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LIST OF ABBREVIATIONS

AMF	Anhydrous milk fat
$BaCl_2 \cdot 2H_2O$	Barium chloride dihydrate
DBE	Double-bond equivalent
C	Carbon
CID	Collision-induced dissociation
cm	Centimeter
COP	Cholesterol oxidation product
Cu	Copper
ESI	Electrospray ionization
etc.	Etcetera
eV	Electron volt
$FeSO_4.7H_2O$	Iron (II) sulfate heptahydrate
FWHM	Full width at half maximum
g	Gram
GC	Gas chromatography
GC-MS	Gas chromatography-Mass Spectrometry
H	Hydrogen
h	Hours
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HPLC-DAD	High Performance Liquid Chromatography- Diode array detector
H_2SO_4	Dihydrogen sulfate (sulfuric acid)
Kg	Kilograms
kV	kilovolt
L	Liter
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LTQ	Linear ion trap
MDA	Malondialdehyde
meq	Milligram equivalents
mg	Milligrams

min	Minute
mL	Milliliter
mm	Millimeter
mmol	Millmolar
mol	Mole
MRM	Multiple reaction monitoring
m/z	Mass-to-charge ratio
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multiple-stage mass spectrometry
N	Nitrogen
NH2	Aminopropyl
$NH_4Fe(SO_4)_2 \cdot 12H_2O$	Ammonium iron (III) sulfate
NMR	Nuclear Magnetic Resonance
O	Oxygen
PFP	Pentafluorophenyl phase
ppm	Parts per million
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
PTFE	Polytetrafluoroethylene
PV	Peroxide value
RP-HPLC	Reversed-phase high-performance liquid chromatography
RT	Room temperature
Si	silica
SPE	Solid phase extraction
SWMP	Simulated whole milk powders
TBA	2-thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEP	1,1,3,3-tetraethoxypropane
TLC	Thin layer chromatography
UHPLC	Ultra-high performance liquid chromatography

UHPLC-QQQ-MS/MS	Ultra-high performance liquid chromatography-triple quadrupole-tandem mass spectrometry
UHPLC-ESI-MS/MS	Ultra-high performance liquid chromatography-electrospray ionization -tandem mass spectrometry
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
VDE	vitamin D equivalent
VDOP	vitamin D oxidation products
WPI	Whey Protein Isolate
wt/vol	weight/volume
%	Percent
°C	Degree Celsius
µL	Microliter
µm	Micrometer
µg	Microgram

CHAPTER 1

Introduction and Research Objectives

1.1. Research background

Vitamin D refers to a group of fat-soluble vitamins responsible for increasing intestinal absorption of calcium, magnesium and phosphate, and multiple other biological effects. Vitamin D is found in two different forms as ergocalciferol (vitamin D2) in plants and cholecalciferol (vitamin D3) in animals. Research conducted over the past decade suggests that vitamin D plays a much broader disease-fighting role than once thought. Vitamin D deficiency may increase the risk of a host of chronic diseases, such as osteoporosis, heart disease, some cancers, and multiple sclerosis, as well as infectious diseases, such as tuberculosis and even the seasonal flu.

Fortification of foods is the practice of deliberately increasing the content of an essential micronutrient such as vitamins and minerals in a food to improve health benefits and reduce dietary deficiencies within a population (Allen, De Benoist, Dary, & Hurrell, 2006). Since only a limited number of foods contain vitamin D, such as cod liver oil, fatty fish, egg yolks and whole milk, the fortification of food with this nutrient has been practiced routinely in certain foods (Byrdwell et al., 2011; Kaushik, Sachdeva, Arora, & Wadhwa, 2014).

Among different foods, milk is a good target for vitamin D fortification as it is a widely consumed product with a high concentration of essential nutrients. Additionally, fat-soluble vitamins like vitamin D may be more stable during processing and storage in a fatty vehicle like whole milk powder (Murphy et al., 2001). Both vitamin D2 and D3 are used for food fortification, however, vitamin D3 is the preferred source because of its superior bioavailability (Trang et al., 1998). Therefore, among countries which fortify foods with vitamin D, vitamin D3 is regulated as a food additive for food fortification purposes. In a successful fortification program, the stability of micronutrients added to the food is one of the most important factors. The amount of micronutrient fortified should be sufficient to prevent deficiency problems. On the other hand, vitamin D is considered to be toxic in excessive levels as it causes serious adverse effects such as hypercalcemia, dehydration, heart damage, kidney damage and soft tissue calcification (Japelt & Jakobsen, 2013; Rubin, Thys-Jacobs, Chan, Koberle, & Bilezikian, 2005; Viswanath, 2013).

Regarding the stability of vitamin D, non-compliance of fortification of vitamin D has been a problem in a few products and some surveys reported under- or over-fortification of vitamin D

(Holick, Shao, Liu, & Chen, 1992; Liu, 2013; Murphy et al., 2001). Vitamins are well known to be unstable to stressors such as light, oxidation, heat and chemicals, thereby making its stability in food products uncertain and its analysis challenging. According to the previous studies, there are conflicting reports regarding the stability of vitamin D and they mostly reported that vitamin D3 was unstable towards these factors (Blanco, Fernandez, & Gutierrez, 2000; Cremin & Power, 1985; Pike & Brown, 1967).

Vitamin D is known to isomerize readily under various conditions to generate vitamin D isomerization products. Heat, light, iodine and acid are the most important physico-chemical parameters which have influences on the generation of vitamin D isomerization products. Thermal kinetics of isomerization of vitamin D to previtamin D is well known (Koshy & Beyer, 1984; Naidoo, 2011). Vitamin D is reported to form 5,6 *trans* isomer by exposure to ultraviolet radiation (Zhang, 2011). In addition, iodine can also be responsible for this isomerization pathway (Verloop, Koevoet, Van Moorselaar, & Havinga, 1959: Zhang, 2011). The iodine induced degradation of ecalcidene, an analogue of vitamin D3, was also reported by Zhang et al., 2006. Vitamin D can be chemically isomerized to corresponding isotachystero1 at low pH (Jin, Yang, Yang, Liu, & Zhang, 2004).

Contradictory reports have also been provided regarding vitamin D stability during processing and storage. Some studies have found that vitamin D is relatively stable during food processing (Stoklosinski, Fong, & Brevitt, 2001) and storage (Hanson & Metzger, 2010; Kazmi, Vieth, & Rousseau, 2007); however, some other studies reported vitamin D losses (Calvo & Whiting, 2003; Kazmi et al., 2007; Pike & Brown, 1967). Vitamin D is known to decline during storage of fortified products and oxidation is suspected as the likely cause. Vitamin D is rather unreactive towards atmospheric oxygen; however, it could be susceptible to reactive oxygen and/or oxidation products (Charlton & Ewing, 2007; Pearce & Wewala, 1993; Riaz, Asif, & Ali, 2009). Fatty acid lipoxidation, which produces reactive oxygen species, could be one of the ways which causes vitamin D degradation in fortified products containing fat. In addition, the reaction of lipid oxidation products with other essential nutrients such as vitamin D can have an impact on the nutritional value of the fortified foods.

Lipid oxidation is known as a major cause of quality deterioration during processing or storage of food products containing high levels of lipid such as whole milk powder. In milk powder production, heat treatment prior to drying and storage conditions are two factors which could have an effect on the lipid oxidation. For example, pasteurization of milk at high temperature heat treatment improved oxidative stability and result in lower cholesterol oxidation products compared to low-medium temperature heat treatment (Chan et al., 1993; Cluskey et al., 1997).

Another challenge in vitamin D study is its determination in natural foods due to the low amount of its content, even in vitamin D fortified foods. Since vitamin D is fortified at very low concentrations (10 to 100 parts-per-billion, ppb), any degradation products will be less abundant, and as well as that, there may be several breakdown products and isomers of each breakdown product (Kasalová, Aufartová, Krčmová, Solichová, & Solich, 2015; Luque-Garcıa & de Castro, 2001). Any method to extract vitamin D has to ensure that all breakdown products are captured and that they are not further altered by the extraction process.

Regarding the inconsistency of vitamin D content in fortified samples, it is unclear whether this is due to the analytical variability, the poor fortification practices or instability of the vitamin during processing or storage. AOAC has provided collaboratively studied and performance reviewed reference methods for vitamin D3 in infant formulas and milk powders (Gill, Abernethy, Green, & Indyk, 2016), so there is better confidence in the analytical results than in the past. However, fortification and stability of vitamin D3 remains areas of concern. In this respect, there are only a few literature references to the fate of degradation and oxidation of vitamin D3 in whole milk powder. Therefore, the paucity of literature on the stability of vitamin D3 in milk powder suggests that more studies might be needed to understand the fate of this vitamin in fortified foods, especially in milk powders.

1.2. Research objectives

There are two major objectives for this study. One of the main objectives of this thesis research is to isolate and identify vitamin D3 degradation products including isomerization and oxidation products. To achieve this main goal, the first step is to produce or synthesise vitamin D3 degradation products under different conditions as these compounds are not available from the

usual commercial chemical providers. A systematic study is needed to investigate the effect of different physico-chemical parameters on vitamin D3 stability. Learning how to identify these degradation products using the available analytical instrumentation will provide tools necessary for their identification, should they exist in degraded milk powders. The second step is to find methods to extract these products as effectively as possible. As mentioned before in this chapter, identification of vitamin D and its degradation products has been a challenge because of their low concentrations in the samples, and potentially relatively large quantities of interfering substances. Moreover, vitamin D3 has similar structure to its degradation products and also to other expected compounds in milk powder such as steroids, cholesterols and plant sterols which make their identification more challenging. Therefore, use of more sophisticated instruments was needed to analyse these products. In addition, optimization of the sample preparation process plays an important role to enhance sensitivity and reduce matrix interference in samples.

The second major objective was to study the vitamin D3 stability in whole milk powder during storage period. As it was mentioned earlier, reactive oxygen or oxidation products could be a way to oxidize vitamin D3. In whole milk powder, milk fat can react with oxygen to cause oxidation especially at long-term storage and higher temperatures. In this research, a model system with a milk powder matrix was developed to study the vitamin D3 degradation with reduced interference from other compounds. The influence of heat treatment and storage conditions on lipid oxidation and vitamin D3 degradation was investigated in these simulated whole milk powders. An increase in the concentration of added vitamin D3 over what is commonly used in commercial fortification practice was used to increase the sensitivity in isolating and identifying these products.

1.3. Thesis framework

In this thesis, the work is presented following the outline below.

Chapter 1 provides a background to the research and the objectives of the thesis.

Chapter 2 introduces the background knowledge of vitamin D3 and its degradation products including their structures and properties. This chapter highlights the effects of different parameters on the stability of vitamin D in different food products. It also reviews relevant studies on the analysis of vitamin D and related products.

Chapter 3 focuses on the production and characterisation of vitamin D3 isomerization products. These products were isolated and identified using a combination of HPLC-DAD, UHPLC-QQQ-MS/MS and chemical derivatization using a dienophile (PTAD).

Chapter 4 investigates the effects of processing condition (heat treatment) and storage conditions (time and temperature) on lipid oxidation and vitamin D3 degradation in a model system comprised of simulated whole milk powder. The most abundant of the vitamin D3 oxidation products in this study were identified and quantified in stored samples.

Chapter 5 presents a detailed study to isolate and identify vitamin D3 oxidation products (VDOPs) under oxidative conditions. Sample preparation and extraction process optimization were significant steps to reduce matrix interference in samples containing fat. VDOPs were identified using LC-MS and LC-MS^n techniques and fragmentation modelling gave reliable methods to identify these compounds.

Chapter 6 summarizes and concludes the findings in this thesis. Further work for the future development of this study is also discussed.

CHAPTER 2

Literature Review

2.1. Structures and properties of vitamin D and its isomerization products

2.1.1. Vitamin D

Vitamin D is a group of fat-soluble seco-steroid compounds with relatively low polarity. Vitamin D, including cholecalciferol (D3), (5Z,7E)-(3S)-9,10-seco-5,7,10(19)-cholestatrien-3-ol, and ergocalciferol (D2), (5Z,7E,22E)-(3S)-9,10-seco-5,7,10(19),22-ergostatetraen-3-ol, are originated from their precursors 7-dehydrocholesterol and ergosterol in animals and fungi, respectively. Whereas vitamin D3 is produced naturally in the skin by ultraviolet (UV) irradiation of 7-dehydrocholesterol (Figure 2.1), vitamin D2 is produced in fungi and yeast by a UVB-exposure of ergosterol (provitamin D2) (Figure 2.2) (DeLuca & Schnoes, 1976; Japelt & Jakobsen, 2013).

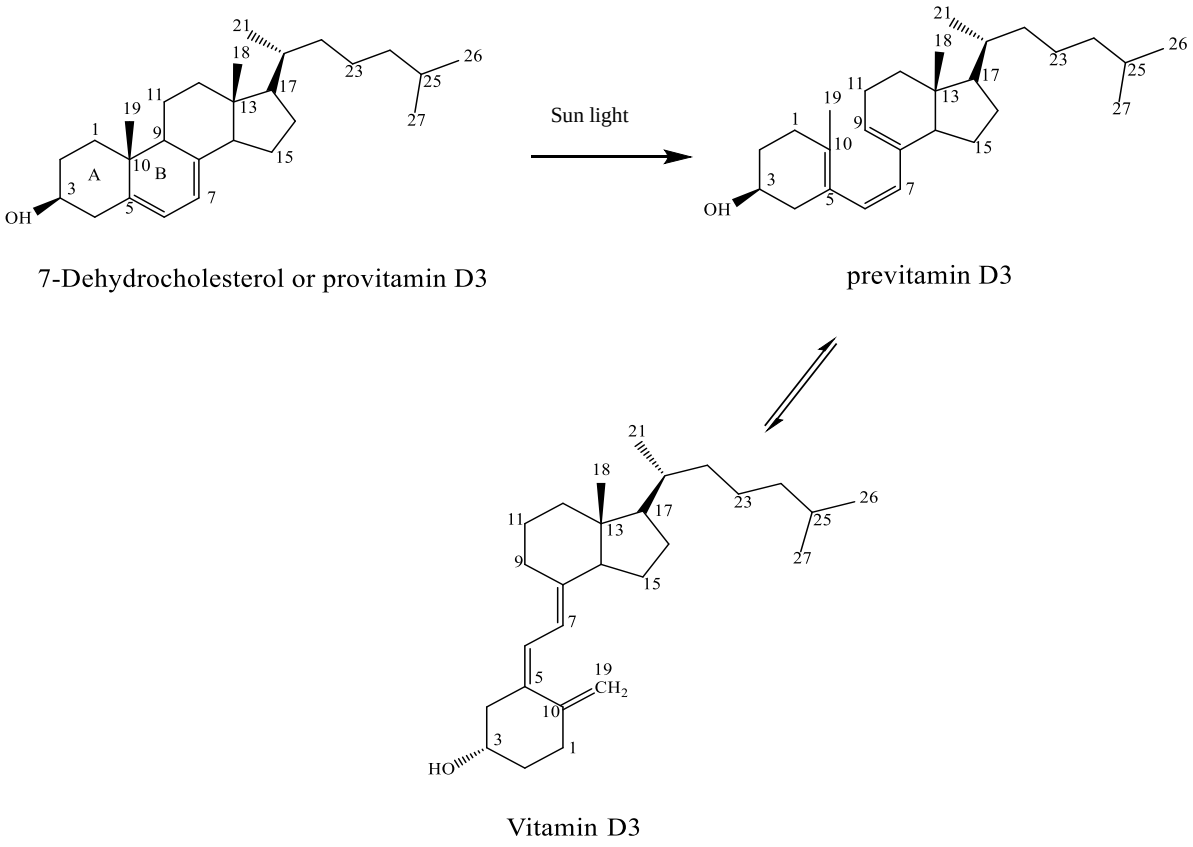


Figure 2.1. Structures of vitamin D3

The cleavage of B-ring between C9 and C10 of vitamin D is central to the creation of the 5, 6, 7 *cis*-triene structure of previtamin D. These compounds have an open B-ring and a conjugated triene system which plays an important role not only in the biological activity of vitamin D but also have an interestingly parallel synthesis from the chemistry point of view. Both vitamin D2 and D3 have a methylene group at C10 and the 5, 6 double bond is considered to be *cis*, and vitamin D2 has an extra methyl group at C24 and an additional double bond in its side chain at C22 (Figure 2.2) (Koshy & Beyer, 1984; Martínez-Núñez, Cabaleiro-Lago, Fernández-Ramos, Hermida-Ramón, & Peña-Gallego, 1999).

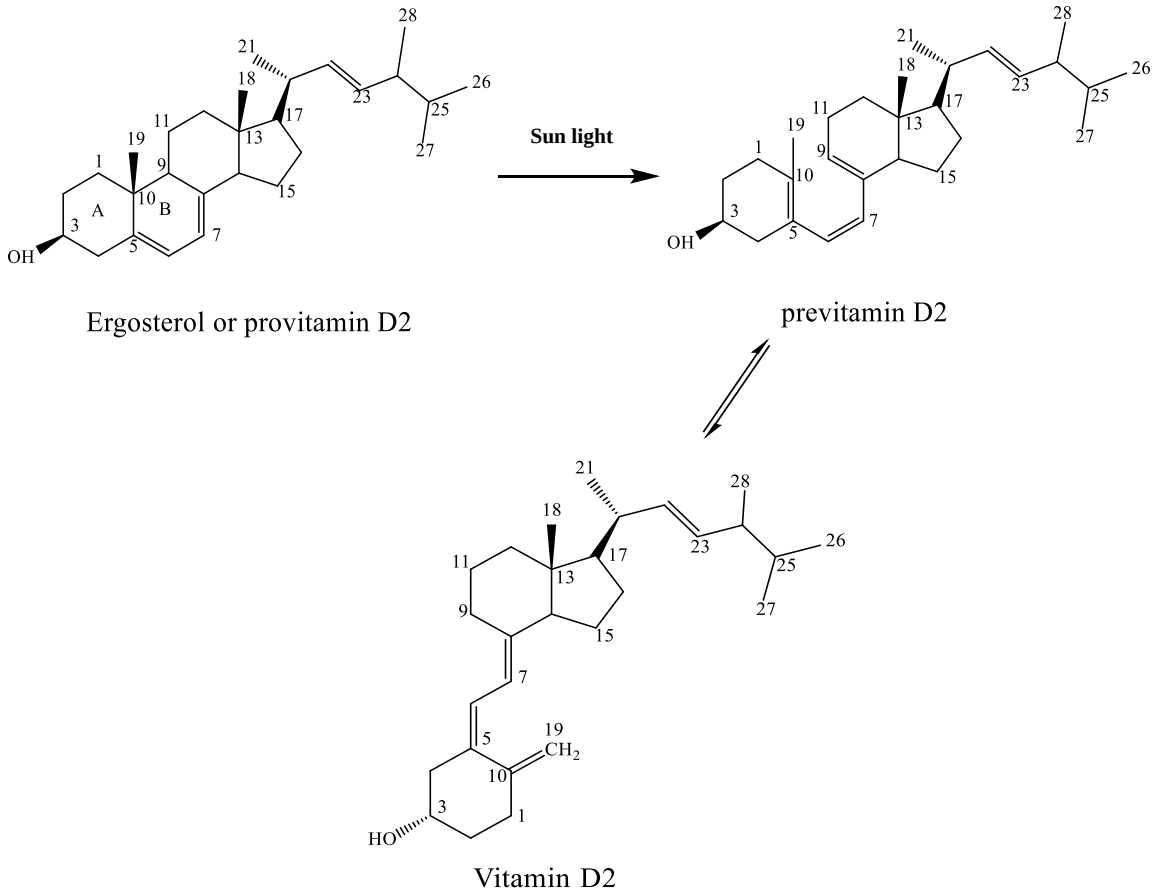


Figure 2.2. Structures of vitamin D2

2.1.2. Previtamin D3

Previtamin D3, (6Z)-(3S)-9,10-seco-5(10),6,8-cholestatrien-3-ol, is an intermediate in the production of vitamin D. In terms of vitamin D formation, the first step is the photolytic opening of the B ring to form previtamin D, followed by the second step, which is thermally favoured transformation of previtamin D into vitamin D (Figure 2.1 and 2.2).

It is well known that vitamin D in solution exists in an equilibrium between vitamin D and previtamin D (Mulder, Vries, & Borsje, 1971). In this regard, Johnsson & Hessel (1987) reported partial isomerization of vitamin D into its precursor previtamin D during the saponification process employed for its extraction. The conversion of vitamin D to previtamin D is a thermal equilibrium and it has been shown that the equilibrium rate constant is independent of the nature of the solvent. The percentage of vitamin D3 in equilibrium with previtamin D3 ranges from 98% at -20°C to 78% at 80°C. Thus, temperature is an important factor in the equilibrium of vitamin D3 to previtamin D3 (Koshy & Beyer, 1984; Naidoo, 2011). The UV absorption maxima in the spectrum of *cis*-vitamin D3 after heat treatment changes from 265 nm to 261 nm as a result of the contribution of the UV absorption spectra of previtamin D3 (Figure 2.3) (Verloop at al., 1959). The extinction coefficient also changes for the conversion of vitamin D to previtamin D (Hanewald, Rappoldt, & Roborgh, 1961; Hanewald, Mulder, & Keuning, 1968).

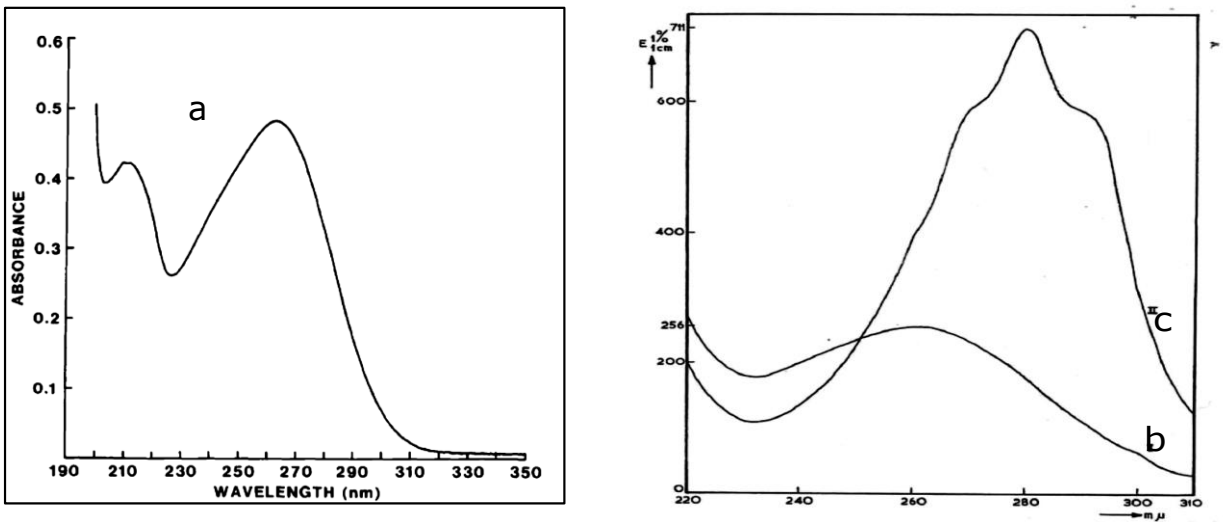


Figure 2.3. The UV spectra of vitamin D3 (**a**) previtamin D3 (**b**) and tachysterol (**c**) (ref. Koshy & Beyer, 1984; Verloop et al., 1959).

