 ˚C) did not make a significant difference in the chromatograms.

Two chromatograms of derivatives (1000 times diluted) are shown in figure 32. The derivatization time was varied from 1 till 24 h.

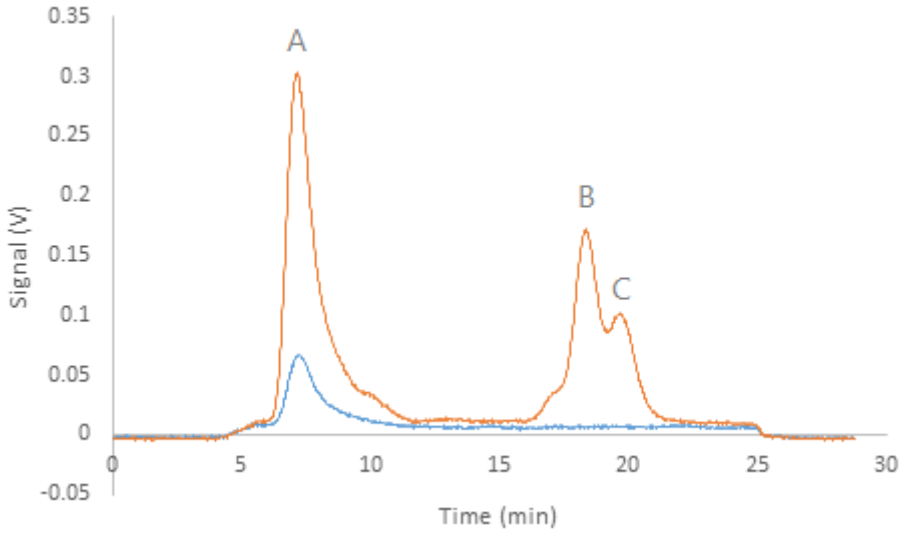

Figure 32. FL chromatograms of the derivatives of lathosterol with dansyl chloride (1000 times diluted), with different derivatization times, with the signal (V) set out against time (min). — : 1 h; — : 24 h. (measured with system B)

The chromatogram measured after one-hour derivatization time showed only one peak, but the after 24 hours of derivatization chromatograms showed multiple signals. Peak B and C are observed, but they did not display baseline separation.

Peak A might indicate the presence of the N-acylpyridinium intermediate. This corresponds to the measurements performed with LC-UV. The derivatization time had influence on the area of this peak, as is expected for concentration of intermediate.

Peak B and C were most likely formed derivative. After a longer derivatization time, both peaks were observed and the retention times correspond to the observed peak with LC-UV. Dansyl chloride is only fluorescent when reacted with a compound and there are no other fluorescent compounds present in the measured derivatives.

As with LC-UV measurements, the contribution of DMAP and DIPEA was examined. Two derivatizations were performed, one with only DMAP and one with only DIPEA. The derivatives were diluted 100 times. The obtained chromatograms are shown in figure 33.

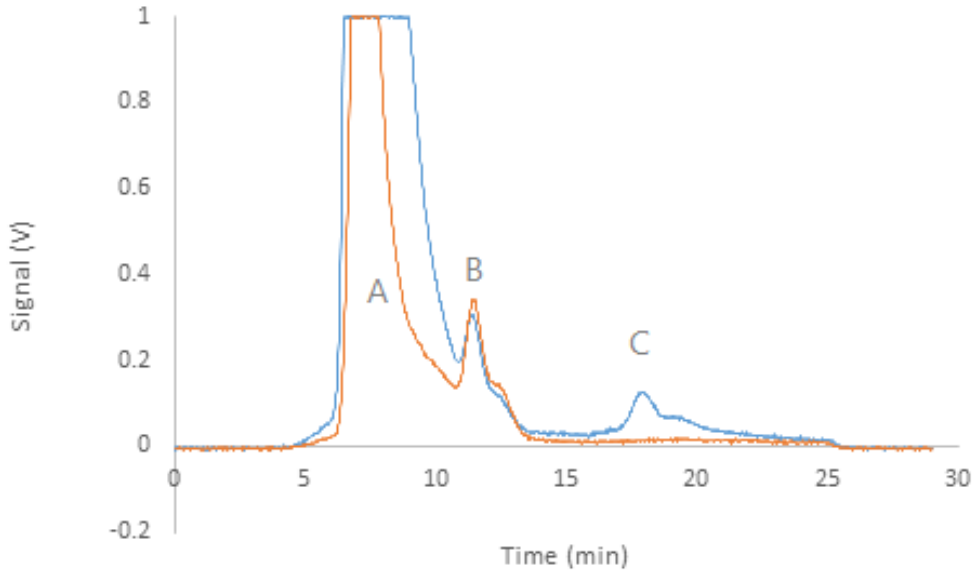

Figure 33. FL chromatograms of the derivatives of lathosterol with dansyl chloride (100 times diluted), one with DMAP and one with DIPEA, with the signal (V) set out against time (min). — : with DMAP; — : with DIPEA. (measured with system B)

A derivatization with DIPEA resulted in a chromatogram with two observable peaks. The first peak was off scale, and no baseline separation was obtained in respect to peak B. The retention time of peak A corresponds to the retention time of peak A in the LC-UV chromatogram. This peak was expected to be caused by the presence of N-acylpyridinium. However, the peak was also observed with the measurement with DIPEA. An explanation might be found in the high reactivity of Dns-Cl. This high reactivity may have resulted in reaction with any impurity in the mixture or reaction with the solvents. This would explain the observation of peak A in both measurements with only DMAP and only DIPEA.

Dansyl chloride was primarily developed for derivatization of primary amines, which have a majorly high reactivity compared to secondary alcohols. thence, the purity of the reagents, especially DIPEA and DMAP, is extremely important. A possibility is that the purity of DIPEA and/or DMAP was/were not adequate.

Peak B corresponds to the peak obtained from a measurement of dansyl chloride (appendix 5.2). When measuring a derivatization with both DIPEA and DMAP, no peak of dansyl chloride was observed. A possible explanation for this observation is that with only one base present, not all Dns-Cl forms an intermediate and/or derivatization product. This could lead to native Dns-Cl present in the mixture.

In the chromatogram of the measurement with DMAP, three peaks were observed. Peak A and B were equivalent to peak A and B of the measurements with DIPEA. Only peak A has a larger area compared to the measurement with solely DIPEA. The peak might be caused by reaction with an impurity in either solvents or the used chemicals/bases. Peak C seems corresponds to the formed derivative. In comparison with the derivatization with both DIPEA and DMAP, less yield was obtained.

The measurements without DMAP or DIPEA confirmed that more desirable results were obtained when both were added to the derivatization mixture.

As with the results of LC-UV, a longer derivatization time did make a significant difference. Extending the derivatization time increased the area of peak C. Since identification of this peak could not be predicted based solely on the ultraviolet and fluorescence detection, further examination with LC-MS/MS was necessary.

5.3.3 LC-MS/MS measurements

To identify the formed derivative, an LC-MS/MS system was used. A derivatization with a reaction time of one hour was performed and measured. The total ion chromatogram is shown in figure 57 in appendix 5.3 and the extracted ion chromatograms are shown in figure 34. Three masses were selected for the XIC, namely the mass of lathosterol (m/z 386.7), dansyl chloride (m/z 269.7) and the preferred derivative (m/z 619.4) (all with a deviation of m/z ± 0.5). The grey trace was an unexplained feature of the software.

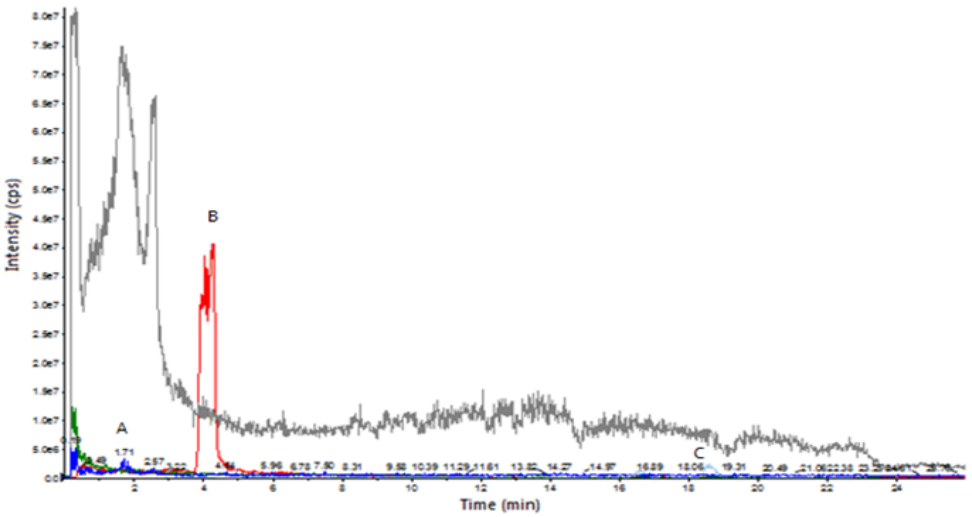

Figure 34. XIC of derivative of lathosterol with dansyl chloride (1h) with intensity (cps) set out against time (min). Three different masses were selected; — : preferred derivative; — : lathosterol; — : dansyl chloride. (measured with system C)

In the XIC three peaks were observed, at 1.6 (A), 4.2 (B) and 18.6 (C) minutes. Spectra of the three peaks were obtained. Interpretation of all three spectra is shown in table 4. Not all fragments could be readily interpreted, all non-interpretable peak are omitted from the table below.

Table 4. The interpretation of the spectra, obtained from XIC (figure 34), of peaks A, B and C.

Spectrum peak A		**Spectrum peak B**		**Spectrum peak C**	
m/z	**Description**	**m/z**	**Description**	**m/z**	**Description**
107.1	DMAP without methyl $[M\text{-}CH_3]^+$	107.3	DMAP without methyl $[M\text{-}CH_3]^+$	123.2	DMAP $[M+H]^+$
123.3	DMAP $[M+H]^+$	123.2	DMAP $[M+H]^+$	170.4	Fragment derivative and/or Dns-Cl (Dns-Cl $[M\text{-}SO_2Cl]^+$) (same fragment)
130.2	DIPEA $[M+H]^+$	130.2	DIPEA $[M+H]^+$	252.5	Fragment derivative (Dansyl with O of lathosterol)
170.2	Fragment Dns-Cl $[M\text{-}SO_2Cl]^+$ and/or intermediate (same fragment)	255.2	Dns-Cl without methyl $[M\text{-}CH_3]^+$	356.3	Fragment derivative $[M\text{-Dns-Cl-}C_2H_6+H]^+$
234.2	Dns-Cl $[M\text{-Cl}]^+$	270.1	Dns-Cl $[M+H]^+$ or intermediate without nitrogen and methyl groups $[M\text{-}NC_2H_6]^+$	399.3	Fragment derivative (rings Dns-Cl and lathosterol with S-O)
252.3	Intermediate without nitrogen and methyl groups and without H_2O	342.4	Intermediate without methyl $[M\text{-}CH_3]^+$	546.4	Fragment derivative $[M\text{-}NC_2H_6\text{-}C_2H_6+H]^+$

(Continued table) Table 4. The interpretation of the spectra, obtained from XIC (figure 34), of peaks A, B and C.