2.1.3. 5,6-*trans*-vitamin D3

The *cis* configuration of vitamin D is important for its biological activity but the *trans* from has very low activity. Vitamin D could be rearranged by exposure to ultraviolet radiation to form 5,6 *trans* isomer, (3S,5E,7E)-9,10-Secocholesta-5,7,10-trien-3-ol, (Figure 2.4). Moreover, the *trans* isomer can irreversibly undergo further isomerization mostly to isotachysterol through exposure to heat or acids (Havinga, 1973; Jin et al., 2004). The *cis* and the *trans* forms of vitamin D3 display different UV spectra. While the *cis* form has a UV maxima at 265 nm (Figure 2.3 a), the *trans* form shows a UV maxima at 273 nm (Figure 2.5) (Holick, Garabedian, & DeLuca, 1972; Verloop, Koevoet, & Havinga, 1955).

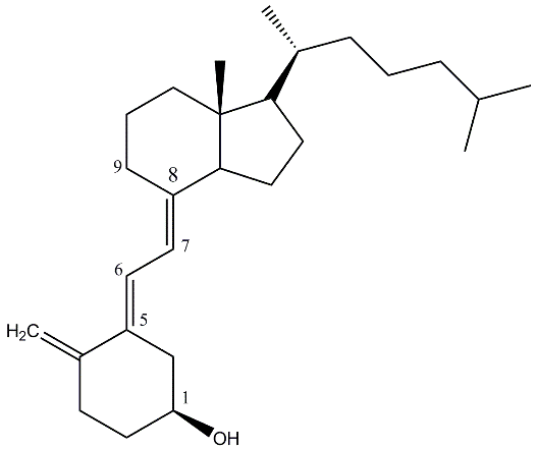


Figure 2.4. Structure of 5,6-*trans*-vitamin D3

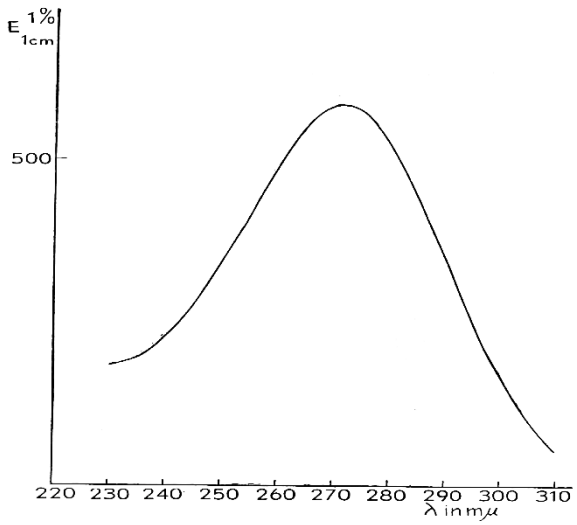


Figure 2.5. The UV spectrum of 5,6-*trans*-vitamin D3 (ref. Verloop et al., 1955).

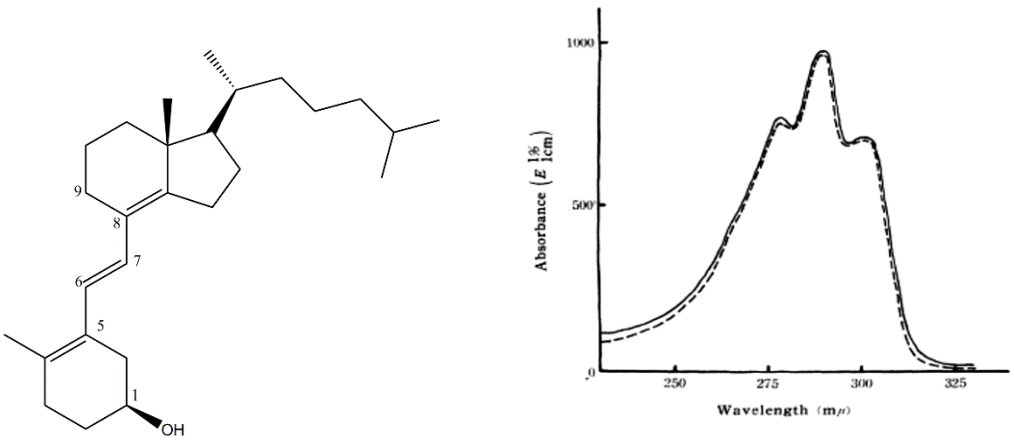
2.1.4. Isotachysterol

Vitamin D3 can be isomerized to corresponding isotachystero1$_3$ derivatives, (6E)-(3S)-9,10-seco-5(10),6,8(14)-cholestatrien-3-ol, (Figure 2.6 a). In this reaction, the double bond system of the secosteroid is rearranged and the A-ring rotated to expose the hydroxyl group of C1. The spectral properties and the polarity of the molecule are thus altered (Trafford, Seamark, Turnbull, & Makin, 1981).

Low pH is known to isomerize vitamin D3 to isotachysterol which exists as a pale yellow oily liquid (Jin et al., 2004). It was reported that vitamin D, previtamin D and tachysterol can be converted to isotachysterol in various acidic conditions such as HCl, BF_3 and H_3PO_3, but the HCl procedure is the most efficient (Seamark, Trafford, & Makin, 1980; Verloop, Corts, & Havinga, 1960; Zhang et al., 2006). It was also reported that in the preparation of isotachysterol, the isomerization yield is affected by the temperature and volume of the reaction mixture and the duration of the reaction (Agarwal, 1990). Zhang et al. (2006) suggested a reaction model by which the acid induced formation of isotachysterol from vitamin D may sequentially proceed through the intermediates of previtamin D and tachysterol. Agarwal, 1990 reported that isotachysterol has absorption maxima at 278, 288 and 301 nm with its molar absorption being more than twice that of vitamin D at 265 nm (Figure 2.6 b) (Kobayashi, 1965).

2.1.5. Tachysterol

Previtamin D3 can be isomerized by ultraviolet (UV) radiation to tachysterol$_3$, (6E)-(3S)-9,10-seco-5(10),6,8-cholestatrien-3-ol, (Figure 2.7). Tachysterol, which is a 7,8 *cis*-isomer of isotachysterol and a *trans*-isomer of previtamin D, could also be generated by chemical isomerization (Verloop et al., 1960). The irreversible isomerisation to inactive products has a major impact on formulation of vitamin D so that all aspects of isomerisation need to be considered. The UV absorption of tachysterol was considered to agree more with a *trans* structure with an absorption peak at 281 nm and shoulders at 272 and 289 nm (Figure 2.3 c) (Lugtenburg & Havinga, 1969; Verloop et al., 1959).



a) **b)**

Figure 2.6. Structure of isotachysterol (**a**) and the UV spectrum of isotachysterol (**b**) (ref. Kobayashi, 1965)

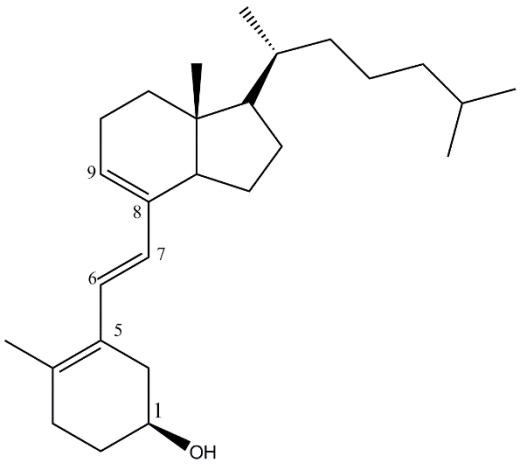


Figure 2.7. Structure of tachysterol

2.2. Food fortification

2.2.1. Background

Food fortification, defined by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations (Allen et al., 2006), refers to "the practice of deliberately increasing the content of an essential micronutrient, ie. vitamins and minerals (including trace elements) in a food irrespective of whether the nutrients were originally in the food before processing or not, so as to improve the nutritional quality of the food supply and to provide a public health benefit with minimal risk to health". WHO and FAO (2006) have identified food fortification as the second strategy of four, in decreasing the incidence of nutrient deficiencies at the global level. As micronutrient malnutrition (MNM) has many adverse effects on human health, food fortification has had a long history of controlling the deficiencies of different micronutrients such as vitamin D, A, B, iodine and iron. The foods that are most commonly fortified include milk and milk products, infant formulas, flour, salt, and oils and fats. There are several forms of food fortification: foods that are widely consumed by general population, foods fortified for specific populations, and foods which are fortified by food manufacturers. Food fortification carries a number of advantages and limitations over supplementation as follows (Allen et al., 2006):

- Consuming fortified foods regularly maintains nutrients more efficiently and effectively in the body. However, it has a less immediate impact but is usually the shortest way to treat deficiencies compared to supplementation.
- One of the most important advantages of food fortification is a prolonged impact for a population who needs a continual supply of micronutrients for growth and development. On the other hand, supplements are needed to satisfy the requirements of selected population groups such as infants and pregnant women who need adequate amounts of some micronutrients.
- In contrast to supplements, which are consumed by individuals who are willing to take them and can afford to buy them, fortified foods are widely distributed and consumed. Thus, a large proportion of the population can be accessed by food fortification. Since broad access to fortified foods may cause increase levels of micronutrients for all population groups, it could be harmful for those who do not suffer from malnutrition.

- Since foods are complex mixtures, some technological issues relating to fortification including appropriate levels of nutrients, homogeneous distribution of the nutrients within the food matrix, stability of fortificants, nutrient interactions, as well as accessibility by consumers have to be resolved. Additionally, due to the likelihood interactions between the added micronutrients and the food vehicles, and with other nutrients (either added or naturally present), the fate of fortificants should be investigated in fortified foods.
- Although multiple micronutrients fortification is technically possible, fortified foods alone may not be sufficient to supply all micronutrients, but, supplement intake might be able to resolve this issue.
- While fortification carries a minimal risk of chronic toxicity, it still can cause a risk of excess. Supplement intake, however, is likely to be better regulated by the individual. However, there is a low compliance for using supplements and those who disregard the instructions may have a high risk of, for example, vitamin D deficiency (Holick, 2006). Therefore, fortification of regularly consumed foods may be more appropriate since they do not require individual compliance or changes in purchasing patterns.

Food fortification is a complex task and there are several factors to be considered in this matter:

- Nature of the fortificant: The micronutrient, which is used for fortification, should be safe, stable, and well absorbed. The fortificant should resist processing and storage conditions without significant degradation.
- Nature of the food vehicle: An appropriate food vehicle that protects the fortificant from degradation during processing and storage should be chosen. The food vehicle containing components that could compromise the stability and/or absorption of the fortificant should be avoided.
- Consumer satisfaction: The fortificant should not adversely affect the quality of final product such as chemical composition, cooking properties or sensory qualities (e.g. colour, flavours or texture).
- Non-compliance of fortification: The amount of micronutrient should be safe and sufficient to prevent deficiency problem. Vitamins A and D are considered toxic in excessive levels of consumption. These fat-soluble vitamins accumulate in tissue over time and are not easily eliminated from the body. Excessive intake of vitamin D causes serious adverse effects such as hypercalcemia, dehydration, heart damage, kidney damage and soft tissue calcification (Rubin et al., 2005; Viswanath, 2013).

2.2.2. Vitamin D fortification

Vitamin D is a regulator of calcium metabolism and is involved in the absorption of calcium in the intestines. Moreover, vitamin D is required for bone growth in the mineralization process. Chronic severe vitamin D deficiency in children causes bone deformation due to poor mineralization, which is known as rickets. In adults, vitamin D deficiencies can result in muscle weakness, bone pain, osteomalacia and rapid development of osteoporosis. Apart from the effects of vitamin D on skeletal health, it may play a role for regulating cell growth, the immune system, and other physiological processes (Bischoff-Ferrari et al., 2004; Bischoff-Ferrari, Giovannucci, Willett, Dietrich, & Dawson-Hughes, 2006; Giovannucci, 2005; Lappe, Travers-Gustafson, Davies, Recker, & Heaney, 2007).

Vitamin D3 is mostly produced via the exposure of the skin to the sun. Vitamin D intake from sunlight depends on latitude, season, cloud cover, ozone level, surface reflection, altitude, outdoor practices, skin type, obesity, age and clothing (Engelsen, 2010; Lips, 2010; Rockell et al., 2005; Webb, Kline, & Holick, 1988). Although the human body is able to generate vitamin D through exposure to the sun, individuals who do not receive sun exposure, people with dark pigmented skin and older people are high-risk groups of vitamin D deficiency (Kiely & Black, 2012; Whiting, Green, & Calvo, 2007). Only a limited number of natural foods contain vitamin D. Vitamin D3 is present in animal foods such as eggs, fish species (salmon, herring and mackerel) and liver. Vitamin D2 also has been found in some wild mushrooms, where it appears to be formed from the action of UV on the provitamin, ergosterol (Teichmann et al., 2007). Other plant sources used as food may contain ergosterol that is not converted to vitamin D2 (Lamberg-Allardt, 2006).

Vitamin D malnutrition is likely to be recognized as a significant public health problem in many countries. The importance of vitamin D in human diet has been recognized since the early 1900's (McCollum, Simmonds, Becker, & Shipley, 1922). Few countries fortify foods with vitamin D. Vitamin D3 is now the permitted form for addition to foods in Canada, while the USA and European Union (EU) permits use of either vitamin D2 or vitamin D3. In these countries, foods usually fortified with vitamin D are milk products, margarines and breakfast cereals (Lamberg-Allardt, 2006). In Australia, it is mandatory for margarines to contain no less than 55 µg/kg of vitamin D. This mandatory requirement does not apply to these foods for sale in New Zealand (Food Standards Australia New Zealand, 2016).

Vitamin D also may be sourced from dietary supplements (usually in pill, capsule, tablet or other controlled dosage form). The vitamin D present in supplements can be in the form of both vitamin D2 and vitamin D3. However, vitamin D2 is rarely used as the fortificant in supplements (Rockell, Skeaff, Logan, & Green, 2008).

2.2.3. Vitamin D fortification of whole milk powder

Milk is a widely consumed product and contains a high concentration of essential nutrients such as protein, calcium and phosphorus. The high solubility of vitamin D in the lipid matrix of milk may also increase its bioavailability. Moreover, fat-soluble vitamins may be more stable during processing and storage in a fatty vehicle like whole milk powder. Thus, milk powder is a good source for food fortification (Murphy et al., 2001).

The first step in developing a fortification process is the development of a stable and reliable vitamin concentrate or premix. When two or more vitamins are added to a food product at the same manufacturing stage, this is commonly done using a premix or a blend. A premix is a homogeneous mixture of the desired vitamins in a dry powder form. Suppliers provide different types of vitamin premix including oil based and water dispersible formulations. Cold-water-soluble (CWS) vitamin D concentrate is a solution of vitamin D3 in medium chain triglycerides (MCTs) that is homogenized into a gum arabic and sucrose solution, dried and coated with starch and calcium phosphate. The premixes usually contain antioxidants such as tocopherol and/or ascorbic acid (BASF, 2015).

Production of powdered milk or dried milk powder is a simple process which involves different steps including separation, preheating, evaporation and spray drying. In preheating step, milk is heated to temperatures between 75 and 120°C and held for a specific time. This step causes denaturation of the whey proteins (to expose antioxidant thiol groups) resulting in a decrease of autoxidation rate. It also destroys bacteria and inactivates enzymes. Whole milk powders made from preheated milk are associated with improved quality. During milk powder manufacture, water is removed by boiling the milk under reduced pressure at low temperature in a process known as evaporation. The resulting concentrated milk is then sprayed in a fine mist into a stream

of hot air to remove moisture almost instantly to produce a powder. Milk powders may vary in their composition (milk fat, protein, lactose), the heat treatment they receive during manufacture, powder particle size and packaging.

The point of addition of vitamin D might affect the final fortification level. Vitamin D is mostly added after standardization and before preheating of the fluid milk. Vitamin D is a fat-soluble vitamin and is supplied in an oil droplet in the premix, so it would merge into the fat matrix after standardization process. Since homogenization assists to disperse and stabilize the fortificants, it is recommended that vitamins be added before homogenization. In terms of the method of addition, manual (batch) and injection meter (continuous) addition with metering pumps have been used for fortification. Manual addition is routinely used for batch pasteurized milk products or for small batches of product. The batch procedure requires accurate measurement of the volume of milk to be fortified, accurate measurement and addition of the required amount of vitamin concentrate and proper mixing (Murphy et al., 2001). Metering pumps allow continuous addition of vitamin concentrate based on the flow rate of the high temperature short time (HTST) pasteurization or other continuous flow systems employed. The product pressure at the injection site should be determined to ensure that it does not exceed the capabilities of the metering pump (Hicks, Hansen, & Rushing, 1996). Vitamin D may also be fortified as a dry-blend and premix of vitamin D is blended with oil or butter, and sprayed into warm freshly dried milk powder in a fluid bed dryer. In this case, homogeneity is more difficult to achieve, but the fortificants have not been exposed to higher temperatures during drying (O'Brien & Roberton, 1993).

2.3. Vitamin D stability

The stability of micronutrients added to the food is one of the most important factors in a successful fortification program. Vitamins are well known to be unstable to stressors such as light, oxidation, heat and trace metals (Deritter, 1982). There are conflicting reports regarding the stability of vitamin D towards light, temperature, humidity and oxygen (Abernethy, 2011). The forms of vitamin D3 available for fortification (crystalline, dissolved and encapsulated) are important and may lead to variable degradation. It was indicated that vitamin D oxidized more rapidly under dry condition than in emulsion (Byrn, 1976). Previous studies reported different vitamin D stability under various conditions in solid forms of the pharmaceutical products (Byrn, 1976; Grady & Thakker, 1980; Liu, 2013). However, there are only a few studies published on the stability of vitamin D in liquid form or in foods.

2.3.1. Vitamin D degradation

Vitamin D degradation products seem to be limited to the isomerization products, which have conjugated dienes that have specific absorbencies in the UV-Visible region. Thus, some of them reported in the early studies, are detectable by UV or are reactive towards dienophile. Some of the early studies reported that vitamin D give a number of isomerization products under different conditions but their structures have not been determined. They also reported the formation of unidentified products of higher polarity than vitamin D (Byrn, 1976; Grady & Thakker, 1980).

Since the early studies on vitamin D were mostly on assaying anti-rickets activity, rather than measuring vitamin D directly, very few data on non-active degradation products or isomers were reported. Although there are a number of studies on the isomerization of vitamin D2 under different conditions, few of them identified degradation products in formulations and fortified products, which created a gap in the field of vitamin D degradation. From a practical viewpoint, few of the identified isomers of vitamin D3 are or were available as standards from commercial chemical suppliers. In this respect, a range of vitamin D isomers and oxidation products, some listed in this Chapter, were generated in the laboratory for use as analytical standards. Various conditions under which vitamin D3 degradation products formed are listed below.

2.3.1.1. Light-induced degradation

Provitamin D3 is converted to previtamin D3 by UV irradiation, while further irradiation may induce isomerization to other sterols, including toxisterol, suprasterol, tachysterol and lumisterol in a process that is accelerated by heating (Indyk, 2006). While vitamin D3 appears to be photostable, in practice it reacts with light indirectly because of the presence of photolabile contaminants (such as riboflavin, carotenoids, chlorophylls) in biological samples. In the presence of light, riboflavin generates highly reactive singlet oxygen from atmospheric oxygen, which may rapidly oxidize vitamin D. Oxidation of vitamin D2 was observed neither in samples without riboflavin that were stored in the light nor in samples with riboflavin that were stored in the dark (Li & Min, 1998).

2.3.1.2. Thermal degradation

The reversible thermal isomerization of vitamin D to previtamin D in solution is well documented (Zhang et al., 2006). In a vitamin D3 solution, the isomerization rate is not affected by the solvent, acidity, ultraviolet light, catalysts, and free-radical reaction inhibitors; however, it depends on the storage time and temperature (Buisman, Hanewald, Mulder, Roborgh, & Keuning, 1968). Because in biology, both vitamin D and previtamin D are freely interconverting, they have equivalent anti-rickets activity. In determining vitamin D content, the presence of previtamin D should be taken into account. Therefore, quantifying the potential vitamin D content, which is a sum of the vitamin D and previtamin D, is more reliable than the actual vitamin D content.

Vitamin D3 can undergo complex irreversible reaction at higher temperature, pyrovitamin D3 and isopyrovitamin D3 (Grady & Thakker, 1980; Nair, Bucana, de Leon, & Turner, 1965). The conversion of vitamin D3 to the pyro compounds is believed to take place through the intermediate previtamin D as shown in Figure 2.8 (Havinga & Schlatmann, 1961; Pelc & Marshall, 1978; Verloop, Koevoet, & Havinga, 1957). This isomerization, which is based on ring closure, occurs only in *cis*-configuration and the *trans* conformers show no evidence of ring closure (Nair, 1968). Pyrovitamin D3 and isopyrovitamin D3 are not expected to be naturally occurring degradation products of vitamin D3 due to the harshness of the conditions for their generation. In this respect, at high temperature, 5,6-*trans*-vitamin D undergoes double bond rearrangement to the corresponding isotachysterol (Figure 2.9). Apart from that thermal isomerization of vitamin D3 depends on the solvent and a weak acidity solvent like DMSO gives isovitamin D3, (5E,7E)-(3S)-9,10-seco-1(10),5,7-cholestatrien-3-ol, and istachysterol as principal isomerization products at high temperature (Jin, Yang, Yang, Liu, & Zhang, 2003) (Figure 2.9).