Spectrum peak A		**Spectrum peak B**		**Spectrum peak C**	
m/z	**Description**	**m/z**	**Description**	**m/z**	**Description**
270.3	Intermediate without nitrogen and methyl groups $[M\text{-}NC_2H_6]^+$ or Dns-Cl $[M+H]^+$	356.2	Intermediate of DMAP and Dns-Cl M^+	620.4	Derivative lathosterol with Dns-Cl $[M+H]^+$
342.4	Intermediate without methyl $[M\text{-}CH_3]^+$				
356.2	Intermediate of DMAP and Dns-Cl M^+				
391.3	Intermediate with Cl $[M+Cl]^+$				

Multiple fragments (with m/z 104; 132; 149; 163), observed in the spectra, are not included in the table above. These fragments are likely caused by a contamination of phthalates in the measurement. The contamination of phthalates was observed in every measurement. A list of phthalates and their fragments, including corresponding m/z values, is shown in appendix 6.

The spectrum of peak A, presented in figure 35 showed a large number of fragments of the intermediate (N-acylpyridinium). The retention time of the peak was as expected and corresponded to the previous measurements (LC-UV and LC-FL). The molecular ion (m/z 356.2), but also the intermediate with chloride was observed with m/z 391.3.

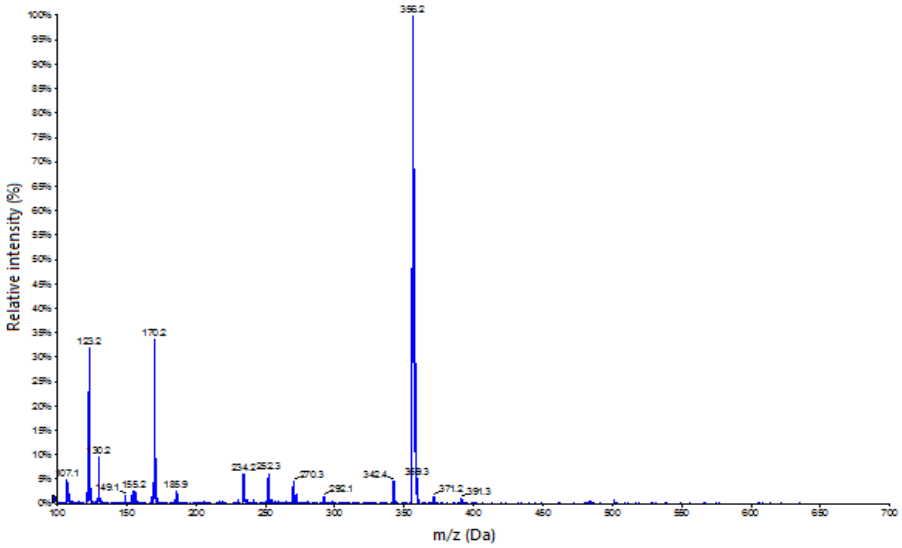

Figure 35. Mass spectrum of peak A of XIC (1 h) at 1.6 min, with relative intensity (%) against m/z (Da).

The spectrum of peak B is shown in figure 36. At first sight, peak B seemed to be caused by dansyl chloride, since the base peak in the spectrum correlates with the mass of the molecular ion of Dns-Cl. If this was the case, the peak at m/z 255 was most likely caused by Dns-Cl without a methyl group. However, the presence of fragments of the intermediate made this explanation less likely. Since all fragments that can be formed by native Dns-Cl can also be formed by the N-acylpyridinium intermediate, it was impossible to determine the cause of peak B with confidence. The lack of a peak at the m/z of Dns-Cl lacking chloride favors the intermediate, since this fragment would be expected if peak B was caused by native Dns-Cl.

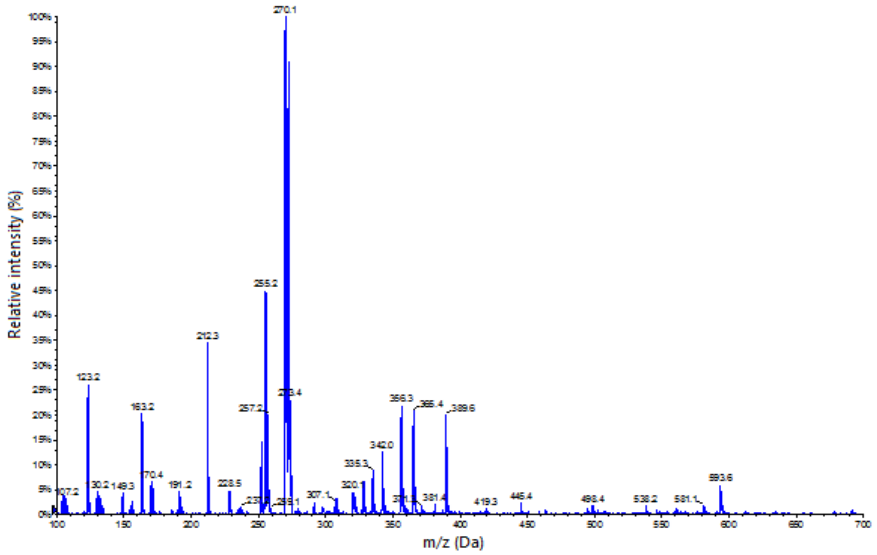

Figure 36. Mass spectrum of peak B of XIC (1 h) at 4.2 min, with relative intensity (%) against m/z (Da). (measured with system C)

In the XIC (figure 34), at approximately 18.6 minutes, a minor peak (C) is observed (light blue trace). Previous measurements suggested that this peak represents the formed derivative. The mass spectrum of this peak is shown in figure 37.

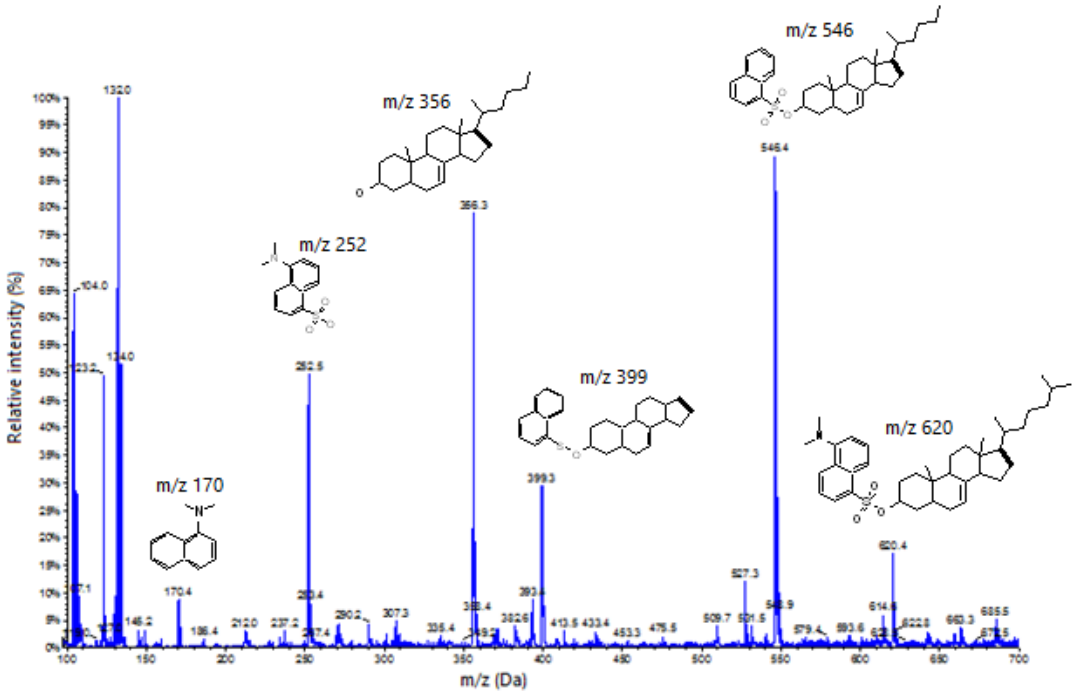

Figure 37. Spectrum of peak C at 18.6 minutes with the relative intensity (%) against m/z (Da). (measured with system C)

Previous measurements (LC-UV and LC-FL) raised the assumption that peak C in the XIC, at approximately 18.6 minutes, was caused by a product of successful derivatization. The spectrum of peak C seemed to confirm this assumption. The spectrum showed a peak at m/z 620.4, which corresponds to the preferred derivative molecular ion. Other fragments also confirmed that the preferred derivative was formed. At m/z 252.5, a fragment of dansyl with an extra oxygen attached was observed, likely the oxygen from a lathosterol hydroxide group. At m/z 546.4 a fragment of the derivative without ethylamine and ethyl groups was observed.

The fragment with m/z 356.3 could represent three compounds, namely a fragment of the derivative $[M\text{-}Dns\text{-}Cl\text{-}C_2H_6+H]^+$, a fragment of lathosterol $[M\text{-}CH_3\text{-}OH+H]^+$ or the molecular ion of the intermediate of DMAP and Dns-Cl. Firstly, a fragment of lathosterol would be unlikely since no other fragments of lathosterol were observed. Representing the molecular ion of the intermediate seemed to be unlikely, for the exact same reason. Furthermore, the spectra of peak A and B show the molecular ion of the intermediate at a m/z differing by 0.1. This leaves a fragment of the derivative as the most likely option. The fragment with m/z 399.3 could also ascribed to a fragment that could be formed out of the preferred derivative. The fragment with m/z 170.4 can be most confidently described to the fragment of Dns-Cl without SO_2Cl. It can be argued that this fragment can be formed out of both a derivative and native Dns-Cl. However, other fragments in this spectrum have been quite confidently assigned to derivative fragments, and this peak is used as an indicator of Dns-Cl derivatives [12]. This increases the likelihood that the fragment with m/z 170.4 is also formed by the derivative, and not native Dns-Cl. Still, it deserves mentioning that there is no certainty considering the origin of this

fragment, thus its contribution to the confidence in successful derivatization is not considered significant.

Native lathosterol was not observed with LC-MS/MS. The molecular ion was not present and no typical fragments were observed. A possibility is that all of the lathosterol had reacted to derivative or N-acylpyridinium, or that the LC-MS/MS was not sensitive enough for the low concentration present after reaction.

Changes made in temperature did not make a significant difference in the results of the measurements. However, extending the derivatization time did.

A measurement of a derivative with a derivatization time of 24 hours was performed subsequently. The TIC is shown in figure 58 in appendix 5.3 and the XIC is shown in figure 38. The same masses as in the previous measurements were selected.