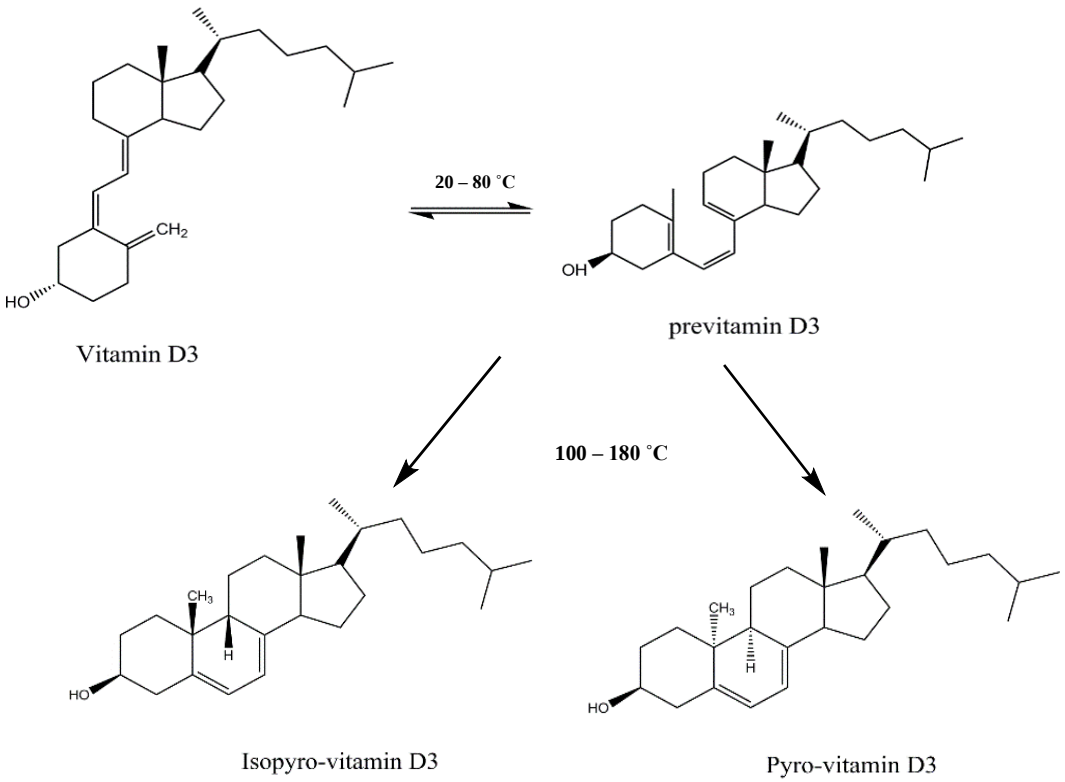


Figure 2.8. The thermal conversion of vitamin D3 to pyro compounds

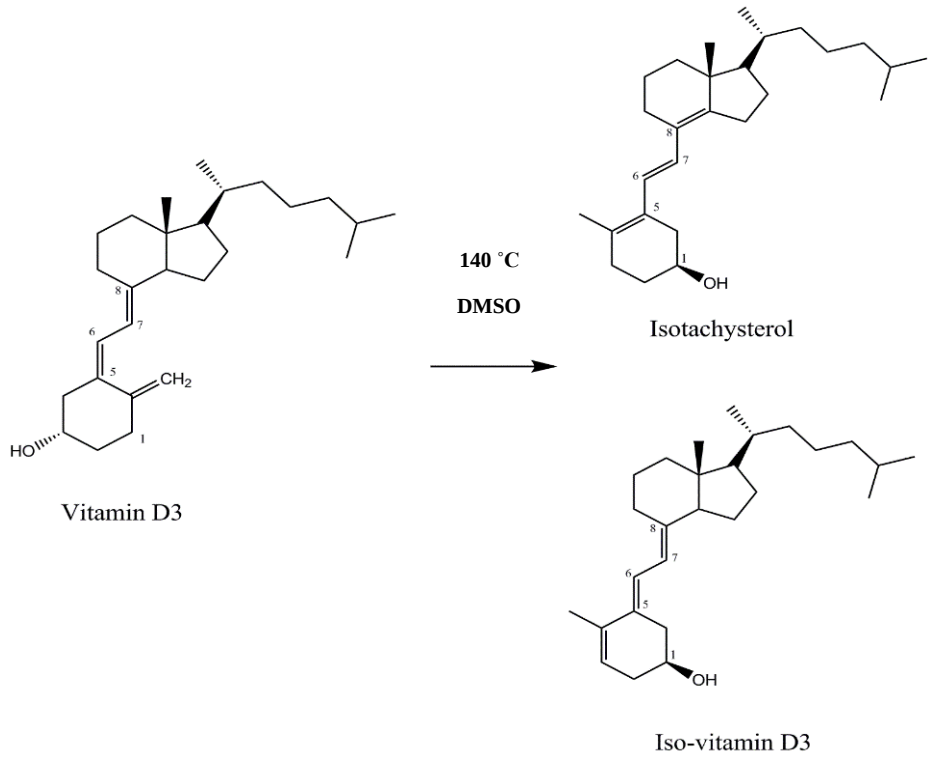


Figure 2.9. The thermal conversion of vitamin D3 to isotachysterol and isovitamin D3.

2.3.1.3. Chemical degradation

Acid-induced degradation

Vitamin D3 is isomerized to isotachysterol by acid treatment. Two studies have reported the presence of a yellow liquid when vitamin D3 was exposed to acid (Grady & Thakker, 1980; Jin et al., 2004). Zhang et al. (2006) also reported the acid induced formation of isotachysterol through the intermediate of previtamin D and tachysterol. According to Agarwal (1990), the temperature, time and volume of solution directly affect the yield of isotachysterol on acid treatment of vitamin D3. Apart from that, it is demonstrated that vitamin D3 is stable in the air at ambient temperature; however, its acid-catalyzed isomerization product seems very labile in the air (Jin et al., 2004). Since isotachysterol could be formed by acidic vitamins, similar autoxidation reaction might take place in the food systems.

Iodine-induced degradation

Although iodine is a natural constituent of milk, the concentration is influenced by iodine intake, season, level of milk production and the use of iodine-containing disinfectants to maintain udder and dairy equipment sanitation. In the case of vitamin D3-fortified milk powder, the iodine levels in the milk are high enough to be involved in the degradation of vitamin D3. In one study the mean iodide levels in milk were approximately 24 µg/200 mL of fresh milk (Cressey, 2003), which is perhaps three times higher than the level of vitamin D3 fortification (Abernethy, 2010).

If iodine is involved in vitamin D3 degradation, it may occur from direct addition to dairy equipment. In addition, iodide, a natural constitute of milk, may be oxidized to iodine by a range of oxidants including sanitizers and free radicals from lipid peroxidation. Thus, iodine should be considered as a potential confounding factor for vitamin D isomerization.

In the case of vitamin D3, the 5,6-double bond isomerizes to the *trans* configuration from the naturally occurring *cis* configuration to become 5,6 *trans* vitamin D3. In early studies, *cis-trans* isomerization of vitamin D2 was reported by iodine. The iodine induced degradation of ecalcidene, an analogue of vitamin D3, was also reported by Zhang et al. (2006). Early studies reported several isomers from iodine isomerization of vitamin D2 which depends on

Iodine/Vitamin D ratio and solvent used. For instance, vitamin D in non-polar solvents or in more polar solvents containing small quantities of pyridine was isomerized to 5,6-*trans*-vitamin D when small quantity of iodine was added, whereas it was isomerized to isotachysterol when a little larger quantity of iodine was added. Apart from that, previous studies reported that there is a significant influence of diffused light on the iodine isomerization of vitamin D. By complete elimination of light, no *trans* isomer and by exposing to bright sunlight, a very low yield of the *trans* isomer could be obtained, while reproducible levels of the *trans* isomer could be formed in diffused daylight (Verloop et al., 1959).

The differences between *cis* and *trans* configuration of vitamin D3 could be distinguished in different ways. While they have the same molecular weight and formula, their UV and NMR spectra vary. On the other hand, it is known that the reaction rates of *cis* and *trans* forms with dienophiles are different, while a *cis* substituent lowers the reaction rate, a *trans* substituent has no such influence (Zhang et al., 2006). Therefore, derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), which is a good dienophile, can be used to distinguish the *trans* forms of vitamin D from the *cis* isomers.

Cysteine-induced degradation

Vitamin D degradation occurs in infant formulas, especially when hydrolyzed proteins are used for their manufacture. Hydrolyzed proteins are proteins which have been broken down into much smaller peptides. Degradation of vitamin D in the presence of hydrolyzed protein might be due to the hydrolyzed protein source, the method of the hydrolysis, the presence of cysteine and its dimer cystine. In the presence of cystine and cysteine, vitamin D3 may undergo isomerization because of thiyl radicals that stem from these sulfur containing amino acids to form beta-thioalkyl free radical intermediates which form reversible reactions with unsaturated organic compounds like vitamin D (Abernethy, 2011). Therefore, cysteine, as thiyl radical precursor, is responsible for the isomerization of vitamin D to 5,6-*trans*-vitamin D and tachysterol. Hill et al. (1995) indicated a need for cystine among different amino acids in the degradation process of vitamin D. In addition, another problem in the presence of cystine is that preservatives such as BHT and BHA, which inhibit free radical reactions, are ineffective in the case of thiyl free radicals.

***Trans*-esterification with triglycerides**

Vitamin D may form esters with fatty acids by a mechanism of *trans*-esterification under heat or prolonged storage (Figure 2.10). As vitamin D premix is supplied in various matrices that may contain a large proportion of triglycerides, *trans*-esterification of vitamin D3 might occur in the ingredient supply chain (Ballard, Zhu, Nelson, & Seburg, 2007). *Trans*-esterification might also occur in dairy products because of common enzymes (e.g. esterases or lipases). The range of fatty acids esterified (oleic, palmitic, stearic etc.) would reflect the relative abundance of the fatty acids in the matrix triglycerides. Since vitamin D3 premix contains MCTs, it would be theoretically possible to distinguish vitamin D3 fatty acid esters formed in dairy product from the vitamin D3 premix ingredient (Abernethy, 2011).

With respect to vitamin D analysis, during sample preparation, the saponification step should also hydrolyze any vitamin D3 fatty acid esters which are formed during the storage to liberate vitamin D3. Thus, the vitamin D3 will be accounted for and will probably not appear to have been degraded; therefore, this degradation route would not be detected. Esterification by fats can logically be discounted as the cause of stronger vitamin D3 degradation.

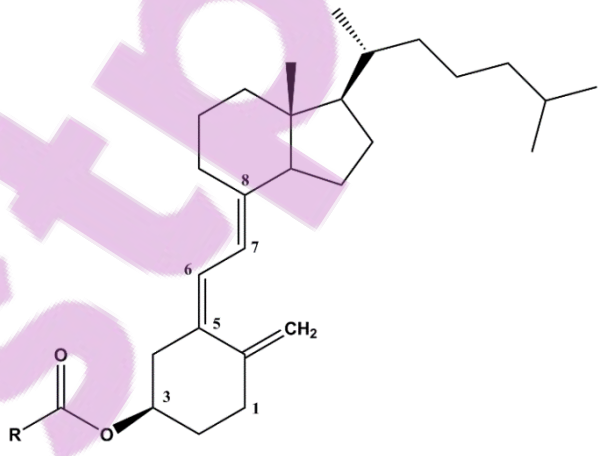


Figure 2.10. Vitamin D3 fatty acid ester. R = alkyl

2.3.1.4. Studies of vitamin D oxidation

Oxidation of vitamin D with singlet oxygen

Vitamin D is known to be sensitive to oxidation, due to the presence of the *cis* diene function in the molecule and can be oxidized by either diradical triplet oxygen or non-radical singlet oxygen. Singlet oxygen is a highly reactive, electrophilic, and non-radical molecule. Highly reactive singlet oxygen could be generated with a photosensitizer pigment from atmospheric oxygen. The singlet oxygen oxidation of food has contributed to the several important chemical reactions in the flavour of soybean oil and milk products, and the rapid losses of vitamin D, riboflavin and ascorbic acid in milk products (Choe & Min, 2005; Min & Boff, 2002b).

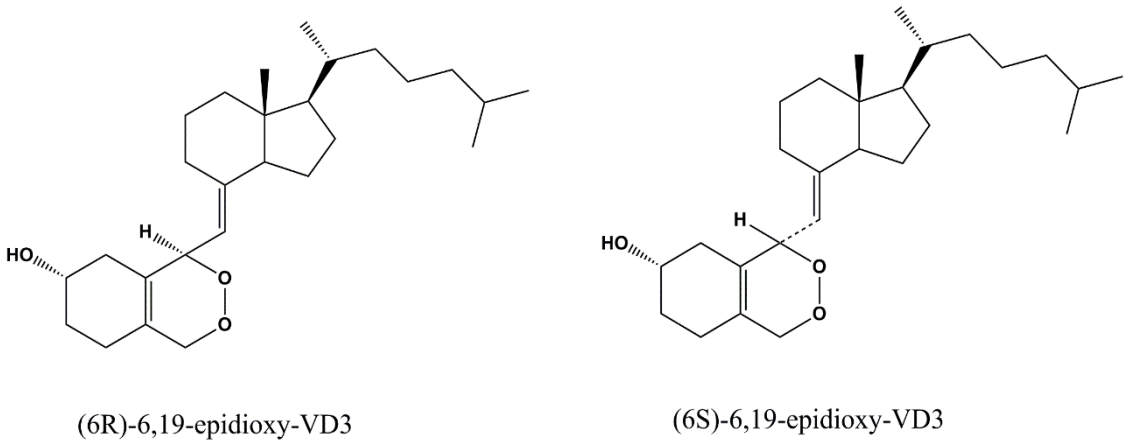
Contrarily to the triplet oxygen oxidation, there is lack of scientific information on the singlet oxygen oxidation in foods. This is because firstly, the detection of short-life singlet oxygen is difficult in complex food systems and secondly, the significant contributions of singlet oxygen to the oxidation of foods have not been well understood (Min & Boff, 2002b).

The singlet oxygen reaction is very fast and it is rapid in foods because of the low activation energy required for the chemical reaction. The lifetime of singlet oxygen is from 50 to 100 μs, depending on the solvent system of the food. As a result, the oxidation of foods by singlet oxygen is very important because the rate of oxidation is much greater than that of triplet oxygen even at very low temperature (Min & Boff, 2002a).

Singlet oxygen participates in reactions such as 1,4-cycloaddition and the ene-type reactions which involve direct reaction with double bonds. It has been demonstrated that the oxidation of vitamin D by singlet oxygen in various solvents using Rose Bengal and tetraphenylporphine as sensitizers, produces 6R- and 6S-epidioxy-vitamin D with the same pathways (Figure 2.11) (Yamada, Nakayama, & Takayama, 1978). If the reaction was under an atmosphere of oxygen, *cis-trans* isomerization would occur. In this regard, since the isomerization is faster than oxidation, oxidation products could be derived from both *cis* and *trans* isomers. The UV spectra of these photooxidation products show no absorption maximum above 220 nm, indicating the absence of

a conjugated double bond. According to Yamada et al. (1978), the molecular ion at *m/z* 416 indicated that two oxygen atoms had been added to the molecule.

Recently researches also studied the effects of light, riboflavin and oxygen on photo-activated singlet oxygen oxidation of vitamin D. Riboflavin accelerates the oxidation of vitamin D by singlet oxygen under light but did not affect vitamin D oxidation in darkness (Min & Boff, 2002b). They reported the formation of 5,6-epoxy-vitamin D3 by the reaction of singlet oxygen with the conjugated triene structure of vitamin D (Figure 2.12).



(6R)-6,19-epidioxy-VD3 (6S)-6,19-epidioxy-VD3

Figure 2.11. Structure of vitamin D3 endoperoxides

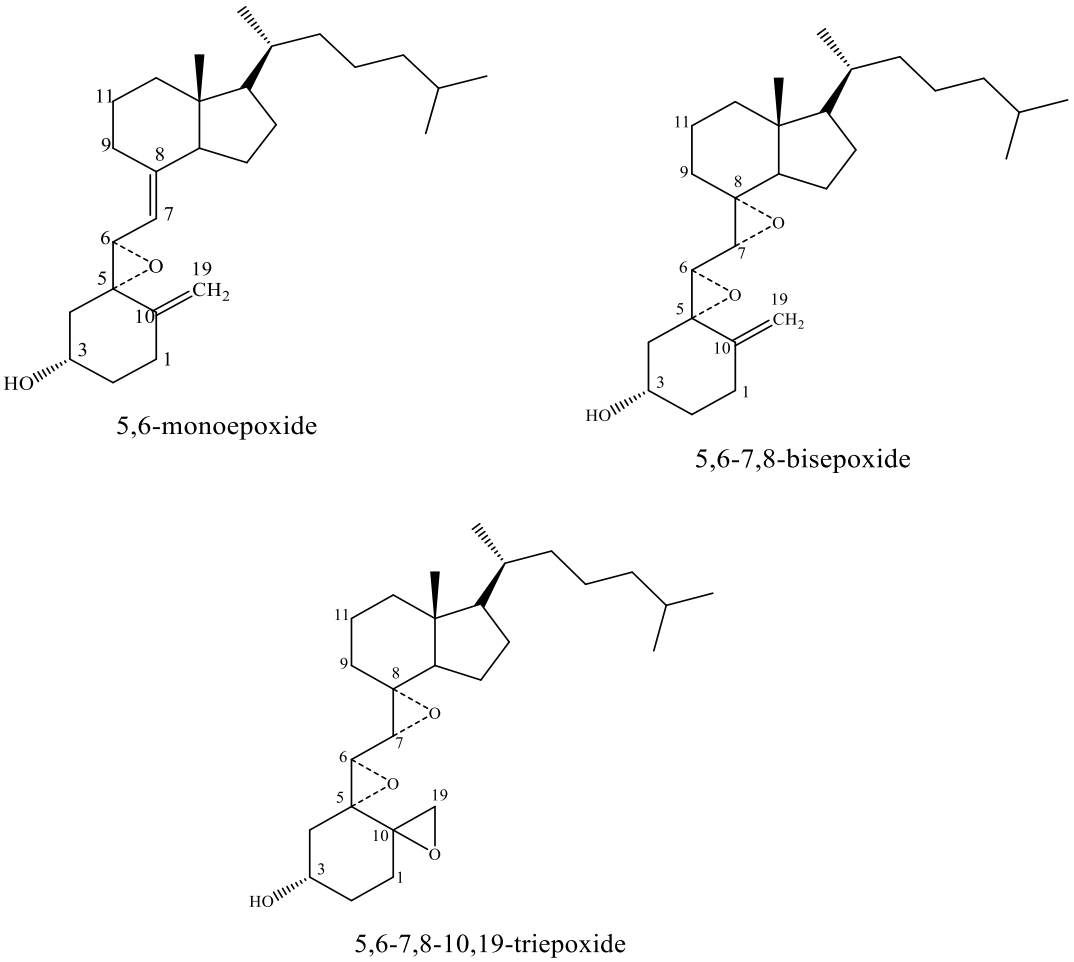


Figure 2.12. Vitamin D3 epoxides

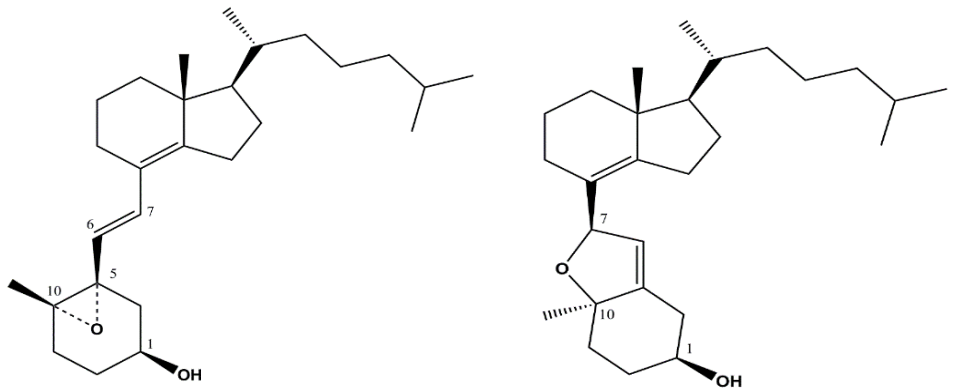
Epoxidation of vitamin D

Structural alterations of vitamin D mostly occur at the 1α-position and the side chain, while the oxidation of the conjugated triene part has scarcely been reported. Regio- and stereoselective epoxidations of vitamin D3 at the 7,8- and 5,6-double bonds were performed by previous studies. According to previous studies, while the epoxidation of 3,5-dinitro-benzoate of vitamin D2 with peracids lead to 7,8-epoxides, epoxidation of vitamin D2 with a combination of hydrogen peroxide and benzonitrile gave 5,6-epoxide (Nakayama, Yamada, Takayama, Nawata, & Iitaka, 1984; Velluz, Amiard, & Goffinet, 1955). Nakayama et al, (1984) also determined the single 7,8-epoxy-vitamin D3 by epoxidation of vitamin D3 with m-chlorobenzoic acid, whereas 5,6-epoxy-vitamin D3 was formed by treatment of vitamin D3 with anhydrous tert-butyl hydroperoxide (TBHP).

The UV spectrum of 7,8-epoxy-vitamin D3 at 227.5 nm shows a conjugated diexo diene chromophore, while 5,6-epoxy-vitamin D3 showed UV absorption at less than 220 nm indicating the absence of a conjugated double bond. Both vitamin D3 epoxides showed the same formula and molecular weight at *m/z* 400, indicating the addition of one oxygen to the molecule.

Bernhard, Kratky, Reischl, & Zbiral (1985) and Reischl, Bernhard, Kratky, & Zbiral, (1985) reported the conversion of vitamin D in methanol with the reagent of benzonitrile/ H_2O_2 to obtain the mono-epoxy compound in good yield (Figure 2.12). It was also reported that if vitamin D3 is converted using two equivalents of Payne reagent, bis-epoxide would be formed, while the remaining C10 and C19 double bond can be further oxidized to tri-epoxide compound using the same reagent (Figure 2.12). Based on this report, the molecular ion at *m/z* 400, 416 and 432 showed the addition of one, two or three oxygen to the molecule of vitamin D3, respectively.

Despite the previous studies on the vitamin D oxidation, there is still some lack of understanding on the autoxidation of vitamin D3 and its isomers. As it was mentioned before, while vitamin D3 is stable in air, the instability of its acid-catalyzed isomer has been reported (Jin et al., 2004). They found that isotachysterol which was kept at room temperature in the dark was oxidized rapidly to a very complex mixture such as 5,10- and 7,10-epoxy-vitamin D3 (Figure 2.13). All compounds showed the same molecular ion at *m/z* 401 with different UV spectra, corresponding to the same molecular formula, but with one more oxygen than isotachysterol.



5, 10-epoxy 7, 10-epoxy

Figure 2.13. Autoxidation of isotachysterol

Action of hydrogen peroxide on vitamin D3

Hydrogen peroxide can be used for disinfection of various surfaces and is recognized as a safe antimicrobial agent for food industries. It is also used in the food packaging industry for containers. Peracetic acid is another sanitizer which is produced by reacting H_2O_2 with acetic acid. It can be used over a wide temperature and pH range in clean-in-place (CIP) processes in various food industries. Hydrogen peroxide is a natural minor component of milk, generated by oxidase enzymes such as xanthine oxidase or glucose oxidase. Lactoperoxidase, another natural milk enzyme and central to the thiocyanate preservation system in milk, has bacteriostatic effect in the presence of hydrogen peroxide and thiocyanate. The lactoperoxidase system can be artificially activated by the addition of sodium thiocyanate and hydrogen peroxide to reactivate the existing lactoperoxidase enzyme in milk to maintain the initial bacteriological quality of the milk without refrigeration until the milk can be processed or pasteurized.

Although H_2O_2 is a powerful oxidizer, stronger than chlorine and chlorine dioxide, it is not by itself a very strong oxidant for organic chemistry reactions. For example, one study used hydrogen peroxide as oxidant to synthesis vitamin K3 (Herrmann, Haider, & Fischer, 1999; Narayanan, Murthy, Reddy, & Premchander, 2002). According to this study, a mix of acetic acid and hydrogen peroxide were used to form peroxyacetic acid which catalytically improved the oxidation power of hydrogen peroxide. In order to provide a selective route for vitamin K3 synthesis, they also used transition metal catalysts to generate peroxy compounds which appear to be more selective and therefore controllable than peroxyacetic acid.

Vitamin D which has a treine system should be sensitive to oxidation by hydrogen peroxide. Like H_2O_2, organic acids and amides are also common in milk at low levels, so peroxy-acids or imino peroxy-acids, would be found in milk, and could therefore be the oxidizers of vitamin D. So, the influence of hydrogen peroxide and the role of organic acids on the oxidation of vitamin D3 need to be studied. Abernethy (2010) reported that H_2O_2 reacted with vitamin D3 under mild conditions and three epoxides, likely to be isomers, were observed. The main product, which was a compound of *m/z* 401, suggest the addition of one oxygen to the molecule.