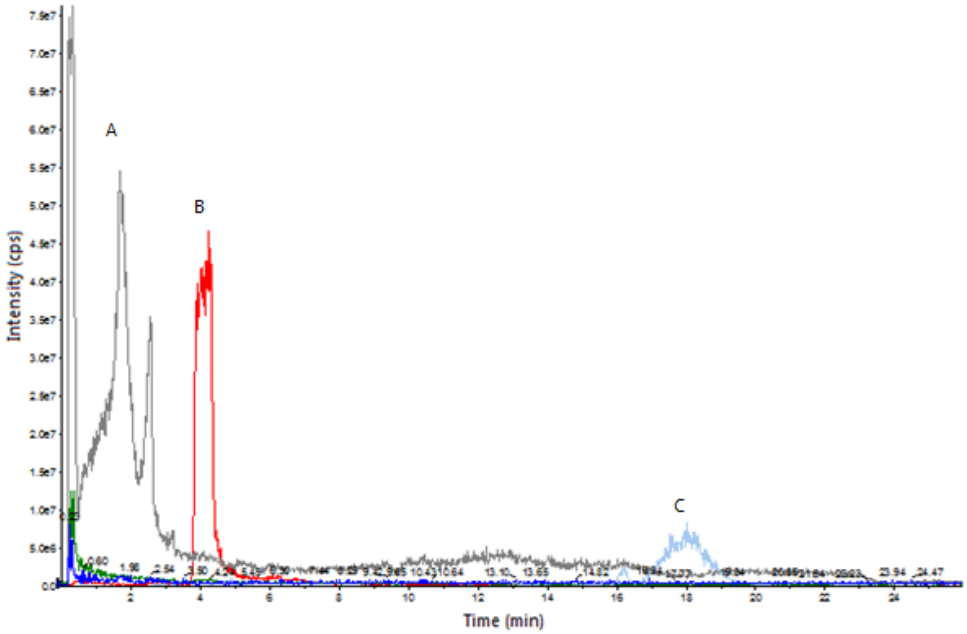

Figure 38. XIC of derivative of lathosterol with dansyl chloride (24h) with intensity (cps) set out against time (min). Three different masses were selected; — : preferred derivative; — : lathosterol; — : dansyl chloride. (measured with system C)

Only one spectrum was of interest from the XIC, at approximately 18.1 minutes. The spectrum is shown in figure 39.

As in the previous measurement, peak C was observed in the XIC, however with an increased area. The same fragments (m/z 252.5; 356.3; 399.4; 546.4; 620.3), that confirmed the preferred derivative has formed, were observed in the spectrum. Also notable is that the relative intensity of the molecular ion (m/z 620.3) has increased with increasing reaction time.

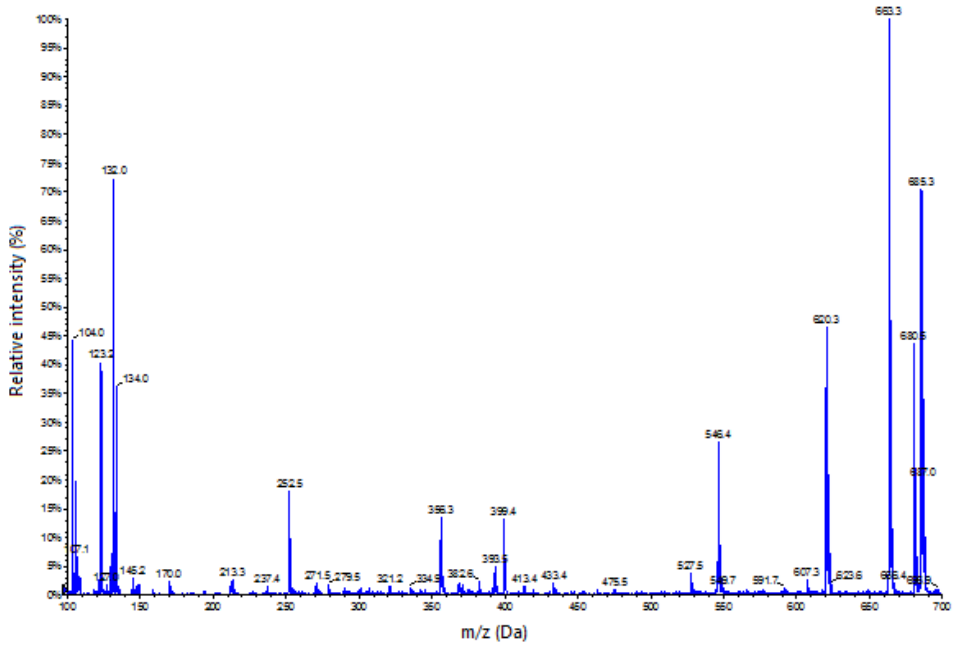

Figure 39. Mass spectrum of peak C at 18.1 min of derivatization of lathosterol with Dns-Cl (24 h), with relative intensity (%) against m/z (Da). (measured with system C)

The interpretation of the spectrum is presented in table 5. Not all the fragments could readily be interpreted, all non-interpretable peaks are omitted from the table.

Table 5. The interpretation of the spectrum, obtained from XIC (figure 39), of peak C.

Spectrum peak C (18.1 minutes)	
m/z	**Description**
123.2	DMAP $[M+H]^+$
252.5	Fragment derivative (Dansyl with O of lathosterol)
356.3	Fragment derivative $[M\text{-Dns-Cl-}C_2H_6+H]^+$
399.4	Fragment derivative (rings Dns-Cl and lathosterol with S-O)
546.4	Fragment derivative $[M\text{-}NC_2H_6\text{-}C_2H_6+H]^+$
620.3	Derivative lathosterol with Dns-Cl $[M+H]^+$

As in the previous spectra, several fragments were observed that are most likely caused by a contamination of phthalates. A list of phthalates and their fragments, including corresponding m/z values, is shown in appendix 6.

A shift in base peak can be observed. Where the base peak in figure 37 is most likely caused by phthalates (m/z 132.0), the base peak after 24 hours of reaction time has an m/z 663.3. The cause of this peak was untraceable with the current measurements.

The preferred derivative was observed, with both ultraviolet and fluorescence detection, as with mass spectrometry. A longer derivatization time resulted in an increase of peak area, which is equivalent to more formed derivative. The results show that dansyl chloride can be a viable option for the derivatization of vitamin D_3 for clinical use. Further research is necessary to enhance yield.

5.4 Overview

In almost every mass spectrum, a repetition of the same fragments (or fragment series) were observed. This likely indicated a contamination. A lot of the observed fragments in the mass spectra were likely caused by phthalates. The fragments of m/z 104 and 132 are typical fragments for phthalates. Observation of these fragments confirmed that there was a contamination. When the intensity of the detected fragments of the compounds was sufficient, the contamination was not observed or disappeared in the background. However, when the intensity of the fragments of interest (especially of the derivative) were relatively low, the contamination proved to be an obstacle. Irreproducible fragmentation could have occurred due to phthalates. The cause of contamination can be from plastic laboratory equipment and tubing. For further research, the origin of contamination should be determined. Another option is to eliminate the peaks of the data sets by using background subtraction software [35-39].

Derivatization using naproxen chloride seemed a viable option derived from the results with LC-UV and LC-FL. Multiple peaks, which possibly were reaction products, were observed using both techniques. However, LC-MS/MS measurements did not confirm the presence of the preferred derivative. A possible explanation might be found in the high reactivity of Nap-Cl. This high reactivity may have resulted in reaction with any impurity in the mixture, or deactivation by reacting with water. Fragments of cholesterol and Nap-Cl were observed in the mass spectra. However, all spectra also indicated the presence of a reaction product of Nap-Cl and diethylamine. This renders it impossible to determine whether Nap-Cl or these reaction products cause the aforementioned peaks. Derivatization using naproxen chloride was eliminated for a viable option in this study.

The second derivatization agent tested was 3,5-dinitrobenzoyl chloride. LC-UV measurements showed an appearance of a peak (at 11.6 minutes) after a longer derivatization time. When the peak appeared or increased in area, the vitamin peak disappeared or decreased in area. LC-MS/MS was used to identify the formed compound. Three peaks were observed in the XIC. The second peak seemed to be caused by a contamination of phthalates. The first peak corresponded to the peak at 11.6 minutes observed with LC-UV. This was expected to be the formed derivative or a degradation product. The spectrum of the peak confirmed it was the degradation product of the vitamin, namely Inhoffen-Lythgoe diol (m/z 212). This is a known degradation product of vitamin D_2 and D_3 [30, 31]. The last peak observed, was caused by native the vitamin (D2 or D3). Multiple fragments of the vitamins were present. However, none of the expected fragments of the preferred derivative were observed. Only one peak was expected to be formed derivative with LC-UV and the peak turned out to be a degradation product of the vitamin. Since there was no indication this derivatization procedure would yield any more results, the use of this procedure was discontinued.

The last derivatization agent tested was dansyl chloride. Both LC-UV and LC-FL measurements showed a peak that appeared to be a formed derivative. A longer derivatization time resulted

in an increase in peak area. Both DIPEA and DMAP had influence on the formed peak. DMAP can form an intermediate with dansly chloride and without DMAP, no peak of the formed derivative was observed. DIPEA likely stimulated the formation of the derivative, since adding DIPEA resulted in an increase of peak area of the product. Measurements with LC-MS/MS confirmed that occurred peak was indeed successfully formed derivative. Multiple fragments in the mass spectrum of the XIC peak confirmed the formation. The observed fragments were m/z 252.5, 356.3, 399.4, 546.4 and 620.3. With extending the derivatization time, the peak area of the derivative increased. In LC-MS/MS measurements the formed intermediate, N-acylpyridinium, was also observed with fragments and molecular ion. Some fragments of dansyl chloride were also observed. It is likely to assume that native dansyl chloride was not present, but it is impossible to say with certainty if all of dansyl chloride had reacted (to intermediate or derivative). Native lathosterol was not observed during the measurements. Using UV and FL detection, lathosterol could not be detected. LC-MS/MS also did not show the presence of native lathosterol. To determine whether all native lathosterol had reacted, further research is necessary.

The first two derivatizations, using naproxen chloride or 3,5-dinitrobenzoyl chloride, did not yield results. Both derivatization agents were eliminated as viable options. However, testing higher derivatization agent : vitamin D_3 ratios may yield better results. The derivatization procedure using dansyl chloride with DIPEA and DMAP yielded the preferred derivative. A longer derivatization time resulted in an increase of peak area/formed derivative. Since degradation of vitamin D occurs at high temperatures, a temperature above 80 ˚C was not tested [27-29]. Degradation of vitamin D can lead to products of a higher polarity than native vitamin D [40]. Further research of the influence of higher temperatures would be recommended. A longer exposure to high temperatures can also lead to degradation. The effects of extended derivatization times at higher temperature on the degradation of the vitamin also has to be examined.

For the MS/MS settings, a higher temperature than 300 °C was tested, but the compounds seemed to be unstable at this temperature and were directly fragmentized before the first quadrupole. Using a higher ionization temperature than 300 ˚C is not recommended.

The aim of this study was the development of a derivatization procedure. A viable procedure using dansyl chloride was developed. However, it is a method for derivatization of lathosterol instead of the main intent of this study, vitamin D_3. Although it might be likely through the chemical similarity between the two, dansyl chloride must still be shown to successfully derivatize vitamin D_3. The expectation is that vitamin D_3 will react in the same way lathosterol reacted. They both have the same functional (hydroxyl) group and the same surrounding area.

Moreover, further optimization of the derivatization reaction has to be performed. Optimizing the derivatization might prove to be challenging, since most of the variables affect each other. Concentrations and concentration ratios of the analyte, dansyl chloride, DIPEA and DMAP must be taken into account. It is recommended to have at least a 100-fold excess of the derivatization

agent relative to the analyte. A maximum yield of derivatization must be determined. To do so, the influence of variation in pH value during derivatization has to be examined.

The settings of the analysis also have to be further optimized. Reversed-phase LC using a C_{18} column seems to be a good separation technique for the assay. The elution of the compounds should be as fast as possible, but sufficient separation has to be achieved. Fluorescence detection has proven to be applicable for the assay when derivatization is successful. Fluorescence spectrometry was selected as the preferred detection method, since some hospitals are not in possession of a mass spectrometer and because of the considerably high costs of MS based assays. The optimization using fluorescence detection should have priority. Developing the method using mass spectrometry could add value to the method.

Further research of the derivatization using dansyl chloride is necessary. The reaction yield must be determined, sample preparation has to be further developed and the assay has to be optimized and (clinical) validated.

All three derivatization agents are highly reactive, which can cause the formation of by-products. A contamination of water (or another compound) can already be sufficient for a reaction to occur with the derivatization agent. Such a reaction might impair the derivatization of vitamin D, also due to the extremely low reactivity of vitamin D. Therefore, contaminations (with water) must be taken into account for further research.

6. CONCLUSION

The aim of this study was the development of a derivatization procedure for the quantitative determination of vitamin D_3 in human samples for clinical use.

Derivatization using naproxen chloride seemed a viable option derived from the results with LC-UV and LC-FL. Multiple peaks, which possibly were reaction products, were observed using both techniques. However, LC-MS/MS measurements did not confirm the presence of the preferred derivative. Fragments of cholesterol and Nap-Cl were observed in the mass spectra. The mass spectra also indicated the presence of a reaction product of Nap-Cl and diethylamine, but no fragments were observed. This renders it impossible to determine whether Nap-Cl or these reaction products cause the aforementioned peaks. However, no significant amount of product of successful derivatization was observed, thus naproxen chloride was eliminated as a viable option.

The second derivatization agent tested, was 3,5-dinitrobenzoyl chloride. Vitamin D_2 and D_3 were derivatized. LC-UV measurements indicated a reaction or degradation product was formed. After a derivatization time above 5 hours a peak appeared and the vitamin peak decreased in area. After 24 hours the vitamin peak had disappeared and the peak of the formed compound had increased in area. LC-MS/MS measurements were performed to identify the formed compound. On the basis of the mass spectrum, it was concluded that a degradation product was formed. The degradation product (m/z 212.4) was Inhoffen-Lythgoe diol, a known degradation product from vitamin D. In the mass spectra only fragments of the vitamins were observed. The lack of observation of DNBC (and fragments) could be due to measuring in the positive-ion mode, thus measuring in the negative-ion mode may prove to be a solution [13]. However, none of the expected fragments of the preferred derivative were observed. Based on the results, derivatization using 3,5-dinitrobenzoyl chloride was eliminated.

At last, a derivatization of lathosterol with dansyl chloride was tested. DIPEA and DMAP were added to the derivatization mixture. LC-UV and LC-FL measurements confirmed that adding DIPEA and DMAP enhanced the derivatization. After derivatization, LC-UV and LC-FL measurements observed the presence of a peak at circa 18 minutes. The peak was expected to represent a formed derivative. LC-MS/MS was used to identify the formed compound. The mass spectrum of the peak confirmed the preferred derivative had formed. Multiple fragments of the derivative (m/z 252.5, 356.3, 399.4, 546.4) were observed and the molecular ion $[M+H]^+$, with m/z 620.3, was also observed. Extending the derivatization time, resulted in an increase of formed derivative. Native lathosterol was not observed with LC-MS/MS. Further research is necessary to determine whether all native lathosterol had reacted to derivative. Derivatization using dansyl chloride with adding DIPEA and DMAP, seems to be a viable option for the development of a derivatization procedure for vitamin D_3.

A contamination of phthalates was observed with LC-MS/MS. The origin of the contamination was not determined. This resulted in irreproducible fragmentation due to phthalates.

7. RECOMMENDATIONS

Further research is required for the method development. First, the origin of contamination observed with LC-MS/MS must be determined, especially when measurements using LC-MS/MS will be continued. When the contamination cannot be discovered, another solution might be applying background subtraction software. A shortcoming of applying software is that this does not alter the initial data, and irreproducible fragmentation due to phthalates will still occur.

The derivatization procedure using Dns-Cl should be tested on vitamin D_3, since only lathosterol was derivatized. The reaction yield will have to be determined. Furthermore, investigating if continuous mixing during the derivatization, possibly enhancing the reaction yield, may be an option.

The occurrence of degradation of vitamin D_3 can be an issue. Before optimizing the method, degradation of the vitamin should be examined. The maximum derivatization temperature should be determined. Also, examining after which period of (derivatization) time degradation of the vitamin will occur.

In general, the derivatization reactions used in this study are nucleophilic substitution reactions of secondary alcohols. These reactions are known for their slow kinetics. Therefore, reaction with solvents, other reagents and impurities often have faster kinetics and all kinds of side reactions can occur. For future derivatization reactions, the use of extremely pure solvents and reagents is of major importance.

Since derivatization using naproxen chloride or 3,5-dinitrobenzoyl chloride was eliminated, there is no further use of both procedures for this study. However, both derivatization agents were not tested with an extremely high amount of derivatization agent with respect to the analyte, so testing higher derivatization agent : vitamin D_3 ratios may yield better results

The derivatization procedure using dansyl chloride must be optimized. Optimizing the derivatization might prove to be challenging, since most variables affect each other. Concentrations and concentration ratios of the analyte, dansyl chloride, DIPEA and DMAP must be taken into account. It is recommended to have at least a 100-fold excess of the derivatization agent relative to the analyte. A maximum yield of derivatization must be determined. To do so, influence of variation in the pH value during derivatization has to be examined. A low pH will most likely interfere with the reactivity of DIPEA and DMAP. Hospitals generally require fast and accurate results, therefore it must be taken into account that the developed derivatization procedure should be quick and reliable.

Sample preparation has to be developed further. As for derivatization, preference goes out to a fast and feasible sample preparation. Since vitamin D_3 is insoluble in water, a liquid-liquid extraction is a possibility. After sample preparation, the method should be tested on clinical

samples and the measurement range must be established. There is a possibility that samples must be concentrated.

The settings of the analysis require optimization. A fast and accurate analysis should be established. This includes, but is not limited to, (further) optimization of the elution gradient, selection of buffer and pH, injection volume, column length, diameter, and particle size.

The elution gradient should be short and effective, meaning all compounds, polar and non-polar, should elute within a short period of time. Different buffers can be tested, such as ammonium acetate or formic acid. Both are volatile buffers and suitable for LC-MS/MS detection. A C_{18} column is suitable for the follow-up study, since separation can be obtained. Moreover, C_{18} columns are widely available and relatively cheap. The column length should stay under 15 cm (if separation does not deteriorate), otherwise measurements will take too long to take place in a hospital. Small particle size and diameter are preferred to improve separation.

For fluorescence detection, the exact optimum of the excitation and emission wavelength of the derivative should be determined. If measurements using LC-MS/MS will be continued, the mass spectrometer settings will also require optimization.

After setting up and optimizing the complete method, (clinical) validation has to be performed. Validation of the method will involve exploring accuracy, precision, sensitivity, specificity, linearity, stability, and the measuring and reference range of the assay. Clinical validation will involve analogue aspects, but focusses on individual patient samples, instead of a pool of samples [41].

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APPENDIX 1: REACTION MECHANISMS

1.1 Naproxen chloride (with cholesterol)

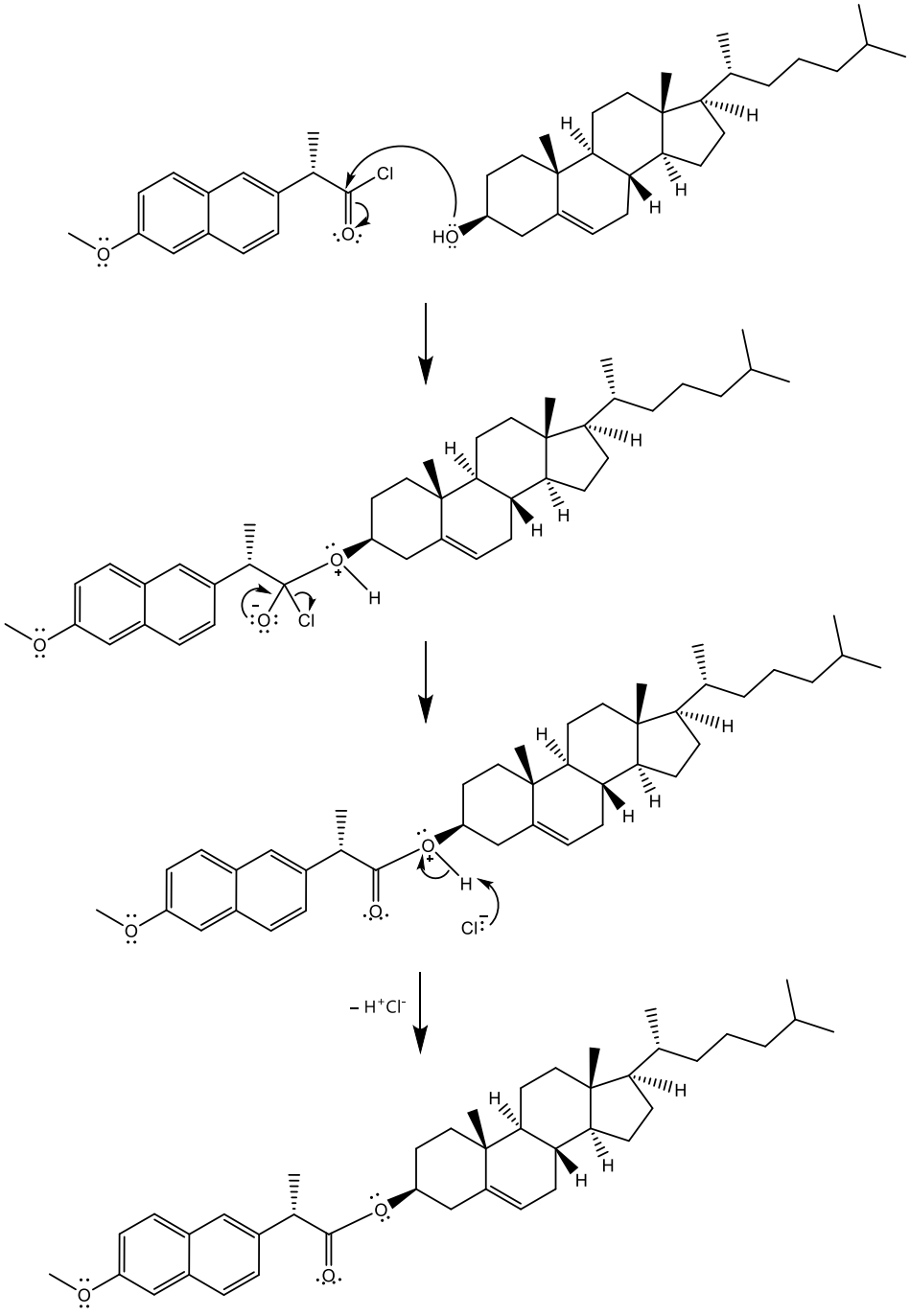

Figure 40. Reaction mechanism of the derivatization of cholesterol with naproxen chloride.