Bland & Craney (1974) also studied the oxidation of vitamin D3 by chemically generated singlet oxygen which is produced by the reaction of hydrogen peroxide with sodium hypochlorite. In this reaction, dimethoxyl product was formed which was not isolated singularly with hypochlorite or hydrogen peroxide. Therefore, the comparisons of the photooxidation product of vitamin D with those obtained by reaction with singlet oxygen generated chemically, could be a good idea.

2.3.2. Stability of vitamin D in foods

From a food processing view, vitamin D is relatively stable during food processing and storage (Hanson & Metzger, 2010; Kazmi et al., 2007), however, some other studies have reported vitamin D loss. Numerous surveys to assess national requirements for vitamin D3 and to help set regulatory guidelines for fortification levels have been conducted. A number of studies suggested the inconsistency between the actual vitamin D content and the claimed amount among fortified milk products (Holick et al., 1992; Murphy et al., 2001). Various studies, in Canada and USA, indicated that a large proportion of the samples were not in compliance with the regulations and most samples had lower levels of vitamin D compared to the declared values on the labels (Calvo & Whiting, 2003). Tangpricha et al. (2003) reported that vitamin D3 in fortified orange juice was stable during 30 days storage at 4˚C. While Kazmi et al. (2007) reported little impact of the acidity of yogurt on vitamin D3 content, Pike & Brown (1967) indicated the instability of vitamin D in an acidic medium. The stability of vitamin D3 during processing and cold storage in making cheese, yogurt and ice cream was also reported by Kazmi et al. (2007). Holick et al. (1992) reported that 60% of fortified milk samples were under-fortified and only 20% of them contained 80-120% of the labelled amount. In another survey, it was reported that more than 70% of all fortified samples were either under or over fortified (Faulkner, Hussein, Foran, & Szijarto, 2000).

Regarding the inconsistency of vitamin D3 content in fortified samples, it is unclear whether this is due to the analytical variability, poor fortification practices or instability of the vitamin during processing or storage. It has also never been clear what the vitamin D3 has degraded into, in foods, and whether these new products have any biological activity or toxicity that should be of concern. The paucity of literature on the stability of vitamin D3 in milk powder and other products suggest that more studies are needed in this area.

2.3.2.1. Stability of vitamin D in whole milk powder

Milk is a complex and variable mixture of a large number of chemical species including prooxidants, antioxidants and oxidizable substances. The impact of light, air and temperature on the stability of vitamin D in an acetonitrile model and skim milk were compared in a previous study (Renken & Warthesen, 1993). In acetonitrile model system, the impact of light exposure was not significant on the stability of vitamin D, while air and high temperature showed significant loss of vitamin D. In this respect, light and/or air contributed the greatest vitamin D3 loss in skim milk. It was reported that the significant effect of light on vitamin D instability might be due to the presence of riboflavin in milk, which is a photo-sensitizer, and trigger the formation of singlet oxygen (Renken & Warthesen, 1993; King & Min, 2002; Li & Min, 1998).

As it was discussed earlier, contradictory observations have been made regarding vitamin D stability. It is clear that vitamin D stability is dependent on the food in which it is present. Vitamin D3, which is rather stable towards atmospheric oxygen, could be susceptible to reactive oxygen species (Charlton & Ewing, 2007; Riaz et al., 2009). Milk powders are more stable than fresh milk primarily due to low water activity, but protection from moisture, oxygen, light and heat is needed in order to maintain their quality and shelf life. In the whole milk powder, vitamin D3 seems to be stable against different processing and environmental factors, but its stability maybe depends on the stability of the milk fat. The fat in whole milk powder can react with oxygen in the air to cause oxidation especially at higher storage temperatures. Studies have found vitamin D3 degradation during the storage of whole milk powder in air and to a lesser extent in nitrogen at various temperatures (Labuza, McNally, Gallagher, Hawkes, & Hurtado, 1972; Pearce & Wewala, 1993).

2.4. Lipids

Food lipids, fat (solid lipid) and oil (liquid lipid) consists mostly of triacylglycerol. Fatty acids, which are esterified with glycerol to form triacylglycerol, are a major part of fats and oils. The characteristics of triacylglycerol such as the melting point, hydrophobicity and oxidation capability depend on the fatty acid composition, especially the double bond or unsaturation content, and also the origin of the oil or the fat.

2.4.1. Milk fat in whole milk powder

Whole milk is composed of roughly 88% water, 3.3-3.5 % fat, 3.3% protein, 4.8% carbohydrate, and some other minor constituents such as vitamins and minerals. However, its composition can vary due to lactation, genetics, season and diet. Milk fat is present in the form of globules, ranging from 0.2 to 10 µm, which are surrounded by a layer of protein and phospholipid termed the milk fat globule membrane (Gutierrez, 2014; Jensen, 2002).

Milk fat is composed of primarily triglyceride (about 98%) and small amounts of mono- and diacylglycerols, phospholipids, cholesterol and free fatty acids. The proportions of fatty acids vary substantially among mammalian species. Milk fat contains predominantly saturated fatty acids ranging from C4 to C18. The unsaturated fatty acid of milk fat consists of mostly oleic acid (C18:1) (18 – 27% of total fatty acid) with small amounts of linoleic (C18:2) and linolenic acid (C18:3). The factors which affect the fat content such as age, diet and season do not alter the fatty acid composition of milk (Gutierrez, 2014; Meurant, 1995).

2.4.2. Oxidation of milk fat

Lipid oxidation is a major cause of food quality deterioration and has always been in the centre of attention by manufacturers and food scientists. Lipid oxidation is responsible for a wide variety of adverse effects such as flavour and colour changes, loss of nutritive value that limit the shelf-life of dried foods. Dried products such as whole milk powders are susceptible to lipid oxidation since they have a large surface area and higher proportion of lipid content than liquid milk. In milk and other dairy products, the presence of volatile compounds produced from the decomposition of primary oxidation products can be responsible for the undesirable flavour. (Addis, 1986; Vazquez-Landaverde, Velazquez, Torres, & Qian, 2005).

Apart from that, lipid oxidation can cause nutritional impacts such as the interaction of lipid oxidation products with other essential nutrients such as vitamins. In dairy products, lipid oxidation reaction can occur by photo-oxidation and autoxidation pathways (Wasowicz et al., 2004). Autoxidation is a free radical chain reaction involving molecular oxygen, also called triplet state oxygen, consists of three steps of initiation, propagation, and termination (Figure 2.14). The initiation step involves abstraction of a hydrogen atom from a fatty acid or acylglycerol to form

the radical known as the alkyl radical (R˙). Alkyl radicals are formed at various positions depending on the initiator, and then triplet state oxygen adds to them to generate peroxyl radicals (Schaich, 2005). Peroxyl radicals are reactive and abstract hydrogen atoms from another lipid to form hydroperoxides and new radicals. Therefore, this process continues indefinitely, providing the basis for the propagation stage. Finally, in the termination step, two radicals can combine to form an almost infinite variety of non-radical products, or alkoxyl radicals (Gutierrez, 2014; Schaich, 2005).

The primary products of lipid oxidation are hydroperoxides, which have little to no flavour. However, these hydroperoxides decompose into low molecular weight fatty acids, alcohols, aldehydes, ketones and hydrocarbons which are volatile and cause rancid off-flavours (Gutierrez, 2014; Schaich, 2005; Wasowicz et al., 2004). The oxidation rate of fatty acids increases by increasing their degree of unsaturation. The fatty acids in milk fat are mainly saturated and their oxidation at ambient temperatures can largely be ignored, however, unsaturated fatty acids are susceptible to oxidation. In the case of oleic acid, a major unsaturated fatty acid of milk fat, hydrogen abstraction at C8 and C11 results in the formation of two possible allylic radicals (Gutierrez, 2014).

1) Initiation: formation of free radicals:

$$RH \xrightarrow{\text{initiator}} R^{\bullet} + H^{\bullet}$$

$$RH + O_2 \xrightarrow{\text{initiator}} ROO^{\bullet} + H^{\bullet}$$

2) Propagation: free radical chain reaction:

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$$

3) Termination: formation of non-radical products

$$R^{\bullet} + R^{\bullet} \longrightarrow R\text{-}R$$

$$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2$$

Figure 2.14. Mechanism of autoxidation reaction

In addition, previous findings indicted that processing and storage conditions of dairy products had an effect on the oxidation rate and the composition of oxidation products. Oxygen is one factor that promotes the autoxidation rate. Therefore, the removal of headspace and/or dissolved oxygen is often required for the long-term storage of food products (Chan et al., 1993; Gutierrez, 2014). Previous studies reported that heat treatment is another factor for oxidative stability of the final products in milk powder manufacturing. The low oxidation rate obtained by heat treatment is attributed to the denaturation of whey proteins, especially β-lactoglobulin, which leads to the increased levels of free sulfhydryl groups (Chan et al., 1993; Cluskey et al., 1997).

2.4.2.1. Oxidation of cholesterol

Cholesterol, a steroid alcohol located in the milk lipid globule membrane, contains a cyclopentanophenanthrene ring and has a very similar structure to vitamin D3. In processed foods, such as milk powder, cholesterol also undergoes autoxidation during processing and storage yielding oxidation products (Cluskey et al., 1997; Dionisi, Golay, Aeschlimann, & Fay, 1998; Rose-Sallin, Huggett, Bosset, Tabacchi, & Fay, 1995). Cholesterol oxidation products (COPs) are similar to cholesterol and contain an additional functional group, such as a hydroxyl, ketone or an epoxide group in the sterol nucleus (Ubhayasekera, 2004).

Oxidation of cholesterol follows the same oxidation patterns such as autoxidation. It is believed that the hyroperoxides derive from oxidation of unsaturated fatty acids play a significant role to facilitate cholesterol oxidation (Guardiola, 2002). Cholesterol autoxidation usually starts at C-7, which is close to a double bond and has more chance to abstract one hydrogen and add one oxygen forming isomers of 7-hydroperoxycholesterol. These 7-hydroperoxycholesterols can further convert into 7-hydroxycholesterol, which are commonly found in food. In addition, 7-ketocholesterol can be formed by dehydration of isomeric 7-hydroxycholesterol in the presence of radicals (Figure 2.15). Oxidation also occurs through the double bond between carbons 5 and 6 in the aromatic ring due to interaction between cholesterol and hydroxyl radical to form isomeric cholesterol epoxides (Figure 2.15) (Guardiola, 2002; Ubhayasekera, 2004).

2.4.2.2. Cholesterol oxidation products in whole milk powder

Cholesterol oxidation products (COPs) has received considerable attention since they have shown a variety of biological activities associated with the certain human diseases. Some of the main sources of COPs are processed animal food products. Storage conditions including time and temperature can have an effect on the formation of COPs in cholesterol containing food (Paniangvait, King, Jones, & German, 1995).

COPs generation is also incorporated with the processing condition such as thermal treatment. Previous studies reported the presence of cholesterol oxides in milk powder products as related to processing technology and storage. They have reported that while no detectable levels of COPs were seen in fresh milk powders, aged products exhibit increased levels of cholesterol oxides. In addition, they also reported low levels of COPs in milk powders with high heat treatment due to the oxidative stability improvements (Chan et al., 1993; Nourooz-Zadeh & Appelqvist, 1988; Sarantinos, O'DEA, & Sinclair, 1993).

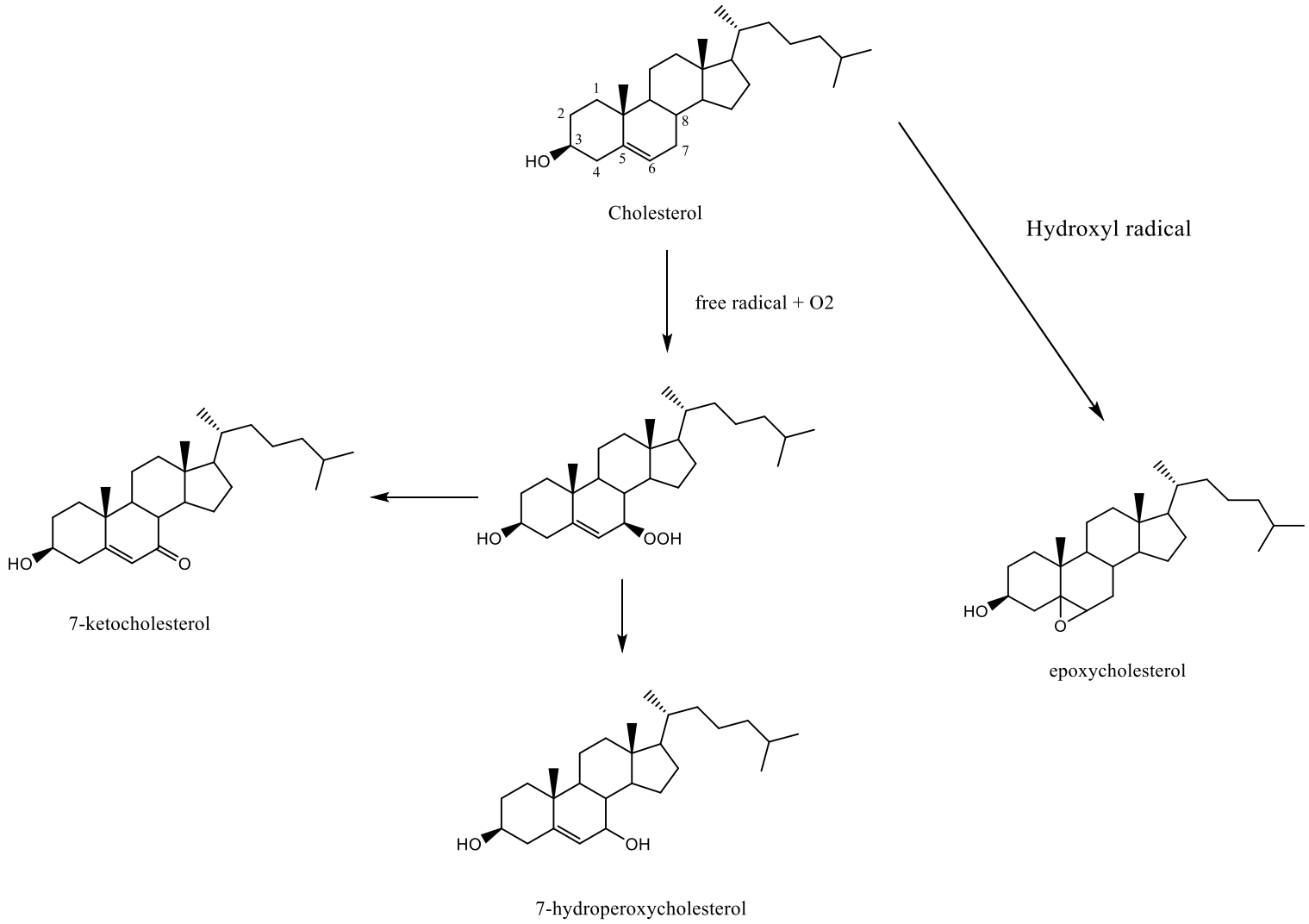


Figure 2.15. Autoxidation pathways of cholesterol

2.5. Analysis of vitamin D and its related products

2.5.1. Introduction

The identification and quantification of vitamin D and its metabolites have always been a challenge due to the low concentration, the presence of interfering compounds and similarity in their chemical structures. The measurement of the levels of vitamin D and its metabolites has been of interest for some time, as it provides important knowledge about the etiology, pathogenesis and treatment of vitamin D deficiencies (Kasalová et al., 2015; Ouweland, Vogeser, & Bächer, 2013). Therefore, any method to extract vitamin D and its related products has to ensure that all products are captured and that they are not altered by the analysis process.

The earliest official methods for vitamin D determination were bioassays, in which the bioactivity of vitamin D was determined by its ability to prevent or improve deficiency symptoms in vivo (Ötleş & Karaibrahimoglu, 2005). The biological methods include curative, prophylactic and calcium absorption. All methods are based on the comparison of the biological responses of animals fed with a standard vitamin D preparation with those fed the test substances. Bioassays can detect vitamin D at very low concentrations; however, this method requires long preparation times and high cost, which make them less convenient as a routine analysis method compared to other methods. In addition, these methods do not distinguish vitamin D3 from its biologically active impurities or metabolites (Koshy & Beyer, 1984).

Vitamin D and its related products are usually analysed in three stages: (1) extraction of the metabolite of interest from the matrix, (2) separation of the metabolite of interest from other metabolites of vitamin D or compounds which may interfere in quantitation of the interest, (3) quantitation or identification. In stage 3, it is necessary to identify the analyte which can be done by a number of physicochemical means such as UV or infrared spectra, nuclear magnetic resonance (NMR) and mass spectrometry (MS).

2.5.2. Extraction

In order to determine vitamin D and its metabolites, sample pretreatment is normally required. The most common extraction technique is saponification, liquid–liquid extraction (LLE) followed by preparative chromatographic cleanup to eliminate contaminants (Musteata & Musteata, 2011). Saponification, which is carried out in various temperature and time combinations, includes the incubated of sample in methanolic or ethanolic solution of NaOH or KOH, and this is followed by liquid/liquid extraction for the concentration of the non-saponifiable fraction (Guardiola, 2002). Time and temperature of incubation have been two important parameters which result in vitamin D isomerization or degradation products. It was reported that the ambient saponification was insufficient and might give uncontrollable emulsions (Johnsson & Hessel, 1987). Official methods normally use the short time high temperature saponification to remove neutral lipids, especially triglycerides (De Vries, Mulder, & Borsje, 1981; Faulkner et al., 2000; Tanner, Barnett, & Mountford, 1993).

After saponification, different solvents can be applied to extract vitamin D from aqueous phase of the samples. For example, hexane is normally used alone as the extracting solvent, but ethanol is sometimes added into hexane to prevent formation of an emulsion (Faulkner et al., 2000). Since vitamin D and its related products are fat-soluble compounds, partition into organic solvents provide significant purification by removal of water-soluble contaminants especially lactose and proteins which amount two-thirds of the milk solids.

The efficiency of extraction depends upon the solvent or solvent mixture used and also upon the metabolite under consideration. There are two basic categories for solvent systems extraction including; 1) total lipid extraction and 2) selective lipid extraction. Although the selective lipid extraction could minimize contamination of the vitamin D analyte extract, they provide efficient extraction for one particular metabolite or group of metabolites and poorly extract those with different polarity due to the selectivity. When simultaneous analysis of several vitamin D compounds with a wide range of polarities is required, a total lipid extraction may be necessary (Bligh & Dyer, 1959).

The direct extraction of vitamin D has also been used in several studies without previous saponification, which could alter the breakdown products, with organic solvents. Vitamin D recoveries using direct extraction have achieved similar results as saponification procedure (89–107%) (Blanco et al., 2000; Gomis, Fernandez, & Alvarez, 2000).

Another sample preparation process is Solid Phase Extraction (SPE) by which compounds are separated in a matrix according to their polarity differences. This technique is rapid, selective and provides concentrated and purified samples. This technique is an efficient way for clean-up and pre-concentration to determine vitamins and has been used for the quantification of vitamin D3 and its metabolites sine the earliest methods (Hollis, 1986; Kao & Heser, 1984). Several SPE methods have been developed for vitamin D and its metabolites isolation prior to analysis. In this respect, the column sorbents used mostly are silica and C18 or its combination for the milk samples (McGraw & Hug, 1990). Apart from that, the use of aminopropyl column has proved to obtain an improved extraction of vitamin D3 and its metabolites (Luque-Garcıa & de Castro, 2001).

2.5.3. Separation

Fat soluble vitamins were originally usually separated by normal phase HPLC columns due to their highly lipophilic nature (De Vries et al., 1981). However, normal-phase chromatography has fallen in disfavour because of some of the complexities involved such as lengthy equilibration times, reproducibility problems and the sensitivity of the technique (Neue, 1997). Therefore, researches have successfully developed methods on reverse phase columns which are currently the more common methods used for routine analysis of fat soluble vitamins. SPE is also applied in some other methods to replace the normal phase column for purification (Faulkner et al., 2000; Hagar, Madsen, Wales, & Bradford, 1994; Mattila, Piironen, Uusi-Rauva, & Koivistoinen, 1993; Renken & Warthesen, 1993).

The chromatographic methods of vitamin D determination usually include Gas Liquid Chromatography (GLC) and High-performance liquid chromatography (HPLC). Although GLC procedures have been used extensively for the determination of steroids and hormones, only a few reports have attempted to develop similar techniques for vitamin D. The major problem with the GLC analysis is due to the thermal rearrangements of vitamin D and its metabolites at higher

temperatures which are observed as multiple peaks from a single compound. Another reason for the lack of interest in GLC analysis is that vitamin D3 concentration is low and the samples generally contain structurally similar compounds requiring extensive sample clean-up. Therefore, in recent years, GLC methods have been replaced by the HPLC methods (Koshy & Beyer, 1984).

HPLC methods have been widely used for the determination of fat soluble vitamins since the mid-1970s and have been adopted as official methods due to good selectivity and detection ability (Ball, 1988; Hofsass, Alicino, Hirsch, Ameika, & Smith, 1978). For different food sources, the suppliers of official methods are the European Committee for Standardization (CEN), the Nordic Committee on Food Analysis (NMKL), and the Association of Official Analytical Chemists International (AOAC). These three methods include quantification by an internal standard (IS), alkaline saponification, extraction with an organic solvent, and different clean-up steps, such as SPE and preparative HPLC for final quantification. The need for sample preparation, extraction, concentration, and partial purification prior to final analysis depends on the nature of the sample matrix and the methods of quantification (Nollet & Toldrá, 2012).

In reversed phase HPLC, the C8 and C18 stationary phases are the most widely used, however, some analysts encounter difficulty in separations, and reproducibility is not easily obtained using these traditional phases. More recent methods take advantage of small-bore columns that significantly reduce solvent usage and run time (Maunsell, Wright, & Rainbow, 2005; Saenger, Laha, Bremner, & Sadrzadeh, 2006). Table 2.1 gives information in terms of stationary and mobile phases used to detect vitamin D2, D3 and their metabolites. Separation of vitamin D and its metabolites may require the use of more selective or novel stationary phases, such as pentafluorophenyl (PFP) bonded to silica for different analysis such as the separation of geometrical isomers. The use of novel selective phases, such as PFP may be assess to reverse an elution order and enable the elution of a minor component in front of a major component, thus making quantitation and identification considerably easier. Novel phases also offer using simpler mobile phases, avoiding ion pair reagents, exotic buffer systems and extreme pH conditions (Grebenstein & Frank, 2012; Saini & Keum, 2016).

Table 2.1. HPLC parameters used for separation and detection of vitamin D and its metabolites.