1.2 3,5-dinitrobenzoyl chloride (with vitamin D_2)

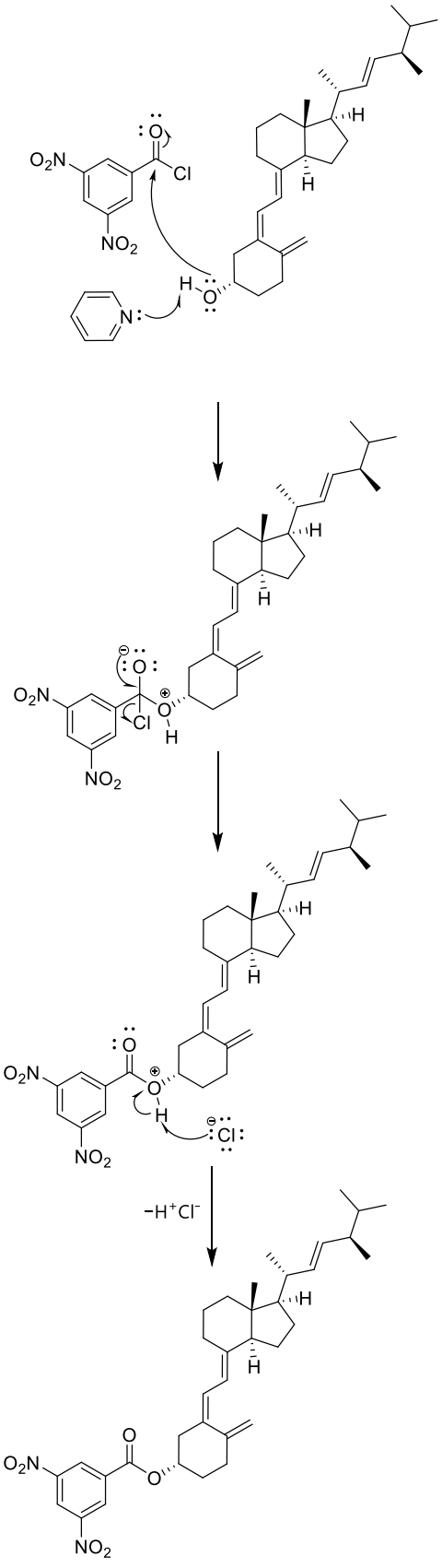

Figure 41. Reaction mechanism of the derivatization of vitamin D_2 with 3,5-dinitrobenzoyl chloride with pyridine.

1.3 Dansyl chloride (with vitamin D_3)

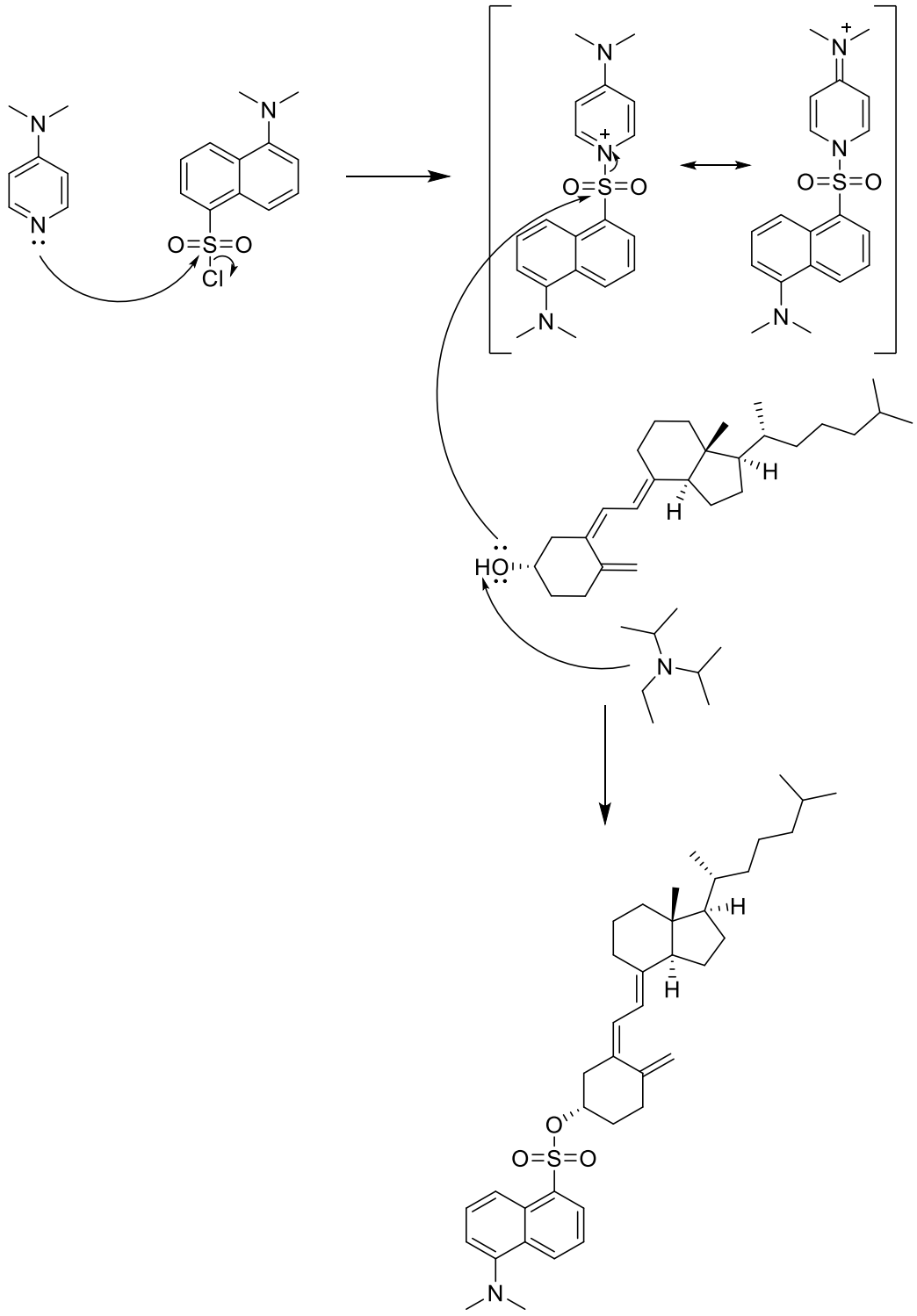

Figure 42. Reaction mechanism of the derivatization of vitamin D_3 with dansyl chloride with DMAP and DIPEA.

APPENDIX 2: CHEMICALS SPECIFICATIONS

Table 6. An overview of the specifications of the used chemicals.

Compound	**Purchased at**	**Purity**	**CAS#**	**LOT#**
Cholecalciferol(D3) (crystalline)	Sigma Aldrich Nederland	≥98%	67-97-0	SLBM6251V
Ergocalciferol (D2)	Sigma Aldrich Nederland	≥97%	50-14-6	SLBM7518V
TetraHydrofuran (THF)	Sigma Aldrich Nederland	≥99%	109-99-9	13596HR
3,5-dinitrobenzoyl chloride	Sigma Aldrich Nederland	≥98%	99-33-2	BCBP3264V
Diethylamine	Sigma Aldrich Nederland	≥99.5%	109-89-7	38947HD
(S)-(+)-naproxen chloride	Sigma Aldrich Nederland	≥97%	51090-84-0	BGBB8051V
Lathosterol (5a-cholest-7-en-3B-ol)	Sigma Aldrich Nederland	≥98%	80-99-9	055M4016V
Dansyl chloride	Sigma Aldrich Nederland	≥99%	605-65-2	BDCB3158V
N,N-diisopropylethylamine (DIPEA)	Sigma Aldrich Nederland	≥99%	7087-68-5	STBF7198V
4-dimethylaminopyridine (DMAP)	Sigma Aldrich Nederland	≥99%	1122-58-3	MKBT6293V
Toluene	Biosolve BV	≥99.8%	108-88-3	1352690
Acetonitrile	Biosolve BV	≥99.95%	75-05-8	2125086
Cholesterol	Sigma Aldrich Nederland	≥99%	57-88-5	SLBM8321V
Methanol absolute	Biosolve BV	≥99.95%	67-56-1	1045511
Formic acid	Sigma Aldrich Nederland	≥98%	65-18-6	BCBN2977V
Pyridine	Sigma Aldrich Nederland	99.8%	110-86-1	01296HK
Chloroform	Biosolve BV	≥99.9%	67-66-3	3561200

APPENDIX 3: NAPROXEN CHLORIDE

3.1 LC-UV measurements

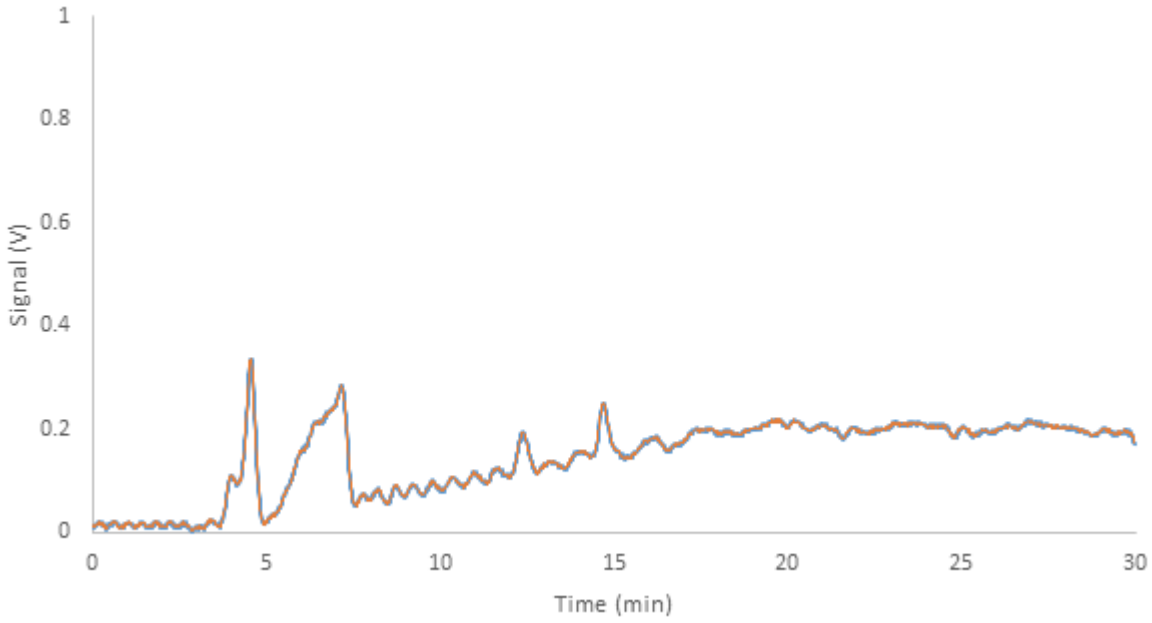

Figure 43. UV chromatograms of cholesterol in THF, with signal (V) against time (min). — : 230 nm; — : 254 nm. (measured with system A.1)

3.2 LC-UV-FL measurements

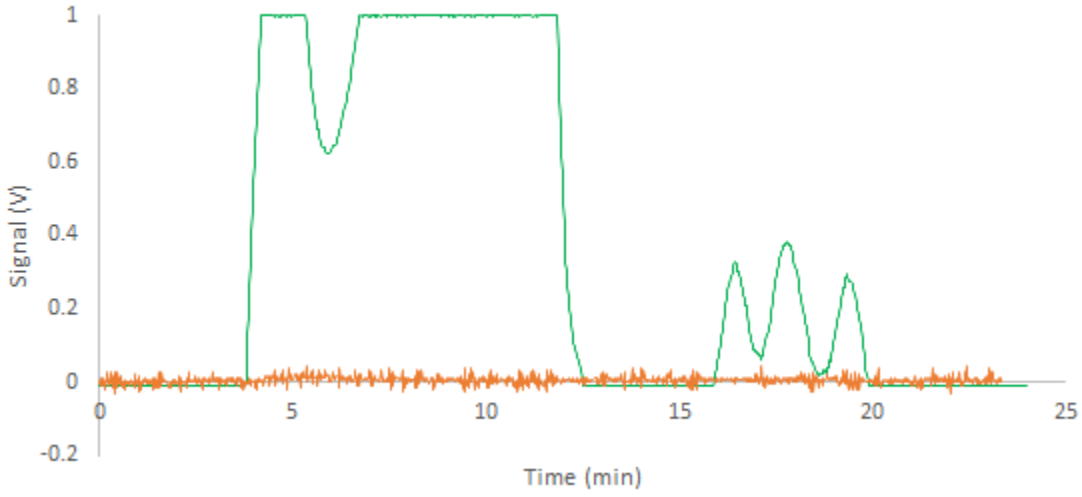

Figure 44. UV and FL chromatogram of the derivatization of cholesterol (10 mM) with Nap-Cl, with signal (V) against time (min). — : UV; — : FL. (measured with system B)

3.3 LC-MS/MS measurements

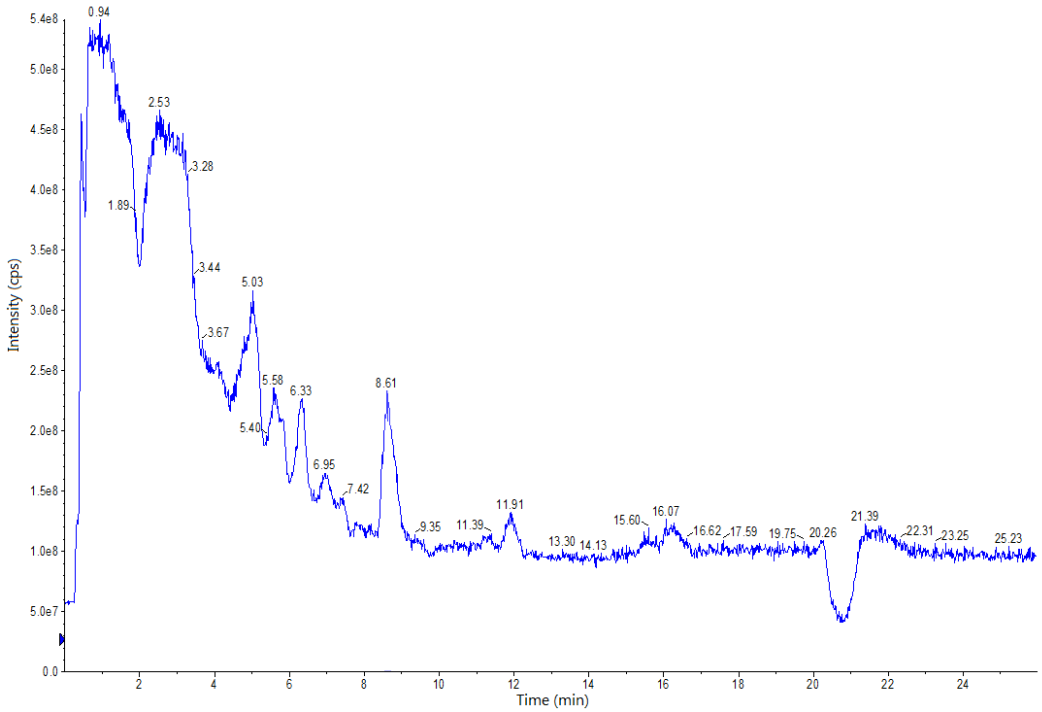

Figure 45. TIC of the derivatization of cholesterol (10 mM), with intensity (cps) against time (min). (measured with system C)

APPENDIX 4: 3,5-DINITROBENZOYL CHLORIDE

4.1 LC-UV measurements

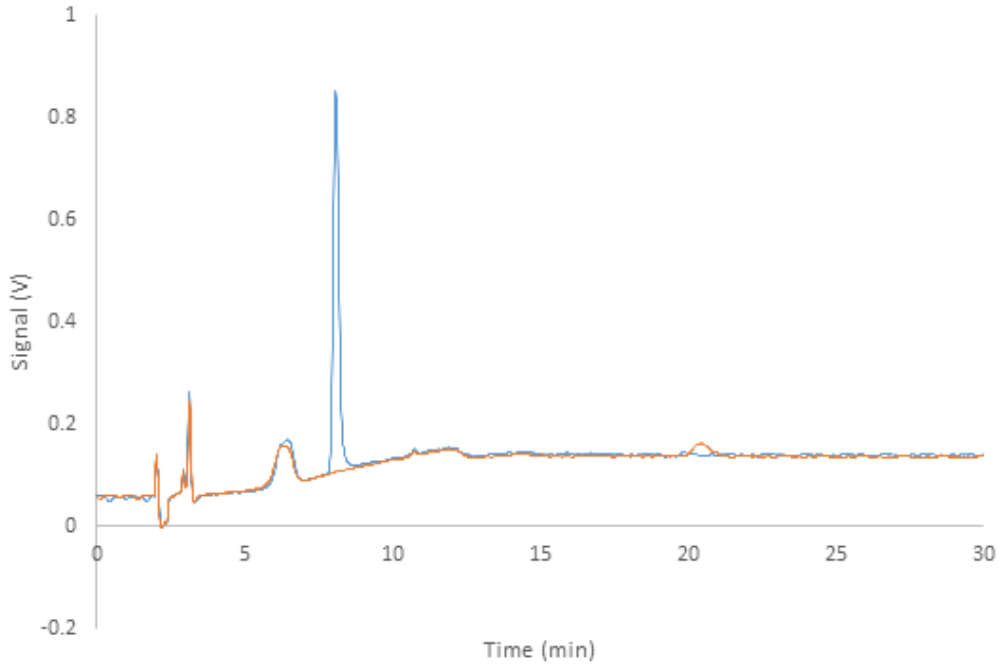

Figure 46. UV chromatograms of standard measurements of DNBC and vitamin D_3, with signal (V) against time (min). — : DNBC; — : vitamin D_3. (measured with system A.2)

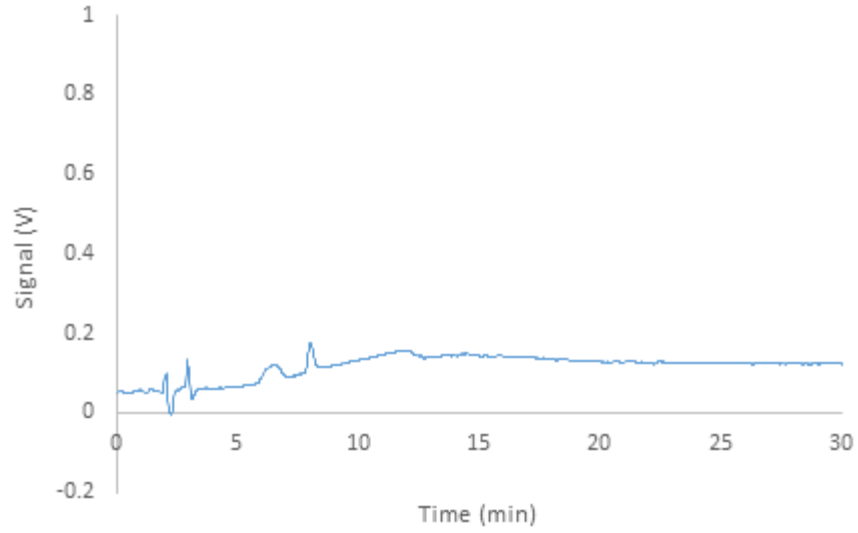

Figure 47. UV chromatograms of chloroform in THF, with signal (V) against time (min). (measured with system A.2)

4.2 LC-MS/MS measurements

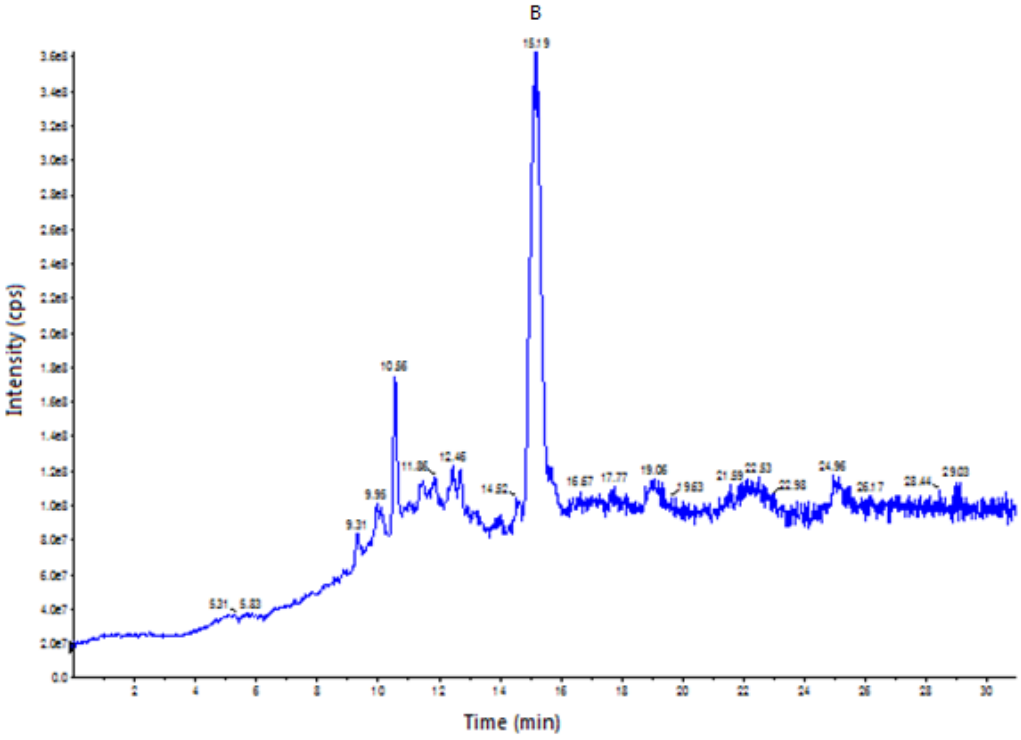

Figure 48. TIC of the derivatization of vitamin D_2 with DNBC, with intensity (cps) against time (min). (measured with system C)