Stationary Phase	Mobile Phase	Detection	Sample	Analyte	Reference
LiChrosorb 10RP18	Methanol:water (95:5)	265 nm	Fish	Vitamin D3	(Egaas & Lambertsen, 1979)
Sperisorb S5 ODS2	Methanol:water (95 + 5/90 + 10)	265 nm	Egg	Vitamin D3	(Jackson, Shelton, & Frier, 1982)
Zorbax ODS, Vydac 201TP54	Methanol:water (96:4)	265 nm	Meat, milk	Vitamin D3	(Mattila, Piironen, Uusi-Rauva, & Koivistoinen, 1995)
VYDAC201TP54	Methanol: Acetonitril (20:80)	265 nm	Meat, milk	Vitamin D3/D2	(Jakobsen, Clausen, Leth, & Ovesen, 2004)
C18 (LUNA)	Acetonitril:water (90:10)	265 nm	Meat, milk	25OHD3/ D2	(Jakobsen & Saxholt, 2009)
Inertil ODS-2	Methanol: Acetonitrol (20:80)	265 nm	fortified milk, cereal, cheese, orange juice	Vitamin D3	(Byrdwell, 2009)

2.5.4. Detection and quantification

2.5.4.1. Ultraviolet Absorbance (UV)

The analysis of vitamin D and its metabolites has been conducted in several steps and quantification using HPLC coupled with UV detection. The determination of vitamin D and its metabolites based on their natural ultraviolet spectrum absorption are principally restricted to conjugated trienes. Due to the relatively short wavelength maxima of vitamin D compounds (λmax 240 to 290 nm), the direct spectrophotometric assay is not suitable in more complex mixtures. Therefore, the application of spectrophotometry can be greatly improved by chromatographic separation or purification of the sample. This detection is not necessarily highly specific since many other non-vitamin D compounds also absorb at this wavelength. Chromatography methods may well provide high-quality separation between vitamin D metabolites but this does not necessarily mean that other UV absorbing compounds are removed from the analyte of interest (Hollis, 1983; Kasalová et al., 2015; Kunz, Niesen, von Lilienfeld-Toal, & Burmeister, 1984).

Using a more sophisticated UV detector based on the diode-array scanning spectrophotometer has increased over the last 10 years. In addition, the diode-array scanning spectrophotometer detector adds another dimension of wavelength, to the conventional absorbance versus time plot. Therefore, diode-array detectors could be a more valuable analytical tool compared to the UV (De Leenheer & Lambert, 2000).

2.5.4.2. Mass Spectrometry (MS)

Introduction

The official methods based on the HPLC-UV that have commonly been used for vitamin D3 analysis have been improved upon, largely in the areas of lower resource requirements, throughput and selectivity. Over the last few years, there has been increased interest in using liquid chromatography mass spectrometry (LC–MS), due to the increased sensitivity and selectivity over the traditional HPLC-UV to determine the levels of vitamin D3 and its metabolites (Byrdwell, 2009; Dimartino, 2009). This has led to the uptake of more sophisticated detection systems like LC-MS and Nuclear magnetic resonance (NMR) (Okamura et al., 2002; Vogeser & Seger, 2008).

A mass spectrometer consists of three fundamental parts: an ion source, a mass analyser, and a detector. This technique is based on the separation of ions in vacuum or gas phase according to their mass-to- charge ratios (*m/z*), with a variety of unique instrumental designs able to achieve this. The analysed molecules have to be first ionized, forming a molecular or pseudo-molecular ion, which is pulled through the instrument via electromagnetic fields. Depending on the instrument design, this analyte ion might induced to fragment, which leads to the formation of characteristic fragment ion(s), some or all of which are again separated in the mass analyser according to their *m/z*. The final stage is detection of the selected ions or fragment ions. In this technique, the sample can be introduced to the ion source directly, or after its previous separation by GC or LC. Several ionization methods are available which have their own advantages and limitations. While in GC-MS, electro ionization (EI) and chemical ionization (CI) represent the fundamental ionization techniques, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) techniques are widely employed in LC-MS (Cajka, Hajslova, & Mastovska, 2008).

In the early methods, the linking of the chromatography output directly to mass spectrometer system was considered as an off-line means of GC detection or/and quantitation, usually via GC-MS (Makin, Jones, Kaufmann, & Calverley, 2010). In the off-line technology, analytes can be loaded directly into the ion source of a mass spectrometry by direct probe. Direct probe insertion requires a reasonably pure sample since the only separation which can be achieved prior to mass spectrometry relies on the temperature at which the sample volatilizes. However, in the early 1990s, methods of connection of the LC eluate directly to the mass spectrometer (LC-MS) began to be developed and this application is considered as an online methodology (Makin et al., 2010). Use of LC prior to MS increases specificity and sensitivity as it presents the sample in a more concentrated form than the peak obtained with the probe (Vicchio et al., 1993).

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS was the first mass technique used to develop methods for the measurement of vitamin D and their metabolites. It has been proved to be a valuable method with high specificity and sensitivity. In GLC, the solute should be in the vapor phase during the process of partition between the mobile gas phase and the stationery liquid phase. Moreover, compounds with molecular weight more than 200 Da tend to be poorly volatile, and analysis is required to be carried out at elevated temperatures. At these temperatures, vitamin D compounds undergo thermal rearrangement involving B-ring closure. Thus, vitamin D and its metabolites are all converted irreversibly to pyro and isopyro isomers (Makin et al., 2010). In this respect, another isomerization process (formation of isotachysterols), which can be carried out prior to GC separation, prevents thermal B-ring closure and thus allows single peaks to be produced during chromatography. Therefore, such procedures have the advantage that they do increase the sensitivity of detection of both after GC and also LC-UV. Apart from that Murray, Day, & Kodicek (1966) overcame the difficulty of the thermal isomerization of vitamin D3 into two peaks by converting it into isovitamin D3 by treatment with antimony trichloride which gave a single peak by GLC. GC-MS has been used as a definitive quantitative tool, however, the formation of pyro- and isopyro- isomers and a requirement for extensive purification prior to analysis make it an inherently more inconvenient analysis method for vitamin D and its metabolites.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Recently, using liquid chromatography coupled to mass spectrometry (LC-MS) has been used for quantifying vitamin D and other metabolites. LC-MS was introduced as a complementary method to GC-MS and one significant advantage is its high sensitivity for identification of low levels vitamin D and its metabolites (Ouweland et al., 2013).

LC-MS using thermospray (TSP) as an ion source was one of the early developments applied to the analysis of vitamin D compounds (Vreeken, Honing, van Baar, Ghijsen, & De Jong, 1993; Yergey, Esteban, & Liberato, 1987). To date, ESI and APCI are the most common ionization sources for the coupling of LC to a mass spectrometry. ESI positive ion mode was used in most cases for the determination of vitamin D and its metabolites. In addition, the most commonly used mass analysers are triple quadrupole (QqQ) and ion trap (IT) (Kasalová et al., 2015).

Besides LC-MS, LC-MS/MS is the preferred choice for more complex sample matrices. This technique allows the sample to be examined, mass spectra obtained, and specific ions subjected to further ionization to give product ions (i.e., MS/MS or MS^2). This process can be repeated to look at fragment ions (MS^3) and so on. This enables a scheme of sequential fragmentation to be drawn up and can be very valuable in structural analysis (Makin et al., 2010). Trenerry, Plozza, Caridi, and Murphy (2011) used liquid chromatography– linear ion trap mass spectrometry ($LC–MS^n$) and liquid chromatography – tandem mass spectrometry (LC–MS/MS) to measure the levels of vitamin D3 in several types of bovine milk. They reported a slightly better limit of quantification (LOQ) for vitamin D3 using $LC–MS^n$ than LC–MS/MS.

Abernethy (2012) developed a faster analytical method for vitamin D3 determination using a dienophile through the Diels–Alder reaction (Figure 2.16). A suitable chemical derivatization can enhance the ionization efficiency of vitamin D and its metabolites and improve fragmentation enabling increased sensitivity of detection, while reducing isobaric interferences (Abernethy 2012; De Vries, Mulder, & Borsje, 1977; Zhang et al., 2006).

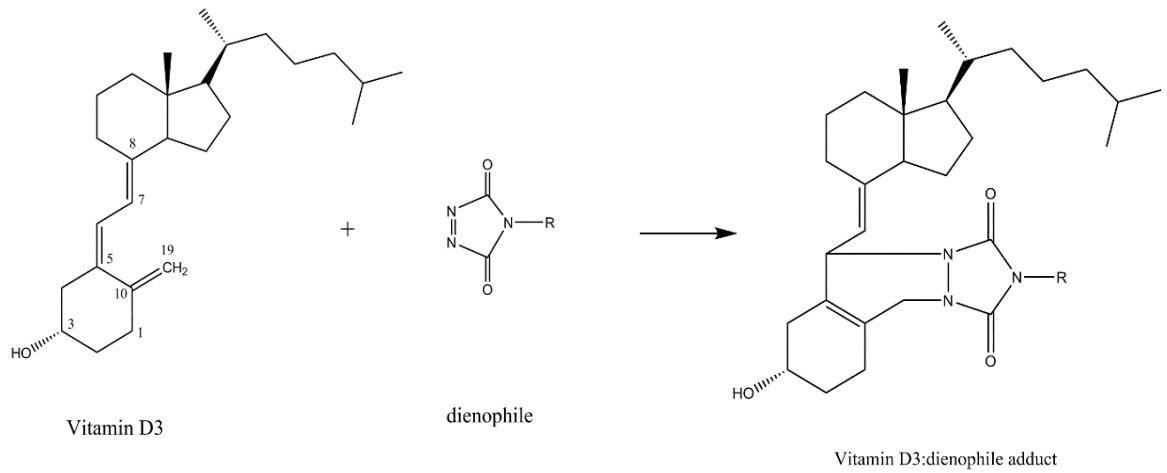


Figure 2.16. The structure of vitamin D adduct via a Diels-Alder reaction

2.5.4.3. Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR is the most informative spectroscopic technique for the elucidation of molecular structure, providing not only information on the connectivity of atoms but also conformational and configurational information. Although larger amounts of sample are needed than that for mass spectroscopy, good data may be obtained from samples weighing less than a milligram with modern instruments. This technique is based on magnetic nuclear spin of H, C, N, F and P. Only 1H and 13C are considered in vitamin D samples. While 1H NMR provides information about the number of protons in the molecule, 13C NMR gives information about the number of carbons in the molecule. By comparison NMR and other analytical methods, it is apparent that the most detailed information to recognize a structure is obtained from NMR spectra. However, this does not mean that NMR is always sufficient for structural analysis. Apart from that the information obtained from NMR spectrum is useful only if it can be interpreted well (Balci, 2005; Elipe, 2003). In addition, NMR could be applied only to reasonably pure compounds and it might be insufficient without purification for the complete characterisation. Previous studies suggested using of thin-layer chromatography (TLC) to purify vitamin D compounds prior NMR analysis (Kobayashi, 1967a; Kobayashi, 1967b; Verloop et al., 1955

Compared to NMR, LC-MS has been the more extensively applied hyphenated technique for vitamin D analysis because MS has high sensitivity and requires much smaller samples size for analysis. HPLC-NMR could be an option to overcome these complexities, but it has not been widely implemented because of its problems such as expensive technique and availability of the equipment (Elipe, 2003). However, recently LC–MS/MS has become the most widely used analytical method for the assay of vitamin D and its metabolites (Kasalová et al., 2015).

2.6. Conclusions

This review has reported the identity, formation, and analysis of all of the known vitamin D isomers and degradation products described in the literature. Most of the previous studies have shown the effects of various physicochemical parameters on stability of vitamin D2 (presumably, because it was historically the more available vitamer for research or supplementation). As mentioned earlier in this Chapter, more recently vitamin D3 has become the predominate vitamer used in fortification of food. Therefore, more studies need to be done to investigate the stability of vitamin D3 under different conditions.

With respect to the identification of vitamin D degradation products, minimally invasive and non-destructive techniques are crucial to isolate them without further decomposition. Due to the typically low concentration of vitamin D, compared to the preponderance of other steroidal or fatty compounds in food, separation is an important stage to identify these structurally similar compounds. Early studies depended on thin-layer chromatography (TLC) to purify vitamin D2 isomerization products prior analysis. TLC had to be carried out under very carefully controlled conditions to prevent or minimize decomposition of the analytes. These methods mostly reported multiple transformation pathways for specific isomers and lacked reproducibility. In order to improve the identification and to quantify vitamin D3 isomerization products, more advanced methods are required to analyse them at high resolution with reproducibility. In this respect, LC-MS has become the most extensively applied hyphenated technique because MS has inherent selectivity, higher sensitivity and requires much smaller samples to analyse these low abundance compounds.

Previous studies have clearly shown that processing and storage conditions have had an influence on cholesterol oxidation in whole milk powders. Therefore, it would be interesting to understand the effect of these parameters on the structurally similar vitamin D3 oxidation in fortified whole milk powders as well. Oxidation of vitamin D, another form of vitamin D degradation, was also studied by early studies in simple matrices. However, there has to date been no study of vitamin D3 oxidation in fortified foods. As a final statement, understanding the stability of vitamin D3 in fortified food and finding the best methods to analyse its degradation products could be useful for industrial monitoring and assurance of vitamin D stability in fortified foods. It may also pave the way for future work in this academic space.

CHAPTER 3

Degradation studies of cholecalciferol (vitamin D3) using HPLC-DAD, UHPLC-MS/MS and chemical derivatization

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3.1. Introduction

Vitamin D, including cholecalciferol (D3) and ergocalciferol (D2), is a fat soluble secosteroid containing conjugated triene systems. Research conducted over the past decade suggests that vitamin D plays a much broader disease-fighting role than once thought. Vitamin D deficiency may increase the risk of a host of chronic diseases, such as osteoporosis, heart disease, some cancers, and multiple sclerosis, as well as infectious diseases. Since only a limited number of foods contain vitamin D, the fortification of food with this nutrient, mostly as vitamin D3, has been the centre of attention (Allen et al., 2006; Martı´nez-Núñez, Cabaleiro-Lago, Fernández-Ramos, Hermida-Ramón, & Peña-Gallego, 1999). Since vitamin D is fat-soluble, milk is an ideal target for vitamin D fortification.

In a successful food fortification program, the stability of added micronutrients is important. Vitamins are well known to be unstable to stressors such as light, heat and chemicals. However, there are conflicting reports regarding the stability of vitamin D3 towards these factors. Some studies have found that vitamin D is relatively stable during food processing and storage (Hanson & Metzger, 2010; Kazmi et al., 2007); however, some other studies reported vitamin D losses (Calvo & Whiting, 2003; Kazmi et al., 2007). The inconsistency between the actual vitamin D3 content and the claimed amount among fortified products that were reported by a number of studies creates the need to understand its degradation properties, in order to develop its potential applications (Hanson & Metzger, 2010; Holick et al., 1992; Murphy et al., 2001). Vitamin D is known to isomerise and/or degrade under various conditions, thereby making its stability in food products potentially uncertain and its analysis challenging. On the other hand, the *cis*-triene configuration of vitamin D is important for its biological activity, as the other types of vitamin D isomers, such as tachysterol and *trans*-vitamin D, have little or no anti-rachitic activity (Borsje, Heyting, Roborgh, Ross, & Shillam, 1977; Parrish & Richter, 1979). Koshy and Beyer (1984) reported that the biological activity is primarily due to vitamin D and previtamin D. Therefore, the study of vitamin D stability is necessary to understand its behaviour under different conditions.

The general aim of this project was to study the isomerisation of vitamin D3 under different conditions and finding the best methods to generate vitamin D3 isomerisation products and to separate them as effectively as possible. The method developed was capable of separating the *cis*-triene structure of vitamin D from other compounds present, including but not limited to

isomerisation products. Therefore, this study not only helps to understand vitamin D3 degradation properties, but also to produce and separate vitamin D3 isomers in reasonably high resolution so they could be used as analytical standards for their quantification.

Based on the early studies, different conditions such as substrate/reactant ratio, solvents and time have various impacts on the pathway of vitamin D isomers generation. These methods mostly reported multiple transformation pathways and other forms of vitamin D isomers in addition to expected isomers. This may be due to additional isomerisation or decomposition during purification using thin layer chromatography (TLC). Moreover, this technique was not successful at separating all forms of vitamin D isomers in a mixture (Kobayashi, 1967a; Kobayashi, 1967b; Verloop et al., 1955; Verloop et al., 1959). They also reported that gas liquid chromatography (GLC) analysis leads to the problem of thermal cyclisation of vitamin D and its isomers resulting in the formation of the corresponding pyrocalciferol and isopyrocalciferol compounds. In order to overcome this problem, extra steps such as chemical treatment prior to analysis was suggested (Kobayashi, 1967c; Koshy and Beyer, 1984).

In terms of the identification of vitamin D3 isomers, those that have specific ultraviolet (UV) absorption, are detectable by high-performance liquid chromatography with diode-array detection (HPLC-DAD). The formation of these products was confirmed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Vitamin D3 and some of its degradation products are reactive towards dienophiles such as maleic anhydride (Zhang et al., 2006). Chemical derivatization with a dienophile could help to distinguish between vitamin D3 and its isomerisation products and also to improve LC-MS sensitivity through increased ionisation efficiency. In this study, the degradation mechanisms of vitamin D3 (cholecalciferol) induced by heat, acid and iodine were studied. The degradation products were isolated and identified using a combination of HPLC-DAD, UHPLC-MS/MS and chemical derivatization using a dienophile (PTAD).

3.2. Material and methods

3.2.1. Chemicals

Cholecalciferol (99%), 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (99%), formic acid (99%), iodine, sodium thiosulfate, sodium sulfate and sodium carbonate (ASC reagent) were obtained from Sigma-Aldrich (Auckland, New Zealand). Methanol (HPLC grade), hexane, diethyl ether and hydrochloric acid (38%) were obtained from Scharlab, S.L (Barcelona, Spain).

3.2.2. HPLC-DAD analysis

An Agilent 1260 Infinity HPLC system with a diode array detector was used (USA). The chromatographic separation was performed using a Kinetex PFP reversed-phase column (5 µm, 150 mm × 4.6 mm; Phenomenex, USA) at ambient temperature. For the optimisation of chromatography, different columns suggest ODS3, C18 and PFP were tried. Based on the efficiency of separation, Kinetex PFP was chosen. Reversed-phase chromatography was used with two solvents as mobile phases, 0.1% formic acid in water (A) and methanol (B) in gradient mode at flow rate 0.6 mL/min. The gradient was changed from 85% B to 90% in 5 min, held for 6 min, then returned to the initial conditions in 15 min. The injection volume was 20 µL.

3.2.3. Liquid chromatography mass spectrometry

A 1290 Infinity II UHPLC Agilent (USA) with 6400 Series triple quadrupole mass spectrometer system equipped with an electrospray ionisation (ESI) source in positive ion mode was used. Detection was carried out in product ion mode, using *m/z* 385 [Vitamin D3+H]$^+$, 560 [Vitamin D3:PTAD+H]$^+$, 401 [Vitamin D3+O+H]$^+$, 417 [Vitamin D3+2O+H]$^+$ and 433 [Vitamin D3+3O+H]$^+$ as precursor ions. The UHPLC conditions were the same as for HPLC-DAD described in Section 3.2.2 except for injection volume, which was 5 µl. The optimised operating parameters of the ESI(+)-UHPLC-MS/MS were as follows: capillary voltage was set at 3500 V, sheath gas and drying gas temperature were 250°C and 300°C, respectively. The nitrogen gas flow rates were 10 L/min for the sheath gas and 7 L/min for drying gas.

3.2.4. Isomerization of vitamin D3 under different conditions

3.2.4.1. Thermal isomerization

A solution of vitamin D3 in methanol (0.2 mg/mL) was heated at different temperatures, i.e. 25, 45, 55 and 65 °C, for 1 h. The reaction was protected from light and it was cooled on ice quickly and injected into the HPLC-DAD and UHPLC-MS/MS, separately.

3.2.4.2. Iodine-induced degradation

The formation of *cis/trans* isomerisation of vitamin D3 was carried out according to the method of Kobayashi, Moriuchi, Shimura, and Katsui (1976), modified as described. To a 10-mL solution of vitamin D3 standard in hexane (0.1 mg/mL), 0.2 mL of iodine solution (0.1 mg/mL) were added. The mixture was stirred at room temperature for 1 h under nitrogen. The reaction was stopped by adding 5 mL of 1% sodium thiosulfate solution. The organic layer was separated and dried over anhydrous sodium sulfate. The filtrate was evaporated to dryness under reduced pressure and dissolved in methanol for HPLC-DAD and UHPLC-MS/MS analysis. This experiment was also carried out after heat treatment of vitamin D3 solution in hexane at 70 °C for 1 h.

3.2.4.3. Acid-induced degradation

The reaction of vitamin D3 with acid was carried out according to the method of Jin et al. (2004), modified as described. A sample of 100 mg vitamin D3 was dissolved in methanol (5 mg/mL) and treated with 50 µL HCl and the solution was refluxed at 70 °C for 5 h. The reaction was protected from light and was under nitrogen. The reaction mixture (pH ~1) was then neutralised with sodium carbonate, extracted with diethyl ether, washed with brine and dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation and the pale yellow oily substance obtained was dissolved in methanol and injected into the HPLC-DAD and UHPLC-MS/MS.

3.2.4.4. Autoxidation of isotachysterol

The pale yellow oily substance prepared by the method described in Section 3.2.4.3 and was kept at ambient temperature in the dark for 2 days without protection from air. Then, it was injected into the HPLC-DAD and UHPLC-MS/MS.

3.2.5. Chemical derivatization

Vitamin D3 isomerisation products were generated as described in previous sections and their reactivity was tested with the dienophile, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), and were analysed by UHPLC-MS/MS. A suitable derivatization can enhance the ionisation efficiency of vitamin D3 and its isomerisation products in UHPLC-MS/MS and improve fragmentation enabling increased sensitivity of detection. Derivatization with PTAD also provides reactivity differentiation or different fragmentation patterns under UHPLC-MS/MS. This may provide a tool to differentiate vitamin D3 isomers, including those that do not react with dienophiles (De Vries et al., 1977; Zhang et al., 2006).

3.3. Results and discussions

3.3.1. Thermal degradation

Heat is responsible for conversion of vitamin D3 to previtamin D3. Since this conversion is reversible, it is not easy to produce an authentic sample of previtamin D3. The HPLC-DAD chromatograms of solutions of vitamin D under various temperature regimes are shown in Figure 3.1. While vitamin D3 eluted at a retention time of about 7.8 min, compound **1** was observed as a degradation product at higher temperatures. Based on Figure 3.1 a, the yield of compound **1** was dependent on the temperature and showed the highest level at the highest temperature tested (65 °C). The UV spectra of vitamin D3 and compound **1** are presented in Figure 3.1 (b and c, respectively). The UV spectrum of vitamin D3 showed a maximum absorption at 265 nm, while the UV spectra of **1** exhibited a shift to 261 nm. A similar phenomenon was also reported by previous studies in terms of vitamin D2 and ecalcidene, a vitamin D analogue (Hofsass, Grant, Alicino, & Greenbaum, 1976; Zhang et al., 2006).