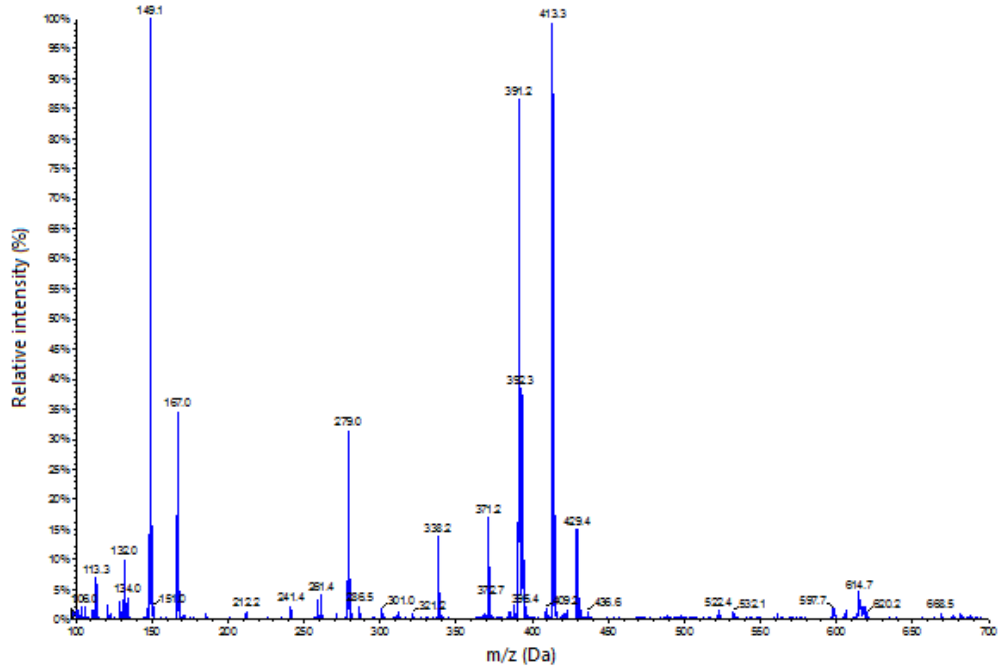

Figure 49. Mass spectrum of peak B of TIC of derivatization of D_2 with DNBC at 15.2 min, with relative intensity (%) against m/z (Da). (measured with system C)

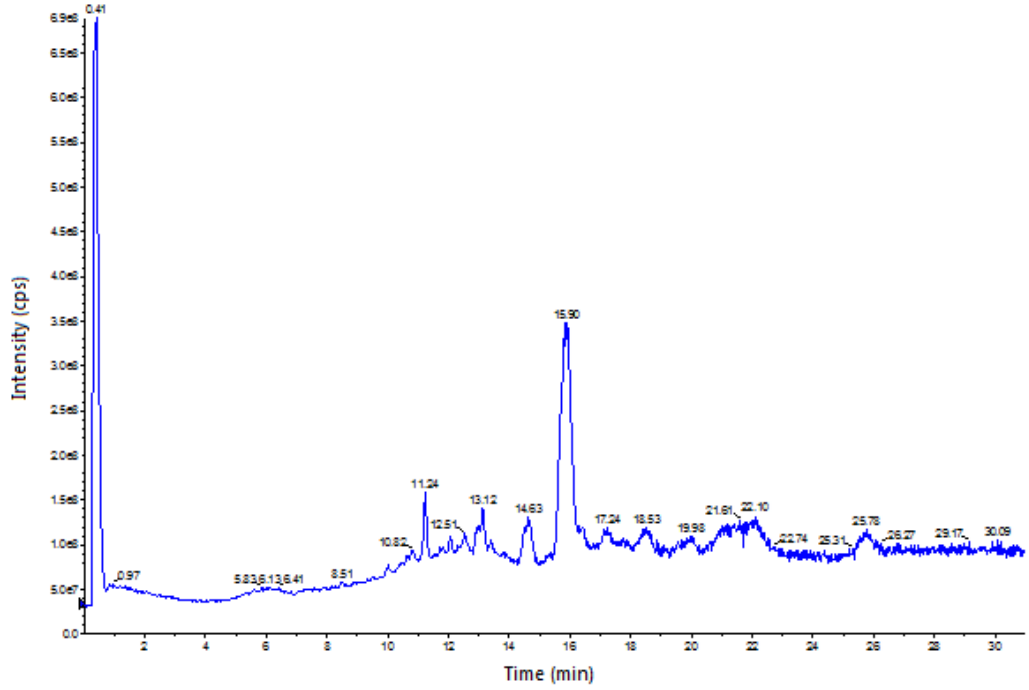

Figure 50. TIC of the derivatization of vitamin D_3 with DNBC, with intensity (cps) against time (min). (measured with system C)

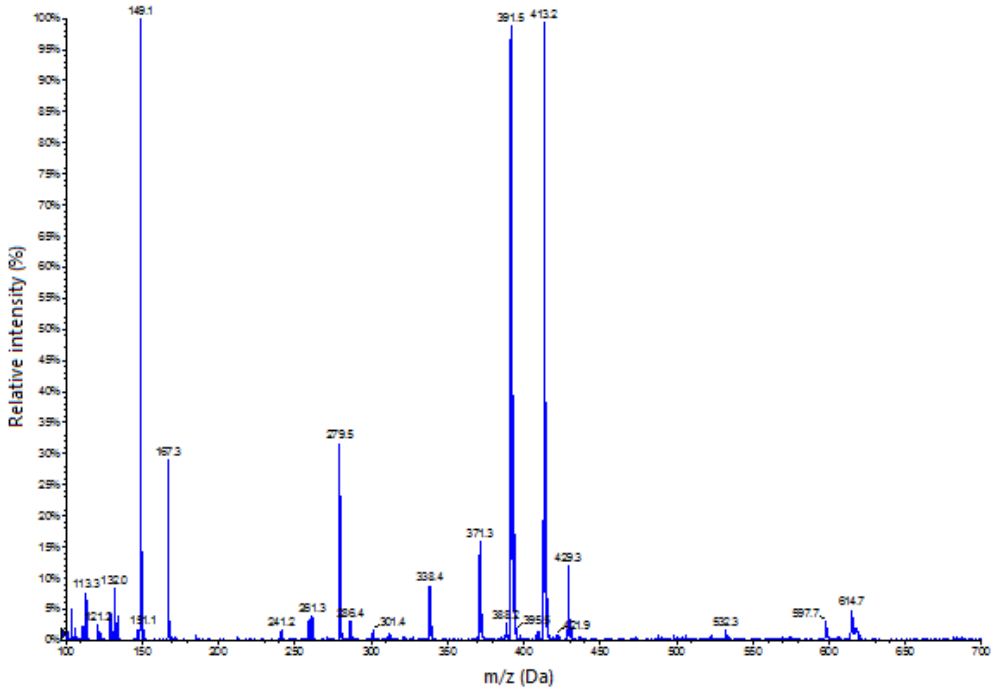

Figure 51. Mass spectrum of XIC derivatization of D_3 with DNBC at 15.8 min, with relative intensity (%) against m/z (Da). (measured with system C)

APPENDIX 5: DANSYL CHLORIDE

5.1 LC-UV measurements

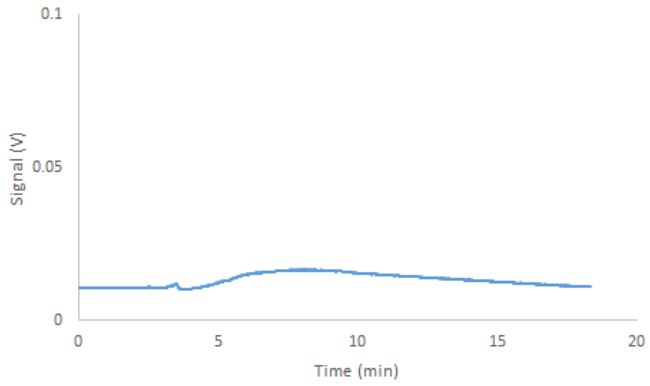

Figure 52. UV chromatogram of DIPEA in THF measured at 330 nm, with signal (V) against time (min). (measured with system A.3)

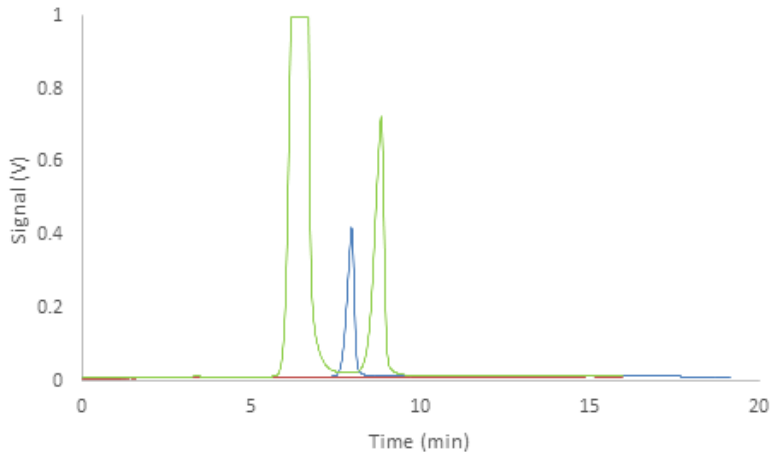

Figure 53. UV chromatograms of standard measurements of lathosterol and Dns-Cl, with signal (V) against time (min). — : lathosterol 254 nm; — ; lathosterol 330 nm; — ; Dns-Cl 330 nm (160 µM). (measured with system A.3)

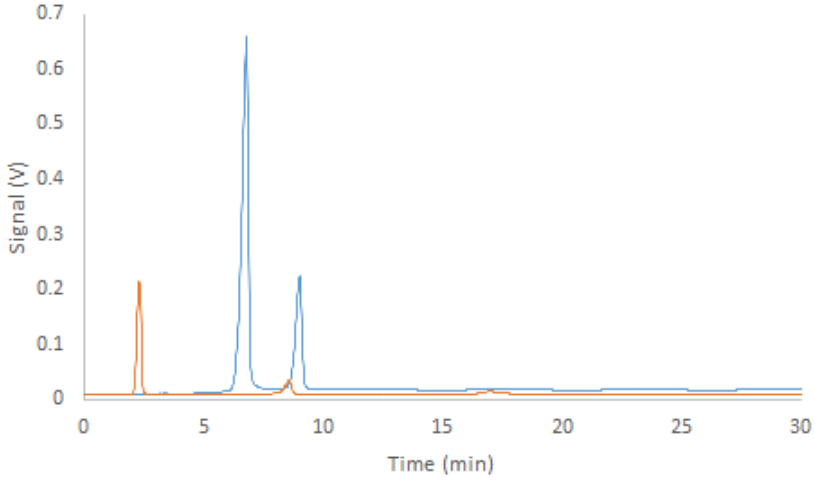

Figure 54. UV chromatograms of derivatization of lathosterol with dansyl chloride, with signal (V) against time (min). — : without DIPEA and DMAP; — : with both DIPEA and DMAP (10 times lower concentration). (measured with system A.3)