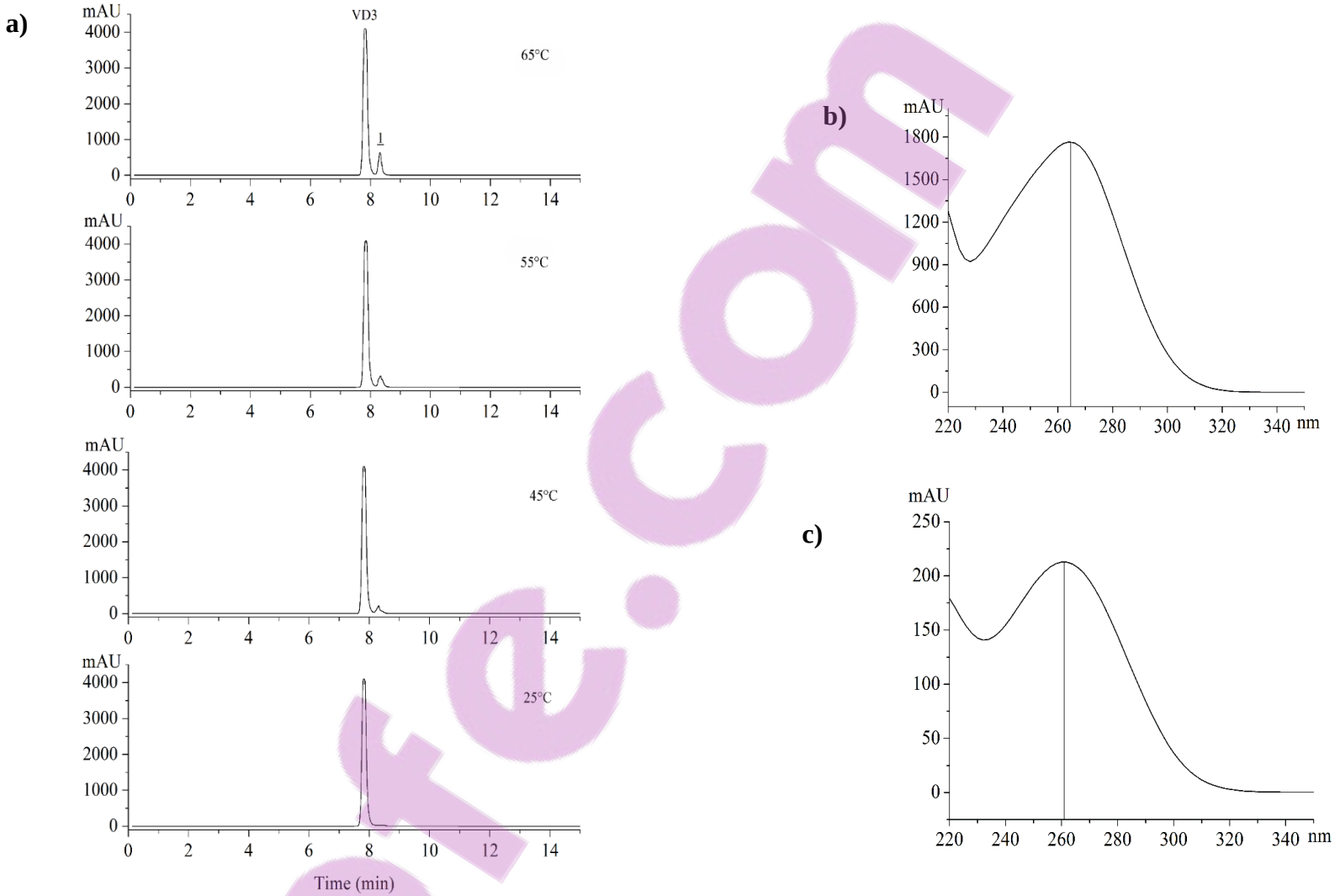


Figure 3.1. HPLC-DAD chromatograms of solutions of vitamin D3 under different temperatures **(a)**, and UV spectra of vitamin D3 **(b)** and compound **1** (previtamin D3) **(c)**

In this study, UHPLC-ESI-MS/MS analyses were also performed and the total ion chromatograms (TICs) of the samples were similar in profile to those of the HPLC-DAD chromatograms. Mass spectrometry studies of vitamin D3 show the protonated molecular ion and the loss of one water molecule from the protonated molecular ion at *m/z* 385 $[M+H]^+$ and 367 $[M+H–H2O]^+$, respectively (Figure 3.2). The molecular ion also was fragmented to *m/z* 107, 159 and 259, similar to the study of Trenerry et al. (2011). The mass spectrum of compound **1** also shows similar molecular ions to those of vitamin D3 indicating that **1** is an isomer of vitamin D3 (Figure 3.2).

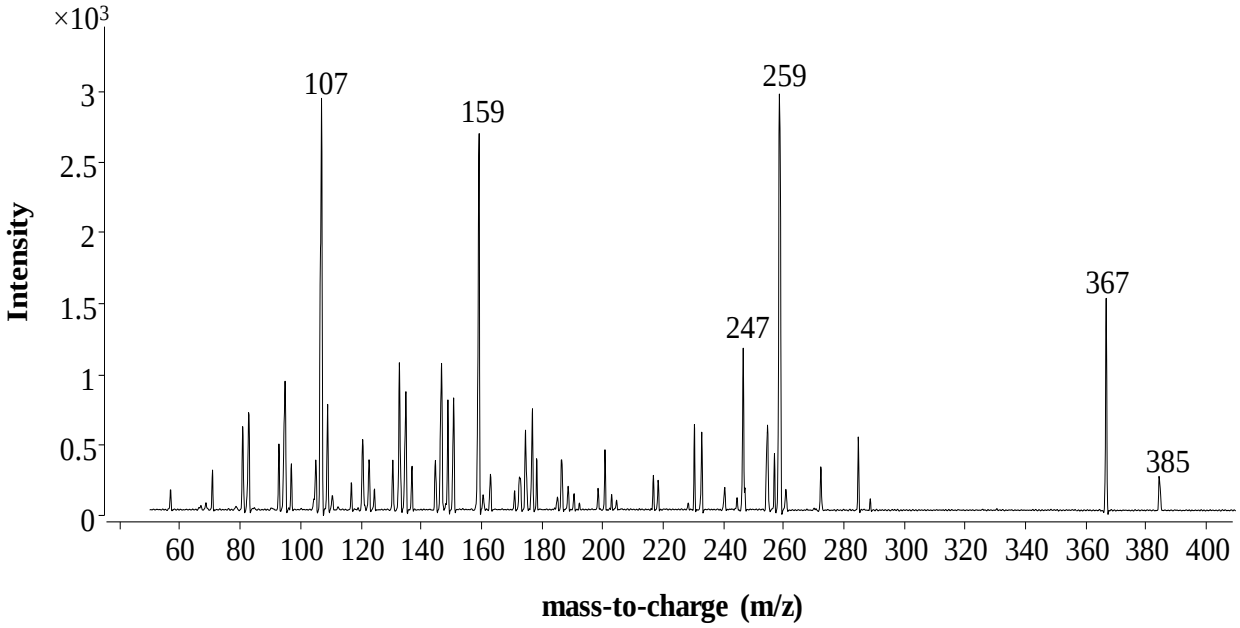


Figure 3.2. ESI+ mass spectrum of vitamin D3 and compounds **1**, **2**, **3** and **4**

3.3.2. *Cis/trans* isomerization

Iodine is an important element which could be available as a natural constituent or added chemical in some food and pharmaceutical products. Iodine also could come in trace amounts as residues of cleaning and sanitation processes in the food industry (Sutcliff, 1990). Iodine even in low concentration could be involved in vitamin D degradation. Iodine-catalysed isomerisation of vitamin D2 was studied by Verloop et al. (1955), and later they found that *cis/trans* isomerisation and the conversions depended on different parameters, such as ratio of iodine and vitamin D, solvent, light and time (Verloop et al., 1959). The effect of iodine on vitamin D3 and its isomers was studied using HPLC-DAD and UHPLC-ESI-MS/MS. In this study, different parameters were used to form *trans* isomers of vitamin D3 and separate them in high resolution with reproducibility.

While the solution of cholecalciferol without iodine exhibits only the peak of vitamin D3, the addition of iodine to the solution resulted in the formation of a new product **2** (Figure 3.3 a and b). Maximum UV spectrum of **2** shows a shift to 273 nm, which is the *trans* form of vitamin D3 (Figure 3.3 d). Previous studies also reported a shift in the UV absorption from 265 to 271 nm after *cis/trans* isomerisation of vitamin D2 (Verloop et al., 1955). Moreover, its ESI + mass spectrum gave a molecular ion at *m/z* 385 $[M+H]^+$ and product ions at *m/z* 367 $[M+H–H2O]^+$ similar to compound **1**, indicating that **2** is an isomer of cholecalciferol.

In order to test the effects of iodine on the other types of vitamin D3 isomers, previtamin D3 was generated by thermal degradation of vitamin D3, as described in Section 3.2.4.1. The addition of iodine to a mixture of vitamin D3 containing the previtamin D type isomer leads to the formation of the *trans* form of vitamin D3 (compound **2**) and a new compound **3** (Figure 3.3 c). This new compound showed another form of UV spectrum with a maximum absorption peak at 279 nm, with two shoulders at 270 and 290 nm (Figure 3.3 e). This is similar to the spectrum of tachysterol, a *trans*-isomer of previtamin D, which was also reported by other studies in terms of vitamin D2 and ecalcidene (Verloop et al., 1959; Zhang et al., 2006). The mass spectrum of compound **3** showed similar product ion mass spectra and fragmentation patterns to those of vitamin D3 and compound **1**, revealing that it is an isomer of **1** and vitamin D3 (Figure 3.2).

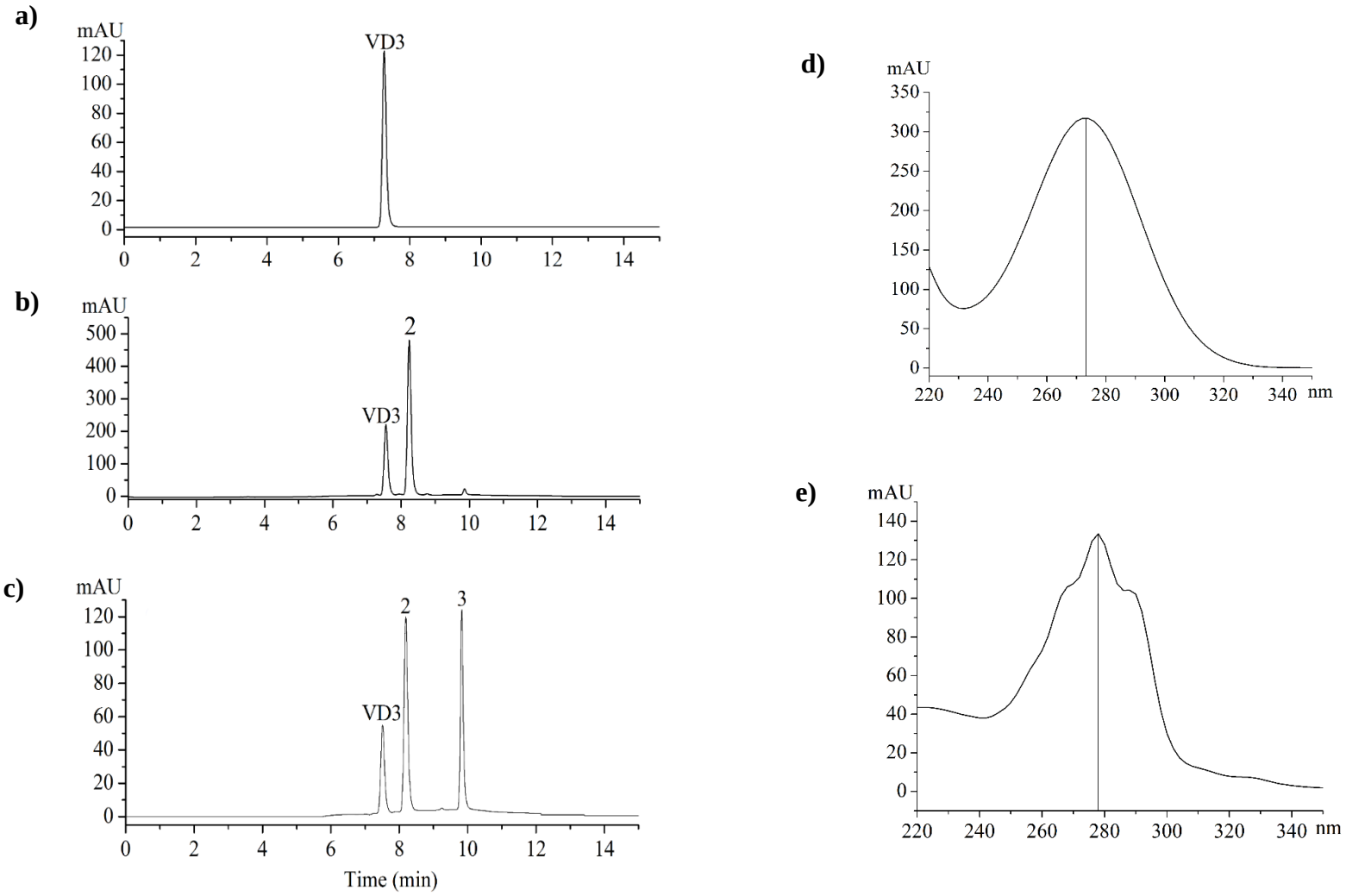


Figure 3.3. HPLC-DAD chromatograms of vitamin D3 **(a)**, vitamin D3 + iodine **(b)** and vitamin D3 + iodine after thermal degradation **(c)**, and UV spectra of compound **2** (*trans*-vitamin D3) **(d)** and compound **3** (tachysterol) **(e).**

3.3.3. Acid-induced degradation

Low pH is known to isomerise vitamin D mostly to isotachysterol (Jin et al., 2004). Isotachysterol, which exists as a pale yellow oily liquid, was prepared by HCl-catalysed isomerisation of vitamin D3 in methanol in high yield. Based on previous studies, isotachysterol can be used as a part of chromatographic identification of unknown vitamin D3 metabolites as well as in the determination of vitamin D3 compounds in different samples (Koskinen & Valtonen, 1985; Naidoo, 2011). It was also reported that in preparation of isotachysterol the isomerisation product yield is affected by the temperature, the volume of the reaction mixture and the reaction time. In this study, different parameters were tested and isotachsyterol in reasonably concentration and resolution was obtained.

As shown in Figure 3.4 a, the HPLC-DAD chromatogram of the reaction sample gave a predominant peak **4** at a retention time of about 13 min. A minor degradation product eluting at a retention time of about 11 min was also observed. Based on the UV spectra of these two peaks, compound **4** is isotachysterol with a maximum absorption peak at 290 nm and two shoulders at 280 and 302 nm (Figure 3.4 c). The other small peak at 11 min is tachysterol, a 7,8 *cis*-isomer of isotachysterol, with a UV maximum at 280 nm. Generation of this isomer also suggests that acid-induction of vitamin D3 may form isotachysterol through intermediates of previtamin D3 and tachysterol. This observation also coincides with previous studies reported by Kobayashi (1965) in terms of chemical isomerisation of vitamin D2 to isotachysterol. The ESI + mass spectrum of compound **4** in Figure 3.2 also exhibits ions at *m/z* 385 $[M+H]^+$ and 367 $[M+H–H_2O]^+$.

It is known that vitamin D3 is stable in air at ambient temperature; however, its acid-catalysed isomerisation product seems very labile in air (Jin et al., 2004). Since this acid-catalysed isomerisation product could be formed by acidic vitamins such as ascorbic acid, similar reaction could take place in systems such as food and pharmaceutical products. In this respect, it was found that the pale yellow oil was oxidized rapidly in air at ambient temperature and a small amount of compound **4** was left. Figure 3.4 b shows that the level of isotachysterol decreased and small peaks with shorter retention time appeared. These small peaks, showing UV maxima at 248 nm, characteristic of conjugated double bonds, could be vitamin D3 oxidation products. The small peak at a retention time of about 8.7 min exhibited a UV absorption maximum at 254 nm, suggesting the existence of a conjugated triene system. Jin et al. (2004) reported that this peak corresponded

to 6,7-*cis*-isotachysterol. It has also been previously reported that isotachysterol could isomerise to *cis*-isotachysterol photochemically (Verloop at al., 1960).

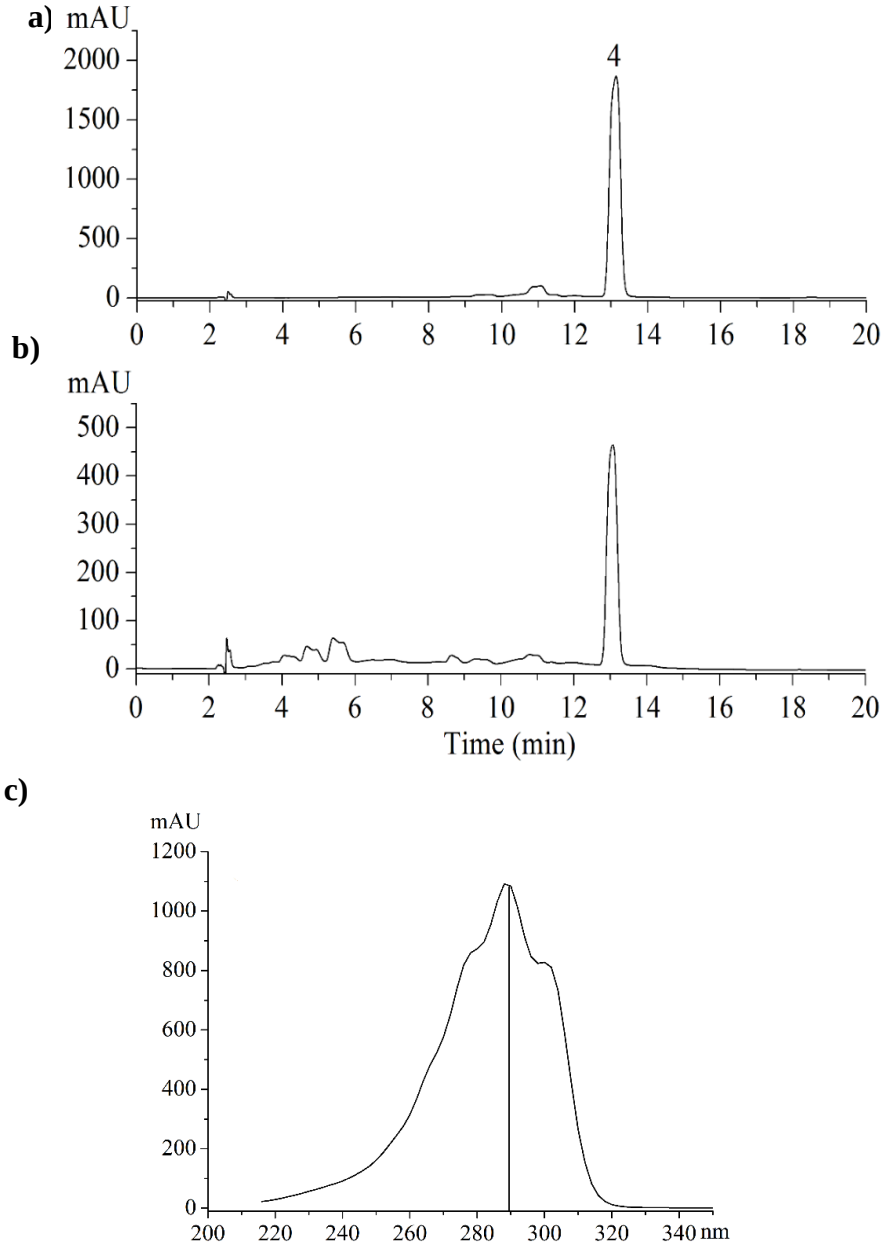


Figure 3.4. HPLC-DAD chromatograms of solution of vitamin D3 in acidic condition (**a**) and oxidation of isotachysterol (**b**), and UV spectrum of compound **4** (isotachysterol) (**c**).

Additionally, UHPLC-ESI-MS/MS determination gave molecular ions at *m/z* 401, 417 and 433 $[M+H]^+$ and product ions losing one water molecule at *m/z* 383, 399 and 415 $[M+H–H_2O]^+$, corresponding to the molecules with one, two and three more oxygen atoms than *m/z* of compound **4** (Figure 3.5). According to Figure 3.5 (b and c), vitamin D3 oxidation products with two and three oxygen atoms showed more product ions, losing two and three water molecules, respectively. All three molecular ions also fragmented to *m/z* 273 and 302. These fragmentation patterns could provide useful information to identify and quantify vitamin D oxidation products in vitamin D fortified products.

The combination of separation technology with spectroscopic technique (UHPLC-MS/MS) was developed to separate all isomer peaks of interest with sufficient resolution. Based on the earlier studies in our laboratory in terms of RP-HPLC, we investigated the effect of elution conditions, particularly flow rate and mobile phase composition, on the separation and resolution of vitamin D3 and its isomerisation products. We optimised the gradient and it was found that the separation of vitamin D3 and its isomerisation products were greatest when gradient reached 90% methanol; the use of other solvents, such as acetonitrile, resulted in poor separation (data not shown). Among different columns tested, the Kinetex PFP column, which has a pentafluorophenyl phase, offered a high degree of steric interactions for improved separation of structural isomers of vitamin D3. The proposed structures of vitamin D3 and its isomerisation products (compound **1**, **2**, **3** and **4**) are shown in Figure 3.6.

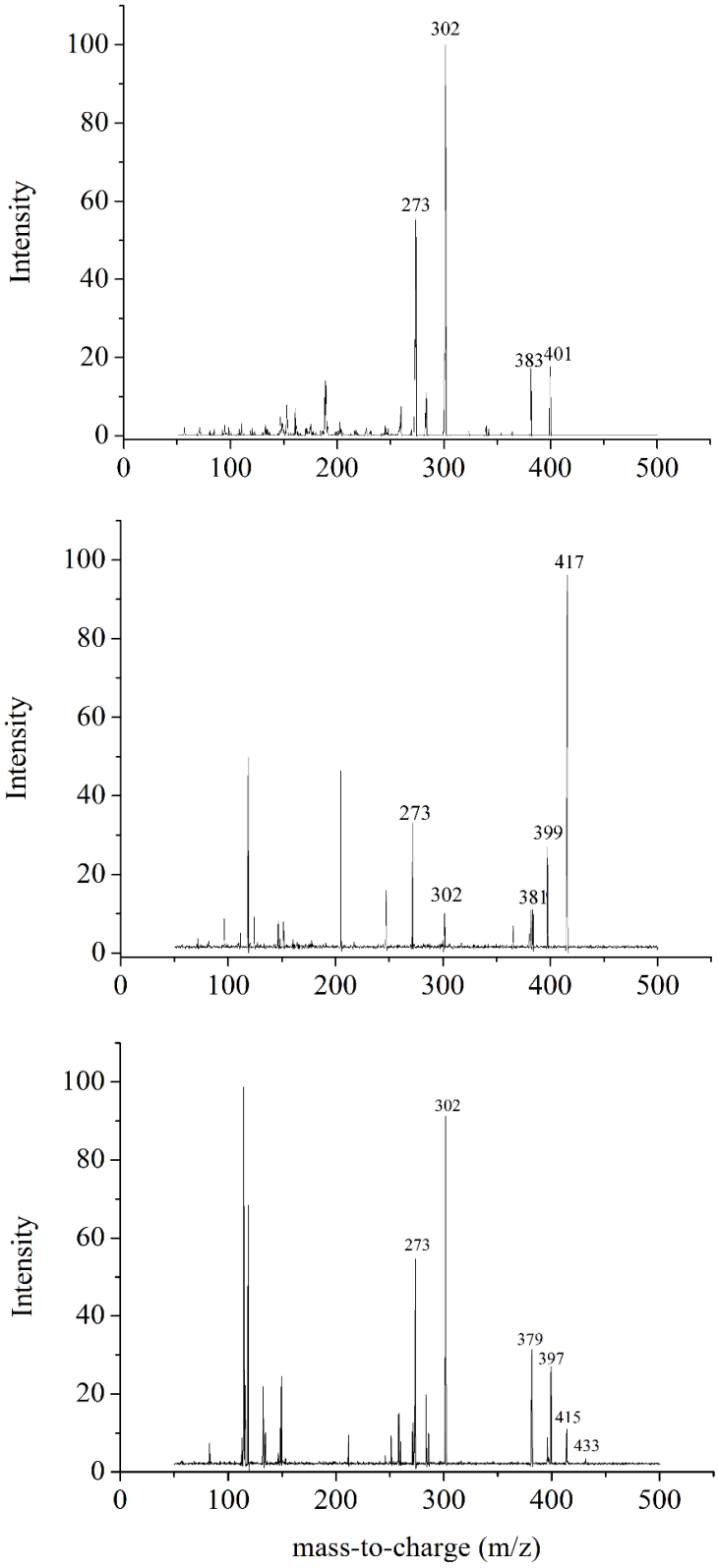


Figure 3.5. ESI+ mass spectra of vitamin D3 oxidation products including one oxygen atom (**a**) two (**b**) and three (**c**) oxygen atoms.

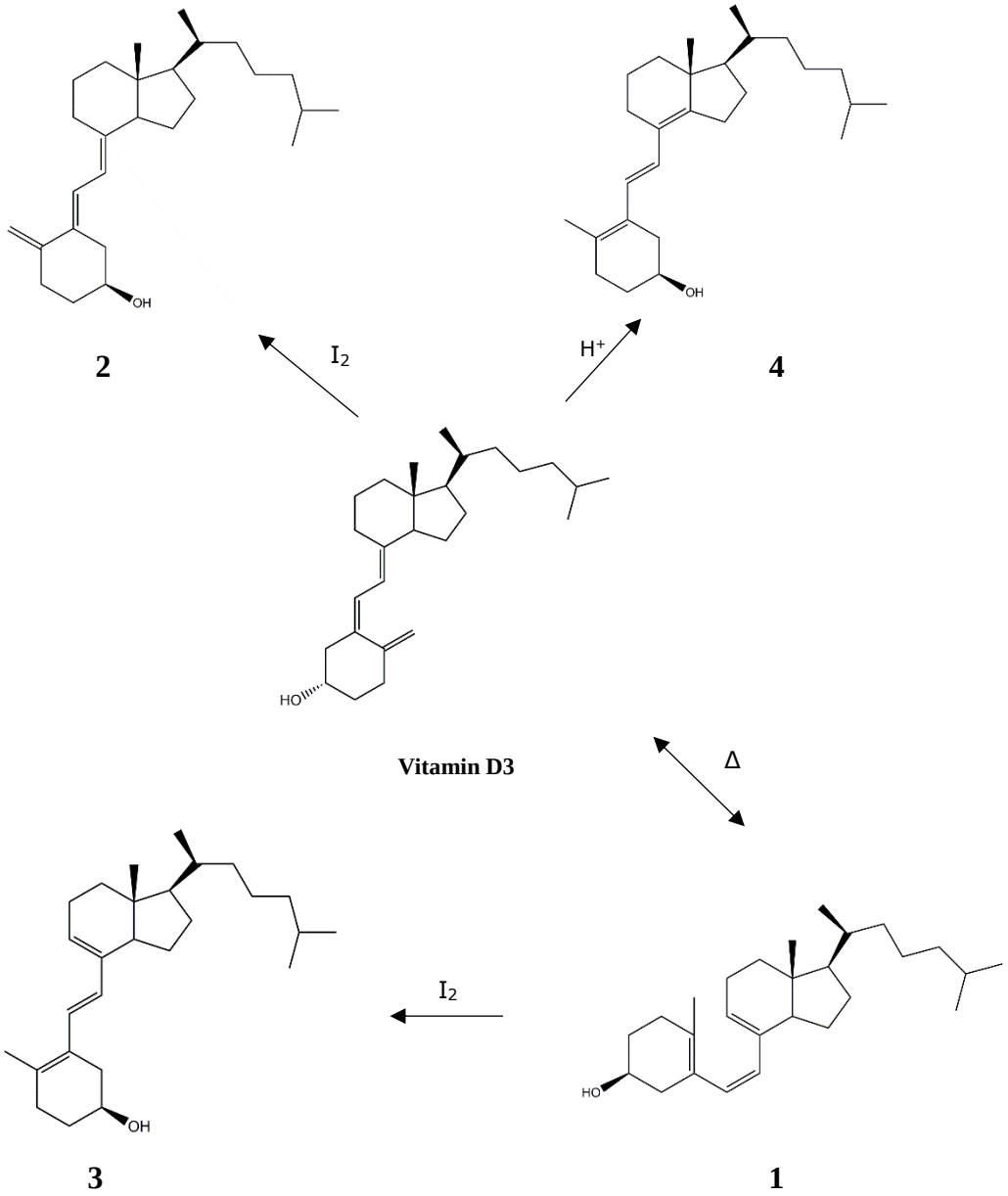


Figure. 3.6. The proposed structures of vitamin D3 and its isomerization products (compounds **1**, **2**, **3** & **4**).

3.4. Chemical derivatization

Vitamin D3 and its degradation products were also identified using chemical derivatization through reacting with PTAD. Derivatization is advantageous because ionisation efficiency is increased and the molecular weight shifted to a higher mass range, where background noise is lower. Ding et al. (2010) reported that the product ion spectrum of vitamin D3:PTAD adduct only exhibits one major fragment, which is beneficial for sensitive selected reaction monitoring (SRM). Moreover, derivatization can be used to distinguish between various vitamin D3 isomers, as they have different reactivity with PTAD. According to previous studies, when compared to the *cis* form, the *trans* form of vitamin D reacted rapidly with maleic anhydride to form Diels-Alder adducts (De Vries et al., 1977). PTAD, which is more reactive than other dienophiles such as maleic anhydride, was chosen for this study.

In the mixture containing vitamin D3, and compounds **1**, **2** and **3**, PTAD reacted rapidly with vitamin D3 and compounds **2** and **3**. Additionally, acid-catalysed isomerisation products did not react with PTAD, which could confirm the formation of isotchysterol. This coincides with the study of De Vries et al. (1977), which reported the non-reactivity of isotachysterol from vitamin D2 with maleic anhydride. The reaction of PTAD with vitamin D3 and its isomers generated the corresponding adducts, which were not detected by UV at the wavelength of vitamin D3 and its isomerisation products, but were detected using UHPLC-ESI-MS/MS. The product ion spectrum of vitamin D3:PTAD adduct only exhibits one major fragment, whereas different fragmentation was observed for its isomerisation products (Figure 3.7). While vitamin D3:PTAD showed a fragment at *m/z* 298 $[M+H–C_{19}H_{33}]^+$ as a base peak, the MS spectra of vitamin D3 isomers showed ions at *m/z* 383 $[M+H–C_8H_7N_3O_2]^+$ and 365 $[M+H–C_8H_7N_3O_2–H_2O]^+$ (Figure 3.7 a and b). These results coincide with the study reported by Abernethy (2012), in terms of cholecalciferol extraction in dairy products. The different fragmentation patterns could provide useful information for the identification of vitamin D3 and its degradation products in complex mixtures.

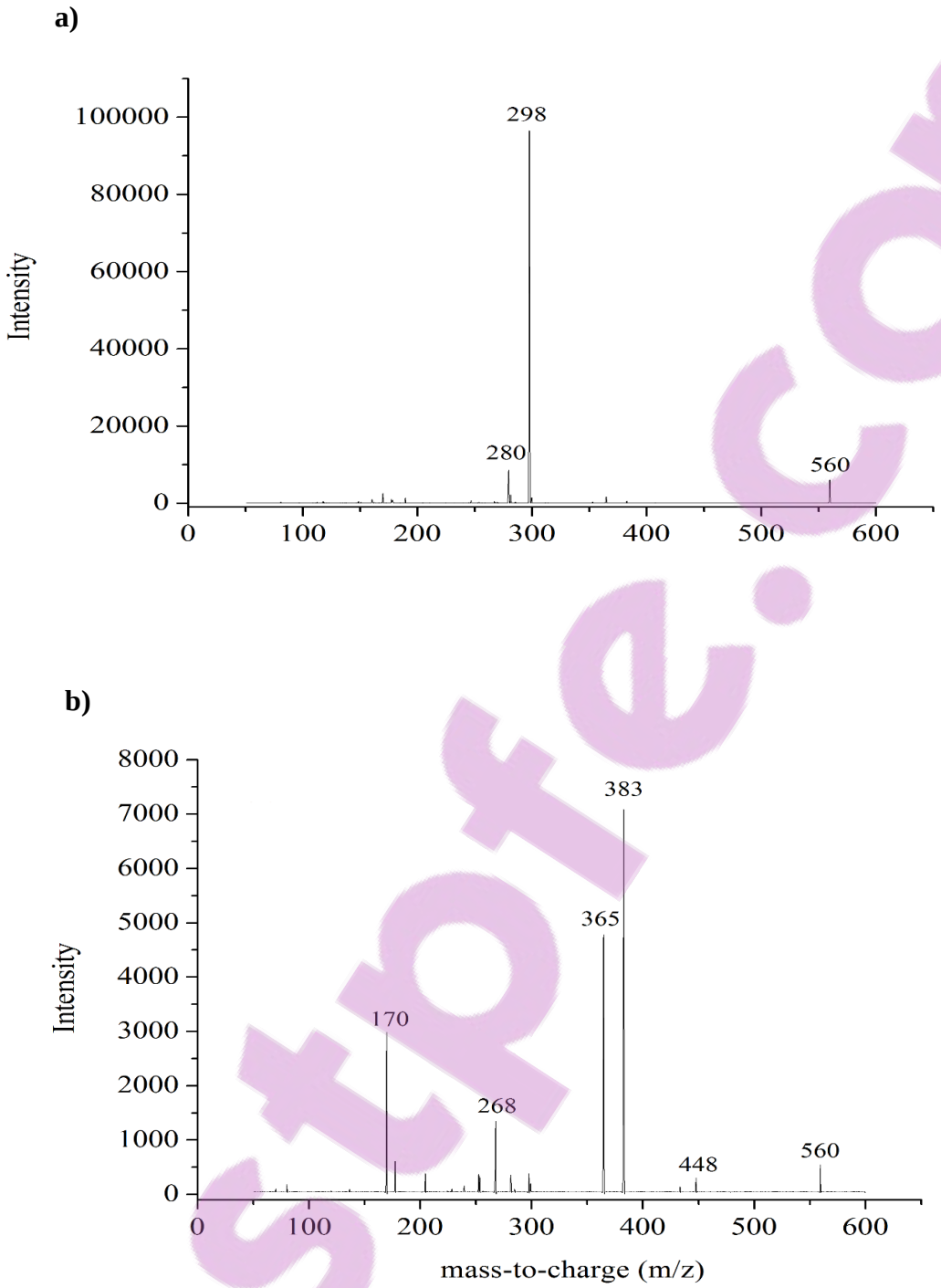


Figure 3.7. ESI+ mass spectra of vitamin D3:PTAD adduct **(a)** and vitamin D3 isomers:PTAD adduct **(b)**.

3.5. Conclusions

The effects of different physicochemical parameters, such as temperature, iodine, acid and oxygen, on the stability of vitamin D3 were tested. Vitamin D3 showed a reversible isomerisation to previtamin D3 isomer by thermal degradation. Iodine-induced degradation showed *cis/trans* isomerisation of vitamin D3 and previtamin D3 to *trans*-vitamin D3 and tachysterol, respectively. In an acidic medium, a pale yellow oily liquid, isotachysterol, was produced from vitamin D3. Apart from that, this work demonstrates that this acid-catalysed isomerisation product is liable to form a variety of oxidation products via autoxidation.

In terms of chemical derivatization, vitamin D3 and all its isomerisation products had different rates of reaction with the dienophile PTAD, while isotachysterol did not show any reaction. Despite the fact that vitamin D3 and its isomerisation products showed the same MS spectra, the vitamin D3:PTAD adduct gave different mass spectra compared to other PTAD product adducts. This data could help to quantify vitamin D3 and its isomerisation products in different complex mixtures with high resolution.

This study suggested modified procedures which were capable of separating vitamin D3 isomerization products in high resolution without the complexities of previous methods, which were time consuming, caused compound decomposition, and lacked sensitivity and reproducibility. This work has also focused on correlating vitamin D3 degradation observed in fortified products with the likely mechanism of degradation. These results may provide useful information for vitamin D3 fortification in order to improve the knowledge of vitamin D in terms of its stability, mechanism of degradation and analytical procedure.

CHAPTER 4

Lipid oxidation and vitamin D3 degradation in simulated whole milk powder as influenced by processing and storage

Contents submitted in *Food Chemistry*. No. FOODCHEM-D-18-00316

4.1. Introduction

Vitamin D3 is an essential nutrient which plays a significant role not only in bone health, but many other roles including modulation of cell growth, immune function and reduction of inflammation. This nutrient can be synthesized in the body through exposure to sunlight. However, seasonal changes, living at high latitude, aging and other factors can impair this process (Holick et al., 2011; Spiro & Buttriss, 2014). There are limited dietary sources of vitamin D, some of which include cod liver oil, fatty fish, egg yolks as well as small amounts in whole milk (O'Mahony, Stepien, Gibney, Nugent & Brennan, 2011). Therefore, vitamin D3 fortification of foods can be beneficial for population who are at high risk of deficiency.

Vitamin D fortification is mandatory in Canada for beverage milk and margarine and optional in the United States for milk, breakfast cereals, and calcium-fortified fruit juices (Sacco, 2013). In Australia, manufacturers must add vitamin D to margarine. There is no mandatory fortification of vitamin D in New Zealand, but vitamin D may be added on a voluntary basis to a range of milk and milk products as well as formulated beverages (Food Standards Australia New Zealand, 2016).

Among the different foods, dairy products may be a good target for vitamin D fortification as they are widely consumed with a high concentration of essential nutrients. Additionally, a fatty vehicle like whole milk may be a proper source for fat-soluble vitamins like vitamin D fortification (Upreti, Mistry, & Warthesen, 2002). In any fortification program, the consistency between claimed and measured values of nutrients is one of the most important as far as the regulations are concerned. In terms of vitamin D3 content, significant disagreement between analyzed values and claimed amounts has been reported by previous studies (Loewen, Chan, & Li-Chan, 2018). In the 1990s, fortified milk products taken in Canada and United States were not entirely consistent with the nutritional labelling (Chen, Shao, Heath & Holick, 1993; Holick, Shao, Liu & Chen, 1993). Murphy et al. (2001) also reported that about 52% of fortified milk tested was not in the acceptable range.

Stability of vitamin D3 has been studied in processed dairy products during processing and storage by several researchers. Their researches have mostly focused on fortified cheese products during ripening in a short storage period at low temperatures (Upreti et al., 2002; Wagner et al., 2008).

They reported that vitamin D3 was not degraded as a result of processing or during storage; however, contradictory reports have also appeared regarding vitamin D stability in some studies (Banville, Vuillemard & Lacroix, 2000; Murphy et al., 2001). Banville et al. (2000) evaluated vitamin D3 reduction in fortified cheddar cheese after seven months of storage. They reported decreasing rates of 11 and 16% for cheese containing vitamin D in cream and water-soluble emulsions of vitamin D, respectively. It was also reported that some vitamin D3 degradation occurred during storage of cheddar cheese for three months (Kazmi et al., 2007). A deterioration of 7% was observed when cheese was fortified with crystalline vitamin D3.

Besides, inconsistencies of reporting of vitamin D stability to different parameters such as oxidation, heat, light and acidity have been known (Liu, 2013; Loewen et al., 2018). Some studies reported that vitamin D3 was unstable to these factors (Cremin & Power, 1985; Liu, 2013; Stewart, Midland, & Byrn, 1984). The stability of vitamin D to oxidation and its instability to heat, moisture and trace minerals were also reported by previous studies (Charlton & Ewing, 2007; Riaz, Asif & Ali, 2009). Vitamin D3 seems stable against different processing and environmental factors, but its stability may be dependent on the stability of the type of food fortified (Loewen et al., 2018; Banville et al., 2000; Renken & Warthesen, 1993). Renken and Warthesen, (1993) reported that air and temperature affected vitamin D3 stability in model systems in acetonitrile. They also reported vitamin D3 degradation in skim milk with light exposure produced the greatest loss. Banville et al. (2000) reported that, while vitamin D3 was stable in yogurts and ice creams during the expected shelf life of these products, it was unstable in cheese. Different stability of vitamin D during cooking of eggs, margarine and bread was also reported which was dependent on the food matrix and the heating process (Jakobsen & Knuthsen, 2014).

In whole milk powder, the unsaturated fat can react with oxygen to cause oxidation, especially when stored at high temperature for long term. One study found vitamin D3 degradation in milk by exposure to air which might have occurred via an oxidation reaction (Renken & Warthesen, 1993). Cholesterol, which is very similar to vitamin D in structure, is highly susceptible to oxidation to form cholesterol oxidation products (COPs). Previous studies have reported the generation of COPs dependent on the processing and storage conditions of whole milk powders (Cluskey et al., 1997; Nourooz-Zadeh & Appelqvist, 1988). They suggested that there is a relationship between lipoxidation and COPs generation in cholesterol containing foods. In milk

powder manufacturing, heat treatments and storage conditions are two factors which could have effects on the rate of oxidation and the composition and percentage of oxidation products (Chen et al., 1993; Cluskey et al., 1997).

The inconsistency of vitamin D3 stability has been associated with several reasons, such as analytical variability, poor fortification practices, chemical breakdown, processing or storage effects and type of foods. In terms of milk powder, there are only a few references to the fate of degradation of vitamin D3. To our knowledge, the stability of vitamin D3 in whole milk powder during processing and storage has never been reported. The paucity of literature on the stability of vitamin D3 in milk powder suggests that more studies might be needed in this area.

The objective of this project was to study the stability of vitamin D3 in whole milk powder. In this study, the aim was to develop a simulated whole milk powders (SWMPs) fortified with vitamin D3 so that vitamin D3 degradation could be studied in a simple matrix. In order to find the relationship between lipid oxidation and vitamin D3 degradation and to identify vitamin D3 oxidation products, samples were fortified with higher concentration of vitamin D3 compared to the real fortification practices. Model samples were used to investigate the effects of heat treatment and storage conditions on lipid oxidation and vitamin D3 degradation under controlled conditions.

4.2. Materials and methods

4.2.1. Chemicals

Vitamin D3 (cholecalciferol, 99%), 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), formic acid, 1,1,3,3-tetraethoxypropane (TEP), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA) and pyrogallol were obtained from Sigma-Aldrich (Auckland, New Zealand). Isotope-labeled vitamin D3 (vitamin D3-d6) was supplied by Chemaphor (Ottawa, Canada). LC grade methanol, ethanol, acetone, acetonitrile, isooctane and hydrochloric acid (38%) were obtained from Scharlab, S.L (Barcelona, Spain). Ammonium thiocyanate, ammonium iron (III) sulfate ($NH_4Fe(SO_4)_2 \cdot 12H_2O$), iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$), barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$), potassium hydroxide and ACS reagent grade chloroform, ethyl acetate, hexane, diethyl ether, petroleum ether, ammonium hydroxide, ammonium acetate and sulfuric acid were obtained from ECP

(Auckland, New Zealand). Bond Elut (2g) aminopropyl SPE cartridge were from Agilent Technologies (CA, USA).

Sodium caseinate, whey protein isolate (WPI), lactose, anhydrous milk fat (AMF) and dry vitamin D3 supplement were supplied by Fonterra Co-operative Group Ltd. (Palmerston North, New Zealand). The specification of milk components are provided in Appendix 4.1. The vitamin D supplement, 2500 µg vitamin D3 per gram, contained vitamin D3 dissolved in medium chain triglycerides in a matrix of gum arabic and sucrose, coated with starch and tricalcium phosphate as a free flowing agent (BASF, 2015). Water used was purified using a Milli-Q system (≥18 MΩ) (Millipore Corp., Bedford, USA).

4.2.2. Laboratory-scale preparation of simulated whole milk powder

Laboratory-scale simulated whole milk powders were prepared with the main milk components. The formulation (w/w) was as follows: 3.5% milk proteins, 4.8% lactose, 3.5% milk fat and 88.2% water. As for milk proteins, the sodium caseinate: whey protein isolate ratio was (w/w) 8:2 in all samples. The proteins and lactose were reconstituted in MQ water at 40°C and the solutions were stirred for 1 h. Emulsions were prepared by adding the milk fat to the oil-free phase, heating to 50°C and allowed to cool to ambient temperature before homogenizing using a laboratory scale homogenizer (Model IKA T25 Ultra-Turax, Malaysia) at 24,000 rpm for 2 min.

Simulated milk under two processing conditions were prepared including high temperature, heating at 95 ± 2°C in a water bath for 30 seconds and cooling in an ice bath, and also without any high temperature heating (referred to hereafter as with and without heating). Model milk samples with and without heat treatment were fortified with the dry vitamin D3 pre-mix at a rate of 1 mg vitamin D3 per 100 g at room temperature. In order to find the relation between lipid oxidation and vitamin D3 degradation and to identify vitamin D3 oxidation products, higher concentration of vitamin D3 was added compared to the real fortification practices. The fortified samples were stirred and stored at -80°C before freeze drying. The simulated whole milk powder preparation procedure is summarized in Appendix 4.2.

The freeze-dried samples were packed in a sealed plastic bag and then sealed in an aluminium package to protect from light (Appendix 4.3). All samples packed similarly, to control any possible interaction of vitamin D and packaging. Samples were stored for 12 months and analysed at zero time and after 1, 3, 5, 7, 9 and 12 months of storage in triplicate. The temperatures selected for storage were room temperature (RT of 20°C) and 40°C. The former is the usual room temperature in markets, while the latter temperature can be experienced under extreme conditions, for instance in shipping the products.

4.2.3. Measurement of fat globule size of emulsions

The particle size distribution of each emulsion was determined using a Malvern Particle Size Analyzer (Mastersizer 2000S, Malvern Instruments Ltd, UK). The optical parameters selected were a particle and dispersant refractive index of 1.46 and 1.33, respectively. Measurements were carried out in triplicate.

4.2.4. Analysis of lipid oxidation products

4.2.4.1. Fat extraction

Milk fat was extracted by Rose-Gottlieb method as described (IDF, 1987). In an extraction tube, 2g of simulated whole milk powder was dissolved in 10 mL water, and 1.25 mL ammonium hydroxide was added to the solution. After mixing, 10 mL ethanol was added and mixed. Then, 25 mL of diethyl ether was added and the mixture was shaken vigorously for about one minute, then 25 mL of petroleum ether was added and shaken for another half minute. The upper layer was separated in a separating funnel and the extraction was repeated twice using 15 mL of each solvent every time. The ethereal extract was evaporated and dried in an oven at 102 ± 2°C for two hours. The fat was weighted and used for peroxide value analysis.

4.2.4.2. Peroxide value

The peroxide value was determined by the ferric thiocyanate method on milk fat samples by the method of Shantha & Decker (1994). A weighed amount of extracted milk fat was dissolved in a mixture of chloroform and methanol (v/v) (70:30) followed by the addition of small quantities of

ammonium thiocyanate and ferrous chloride solutions. Any peroxide present causes the oxidation of the iron (II) to iron (III), which then forms a red complex with the thiocyanate.