5.2 LC-UV-FL measurements

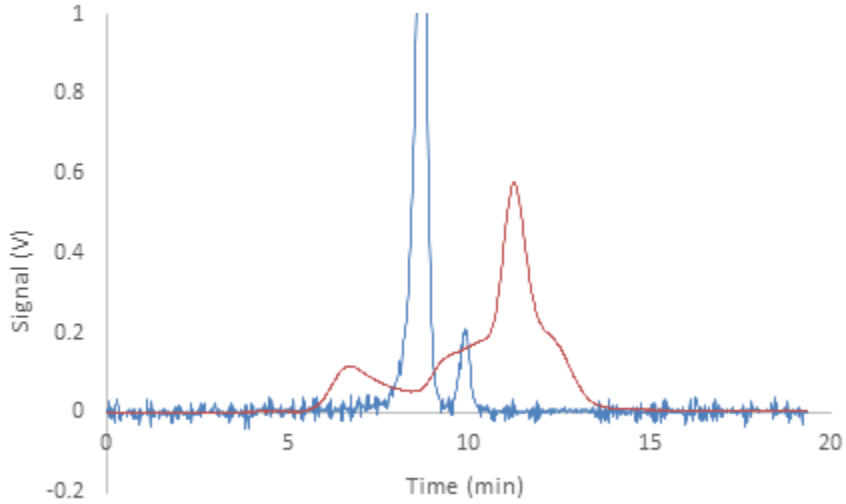

Figure 55. UV and FL chromatogram (330 nm) of Dns-Cl standard, with signal (V) against time (min). — : UV; — : FL. (measured with system B)

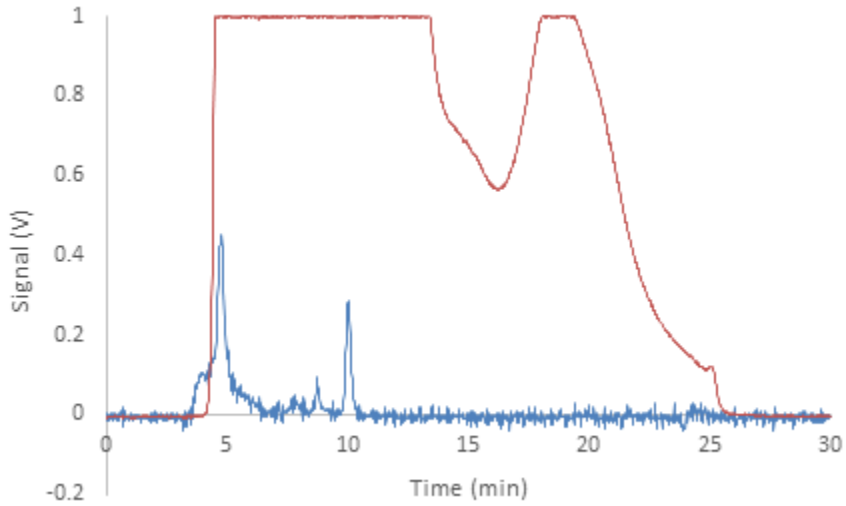

Figure 56. UV and FL chromatogram (330 nm) of a derivatization of lathosterol with Dns-Cl (not diluted), with signal (V) against time (min). — : UV; — : FL. (measured with system B)

5.3 LC-MS/MS measurements

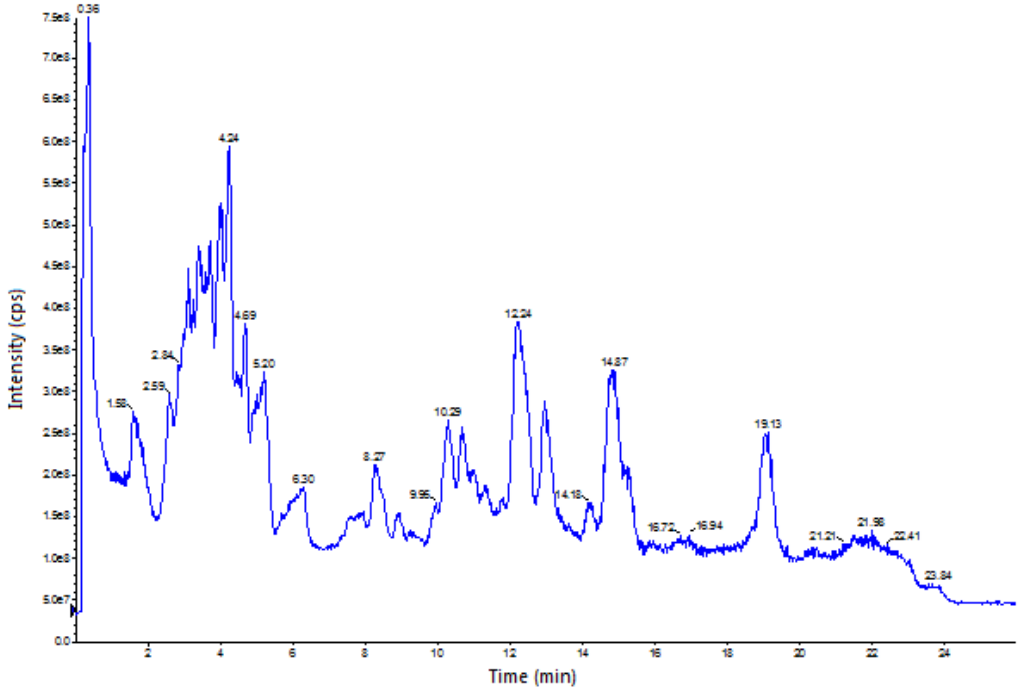

Figure 57. TIC of the derivatization of lathosterol with dansyl chloride (1 h), with intensity (cps) against time (min). (measured with system C)

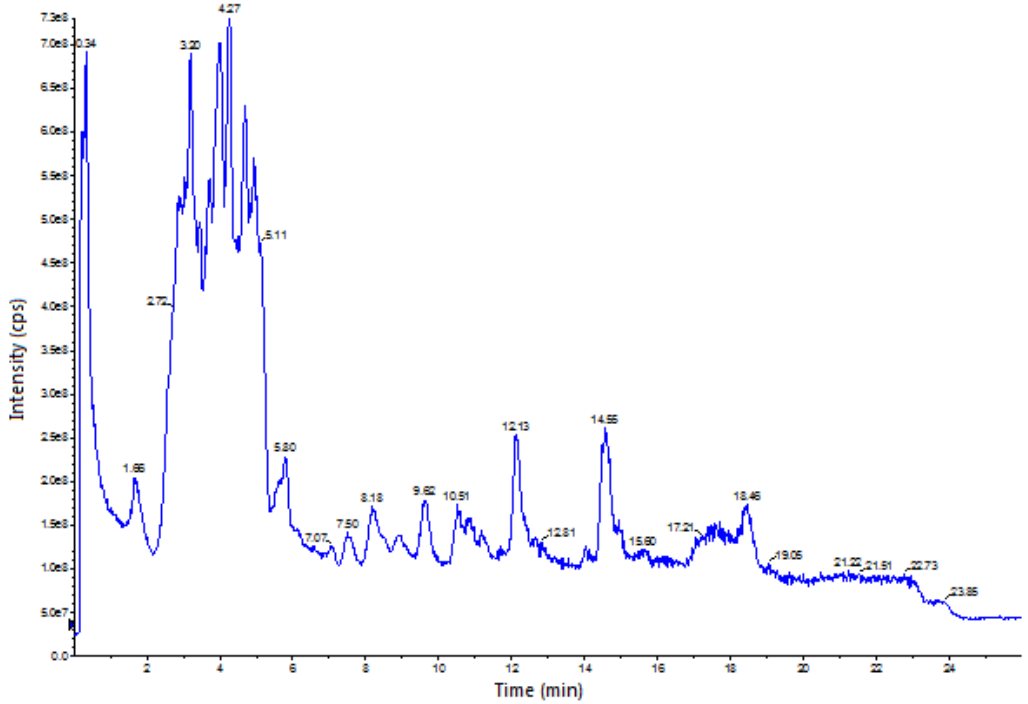

Figure 58. TIC of the derivatization with lathosterol and Dns-Cl (24 h), with intensity (cps) against time (min). (measured with system C)

APPENDIX 6: PHTHALATES CONTAMINATION

Table 7. A list of all the observed fragments, with corresponding fragments, of phthalates in the mass spectra. A mass spectrum with the most observed fragments is shown below in figure 59. Note: m/z values are rounded.

m/z	Description
104	Typical (small) fragment phthalates/dibutyl phthalate/phthalic anhydride
113	Bis(2-ethylhexyl) phthalate fragment
132	Benzylbutyl phthalate
149	Typical fragment phthalates
163	Dimethyl phthalate fragment
167	Bis(2-ethylhexyl) phthalate fragment
261	Bis(2-ethylhexyl) phthalate fragment
279	Bis(2-ethylhexyl) phthalate fragment and/or dibutyl phthalate M^+
301	Dibutyl phthalate $[M+Na]^+$
391	Diisooctyl phthalate $[M+H]^+$ and/or bis(2-ethylhexyl) phthalate $[M+H]^+$
413	Diisooctyl phthalate $[M+Na]^+$
429	Diisooctyl phthalate $[M+K]^+$

All fragments could be derived from known (typical) fragmentations of different phthalates. See references: [35-39].

A spectrum with the most observed fragments of phthalates is shown below in figure 59.

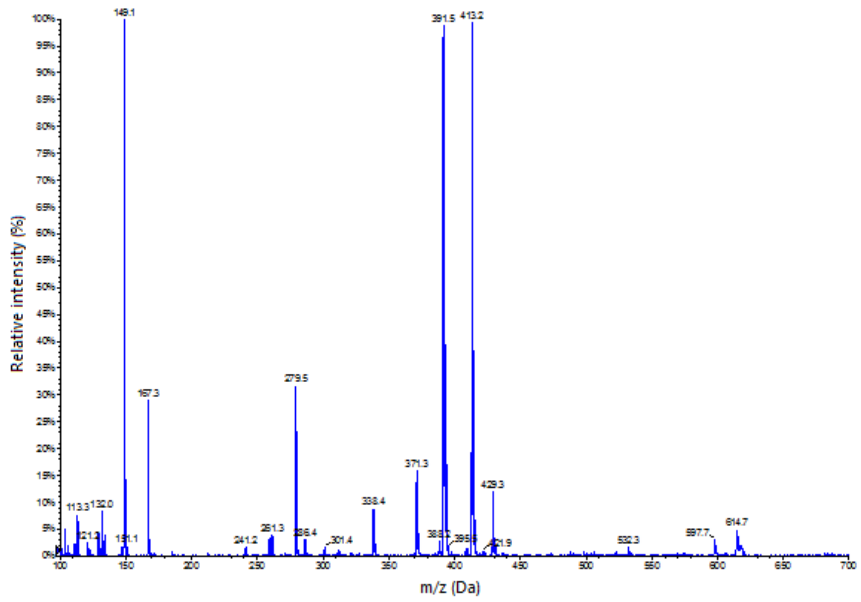

Figure 59. Mass spectrum with most observed fragments of phthalates, with the relative intensity (%) to m/z (Da). Spectrum was obtained from a measurement of a derivatization of vitamin D_3 with DNBC at 15.813-15.931 min.