Ammonium thiocyanate solution (30%) was prepared by dissolving 30 g of ammonium thiocyanate in water and made up into a 100 mL volumetric flask. To prepare ferrous chloride solution, approximately 0.4 g of barium chloride dihydrate was dissolved in about 50 mL water. Then dissolved approximate 0.5 g of iron(II) sulfate heptahydrate in about 50 mL water. Slowly the barium chloride solution was poured with constant stirring, into the iron(II) sulfate solution. About 2 ml of concentrated hydrochloric acid was added and the solution was mixed. Finally, barium sulfate was precipitated and the mixture was centrifuged to separate the upper clear layer.

To prepare the standard solution, 1.079 g of ammonium ferric sulfate ($NH_4Fe\ (SO_4)_2 \cdot 12\ H_2O$) was dissolved in about 70 mL water and mixed with 5 mL of concentrated hydrochloric acid. The solution was made up into a 100 mL volumetric flask with Milli-Q water. An aliquot of 10 mL of this solution was made up to 100 mL with Milli-Q water. Finally, 2 mL of the last solution was diluted with the chloroform-methanol mixture into a 100 mL volumetric flask. Calibration curve: 0, 5, 10, 15 and 20 µg of ferric ion were prepared by pipetting 0, 2, 4, 6 and 8 mL from the diluted solution into 10 mL volumetric flasks. To each solution, 50 µL of ammonium thiocyanate solution followed by 50µL of 0.2 mol/L hydrochloric acid were added and made to volume with the chloroform-methanol mixture.

The absorbance of the sample was determined at 500 nm against blank (chloroform-methanol mixture) by using a spectrophotometer (Shimadzu, UV-Vis spectrophotometer, 1700, Japan). The ferric ion content of samples were calculated from the standard curve in µg and the peroxide value was expressed as milligram equivalents of oxygen per kilogram as follow: PV value (meq O2 / kg of fat) = µg of ferric ion in sample /55.84.

4.2.4.3. TBARS value

Thiobarbituric acid reactive substances (TBARS) are formed as a secondary products of lipid peroxidation which can be detected by the TBARS assay using thiobarbituric acid (TBA) as a

reagent. Assay of TBARS measures malondialdehyde (MDA), which is one of the final products of lipid peroxidation, present in the sample. For TBARS determination, compounds reacting with TBA were determined by measuring fluorescence (Manglano et al., 2005). To 2 mL of reconstituted simulated whole milk powder (10%) (wt/vol), 2 mL of trichloroacetic acid (TCA) (10%) (wt/vol) in water and 2 mL of TBA (0.6%) (wt/vol) in 0.25 mol/L HCl were added. The tubes were shaken and heated at 80°C in a water bath for 1 h, left to cool at room temperature and centrifuged at 3000×g at 4ºC for 30 min.

For standard curve, acid hydrolysis of 1,1,3,3-tetraethoxypropane (TEP), an MDA precursor, was prepared by dissolving 75.6 mg of TEP (equivalent to 24.7 mg of MDA) in 10 mL of 0.1 mol/L of HCl (Sugiarto, Ye, Taylor, & Singh, 2010). The test tube was immersed into a boiling water bath for 5 min and quickly cooled. A stock solution of MDA (247 μg/mL) was prepared by transferring the hydrolyzed TEP solution into a 100 mL volumetric flask and diluting to volume with water. The stock solution was diluted further with water to give MDA solutions of various concentrations (ranging from 0 to 5 μg/mL). A 2 mL aliquot of each of the solutions was reacted with TBA and was processed as described above.

Fluorescence was measured at λ excitation = 515 nm and λ emission = 553 nm, using a PerkinElmer LS-55 Luminescence Spectrometer (UK). The concentration of MDA in samples were obtained as μg per gram sample from the standard curve.

4.2.5. Quantitation of vitamin D3 in simulated whole milk powder

Vitamin D3 extraction and derivatization was carried out by the method of Gill, Zhu, & Indyk (2015) with some modifications. Standard preparation and vitamin D3 extraction are described in Sections 4.2.5.1 and 4.2.5.2.

4.2.5.1. Standard preparation

A stock standard solution of stable isotope-labeled standard (40 μg/mL) was prepared by dissolving 1 mg of vitamin D3-d6 in ethanol and made up into 25 mL volumetric flask. The internal standard solution of vitamin D3-d6 (20 μg/mL) was prepared by diluting the stock solution with acetonitrile and the aliquots of the solution (1.5 mL) were stored at -15ºC.

A Non-labelled stock standard solution (1 mg/mL) was made by dissolving 50 mg of vitamin D3 standard in 50 mL ethanol and the solution was stored in -15ºC for 3 months. A vitamin D3 working standard solution (20 µg/mL) was made freshly by diluting the stock standard with acetonitrile. Five calibration standards contained a constant concentration of the internal standard of vitamin D3-d6 (about 3 µg/mL) and variable concentrations of vitamin D3 (about 0.1–10 µg/mL) were prepared freshly. A 1 mL aliquot of each standard solution was transferred to an HPLC vial and derivatized with 100 µL PTAD reagent (10 mg/mL in acetone).

4.2.5.2. Vitamin D3 extraction and derivatization

Approximately 0.5 g of simulated milk powder was weighted in a boiling tube. To the sample, 10 mL ethanolic pyrogallol solution (1%, w/v) and then 1.5 mL internal standard solution were added. After sample mixing, 2 mL of potassium hydroxide solution (50% w/v) was added to the boiling tube and was vortex mixed. Saponification was completed in a water bath at 70ºC for 1 h with vortex mixing every 15 min. A 10 mL of isooctane solution was added to the mixture after cooling to room temperature and then the mixture was shaken in a horizontal shaker for 10 min. After addition of 10 mL water in the boiling tube, the mixture was centrifuged at 250×g for 15 min. A 5 mL aliquot of the upper phase (isooctane layer) was transferred to a 15 mL centrifuge tube and another 5 mL water was added to wash the isooctane extract. After centrifuging the mixture at 2000×g for 5 min, the lower aqueous layer was removed to waste and 75 µL PTAD solution was added to the sample. The centrifuge tube was vortex mixed and kept for 5 min, then 1 mL acetonitrile was added and the mixture was vortex mixed, then placed in a centrifuge at 2000×g for another 5 min. A 0.5 mL aliquot of the lower layer (acetonitrile) was mixed with 167 µL water and filtered using a syringe filter (PTFE, 0.22 µm, 17 mm) and finally transferred to an amber HPLC vial.

4.2.5.3. Liquid chromatography mass spectrometry analysis

Liquid chromatography analysis was carried out using a 1290 Infinity series UHPLC (Agilent, USA). The LC column used was Kinetex PFP 150×4.6 mm, 5 µm core-shell reverse-phase (Phenomenex, USA) at 30°C. The mobile phases were (A) 0.1% formic acid in water and (B) methanol, containing 2 mmol/L of ammonium acetate. The gradient started from 75% B and moved to 100% B in 4.8 min and held until 6 min, then returned to the initial conditions in 10 min. The flow rate was 0.6 mL/min and the injection volume was 5 µL. Detection was carried out using 6460C Triple Quad Mass Spectrometer system equipped with an electrospray ionization (Agilent Jet Stream ESI) source in positive mode and multiple reaction monitoring (MRM) under the MS conditions presented in Table 4.1. Analyst software (MassHunter, version B.07.00) was used for instrument control and data processing.

4.2.6. Vitamin D3 oxidation products extraction and derivatization

To extract vitamin D3 oxidation products (VDOPs), the methods of Abernethy (2012), Rose-Sallin at al. (1995) and Trenerry at al. (2011) were used with some modifications as discussed in the next Chapter. Sample preparation was based on the direct solvent extraction with an appropriate solvent and a clean-up step using a solid-phase extraction (SPE) cartridge after extraction.

For vitamin D3 and its oxidation products extraction, simulated whole milk powder was solubilized in methanol and partitioned into isooctane. An aliquot of isooctane layer was separated and dried under nitrogen. The residue was subsequently dissolved in 3 mL hexane and loaded on an aminopropyl SPE cartridge, which was pre-conditioned with 10 mL of hexane. Then, the SPE column was washed two times with 10 mL of hexane followed by 10 mL of hexane:ethyl acetate (90:10). Vitamin D3 was eluted with 10 mL of hexane: ethyl acetate (80:20) (fraction F1) and its oxidation products were then eluted using 10 mL of hexane:ethyl acetate (60:40) (fraction F2) and 10 mL of acetone (fraction F3). All fractions were dried under nitrogen and dissolved in 1 mL ethyl acetate containing 0.5 mg/mL PTAD reagent and the fractions were derivatized for 1 hour at room temperature. The PTAD-derivatized fractions were dried under nitrogen and dissolved in methanol for further analysis.

4.2.7. Statistical analysis

Means (n = 3), standard error mean (SEM) and, linear regression analysis were calculated using Microsoft Excel 2016. Data was subjected to a single way analysis of variance (ANOVA) and Tukey's test to determine significant differences (p <0.05) using IMB SPSS Statistics 22.

4.3. Results and discussions

4.3.1. Fat globule size

Different homogenization speeds and times were tried to reduce the fat emulsion size to less than about 1 micron in simulated whole milk. The efficiency of homogenization was tested for fat globule size distribution by MaterSizer are shown in Figure 4.1. No difference in particle size was observed for emulsions with and without heat treatment.

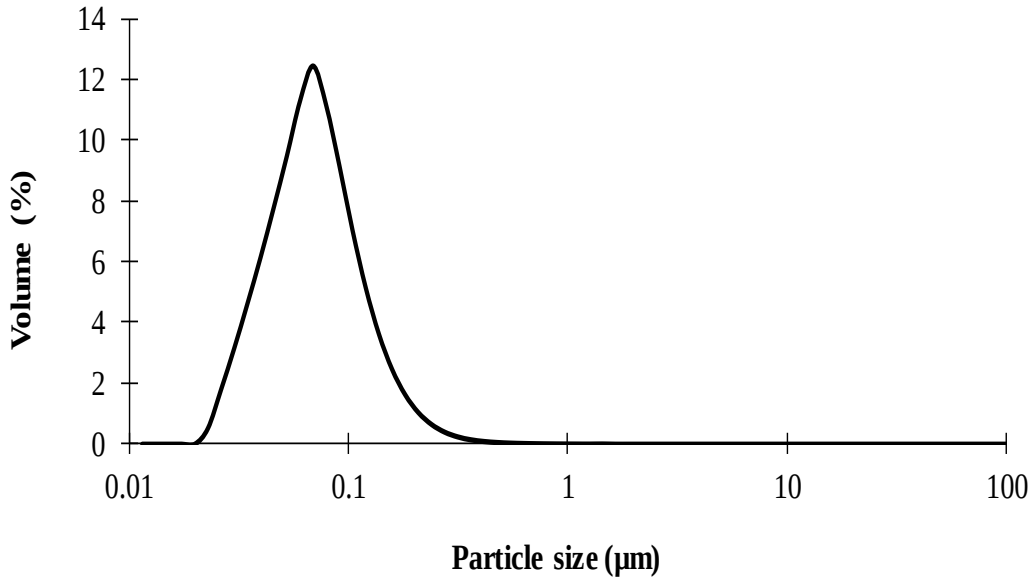


Figure 4.1. Size distribution of fat globules in homogenized simulated whole milk

4.3.2. Lipid oxidation

Peroxide values (PV) of model samples, expressed as meq O2 / kg of fat, in different storage conditions are illustrated in Figure 4.2 a and b. PV values of fresh powders were lower than limit of detection in all samples, but increased rapidly, reaching a maximum at 7-month at both storage temperatures, and declined thereafter. Samples stored at 40°C had higher PV values than those stored at room temperature (RT).

TBARS values, expressed as µg of MDA / g sample, of simulated milk powder at two different storage temperatures are illustrated in Figures 4.2 a (RT) and b (40°C), respectively. The initial TBARS values of all samples at the two storage temperatures were not significantly different, however, during the subsequent storage of powders, the storage temperature had a significant effect ($p < 0.001$) on TBARS development. In fact, general rate of lipid oxidation was obtained by comparing the TBARS values of samples stored at the two different temperatures. As shown in Figure 4.2, TBARS of samples stored at 40°C increased more rapidly than those of samples stored at RT. An average factor of 1.97 per 10°C was estimated for TBARS development in the samples that were not subjected to heating condition, while an average factor of 0.83 per 10°C was observed for those with heating condition over the storage period. These results are in agreement with previous studies which observed higher lipid oxidation rate in whole milk products stored at higher temperatures (Chan et al., 1993; Cluskey et al., 1997).

An inverse relationship was observed between PV and TBARS values after 7-month at both storage conditions, indicating progression of lipid oxidation from a primary to a secondary step. Significant differences ($p < 0.001$) with respect to the storage time were found in both PV and TBARS values in all samples. No significant differences were found between processing condition (heat treatment) and PV, while it showed significant differences among samples in TBARS ($p < 0.001$). For TBARS values, significant interaction ($p < 0.001$) between processing condition and storage temperature, and processing condition and storage time were found. These findings are also in agreement with the findings reported by Cluskey et al. (1997) and Manglano et al. (2005) which showed the effect of storage and processing conditions on the lipid oxidation products.

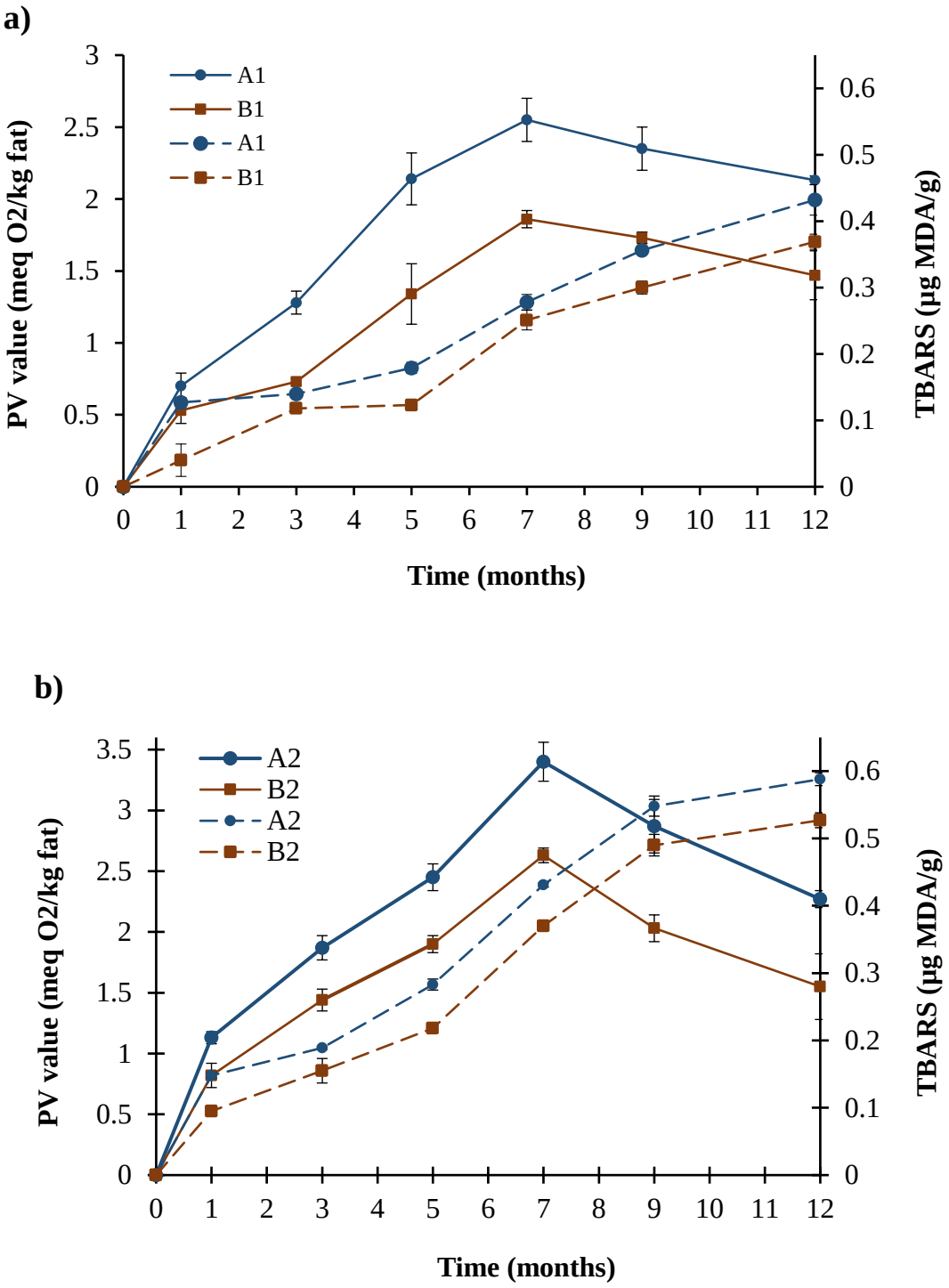


Figure 4.2. TBARS (---) and PV (—) values were monitored in SWMPs during 12 months storage at room temperature (RT) (a) & 40°C (b).

A1 & A2: samples without heating condition at RT & 40ºC, respectively;

B1 & B2: samples with heating condition at RT & 40ºC, respectively.

Based on the observation of 12-month storage, heat treatment resulted in lower levels of PV and TBARS in simulated whole milk powders (SWMP) compared to those without heat treatment. The PV values for stored samples without heating conditions (A1 & A2) were greater (~1.5-fold) than those with heat treatment (B1 & B2) at both storage temperatures (RT & 40°C; Figure 4.2 (a & b)). In addition, TBARS levels were also higher in no heating conditions powders stored at RT (A1) and 40°C (A2) during 12-months storage. This difference was attributed to improved oxidation stability in milk powders with heat treatment (B1 & B2) during subsequent storage compared to samples without heat treatment (A1 & A2). Similar results were reported in previous studies on the oxidation of whole milk powder prepared by heat-treated milk by several authors (Cluskey et al., 1997; Van Mil & Jans, 1991; Walstra & Jenness, 1984). They found that heating leads to the denaturation of whey proteins and consequently increased levels of free sulfhydryl groups, which are important in the antioxidant activity of milk.

4.3.3. Vitamin D3 quantification in simulated milk powders during storage

For vitamin D3 extraction, the method described was based on the strategy of direct saponification, solvent extraction and PTAD derivatization. For MS detection, the two most intense product ions were selected as qualifier and quantifier ions for MRM mode (Table 4.1 and Figure 4.3). The product ions of *m/z* 298 and 280 were generated from both analyte and internal standard, since the location of deuteration was on the side chain in the isotope labelled vitamin D3 (vitamin D3-d6) (Figure 4.4). As it is shown in Figure 4.3, fragment *m/z* 383 was derived from the loss of PTAD and two hydrogen atoms. Fragment *m/z* 179 resulted from loss of a phenyl fragment (119) from fragment *m/z* 298. Finally, fragments *m/z* 161, 280 and 365 were dehydration ions of *m/z* 179, 298 and 383, respectively. All the observed fragments, which were consistent with the expected structures, also coincided with those previously published (Abernethy, 2012). The highest intensity transition was used for quantification of vitamin D3. The analyte and the internal standard co-eluted and showed peaks at a retention time of about 7.5 min (Figure 4.5).

Table 4.1. Parameters for MS/MS step for qualitative and quantitative determination of PTAD-derivatized vitamin D3 and its isotope labelled.

Analyte	Precursor ion *(m/z)*	Product ions *(m/z)*	Collision energy (eV)	Quantitation transition *(m/z)*
D3[a]	560.2	298[b] 280[c]	25	560 → 298
D3-d6[d]	566.2	298[b] 280[c]	25	566 → 298

[a] vitamin D3; [b] quantifier; [c] qualifier; [d] isotope labelled vitamin D3

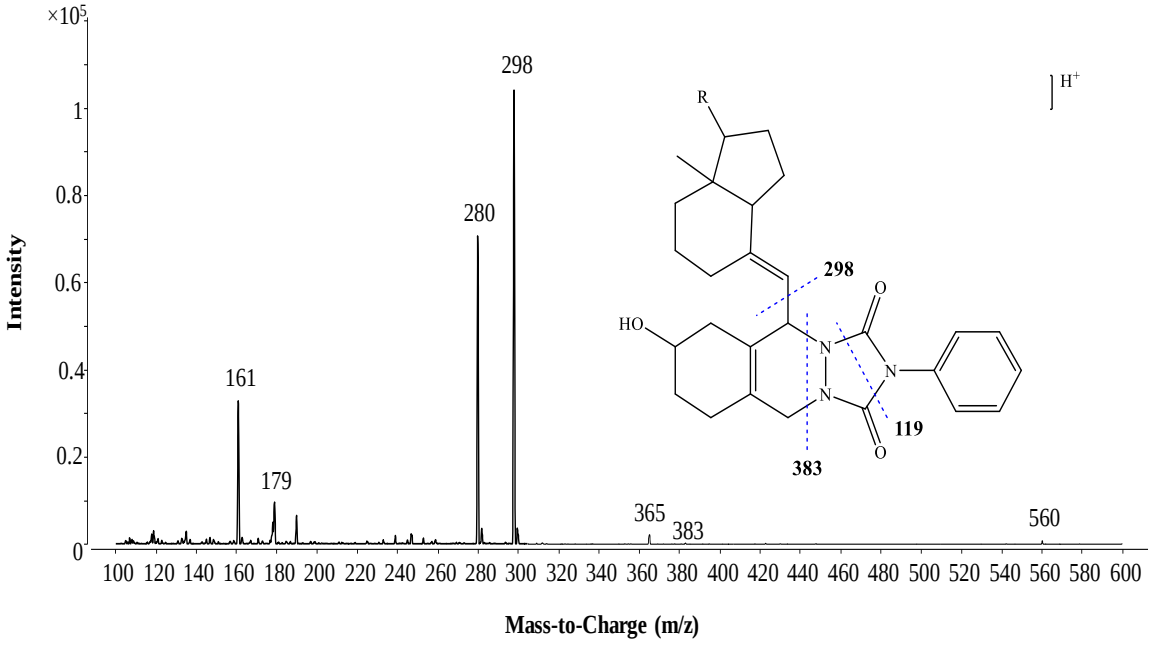


Figure 4.3. Product ion spectrum of PTAD-derivatized vitamin D3 at *m/z* 560.

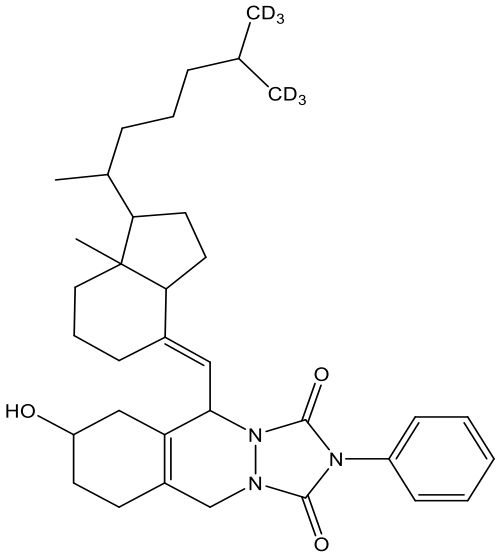


Figure 4.4. Structure of PTAD-derivatized isotope labelled vitamin D3 (vitamin D3-d6-PTAD).

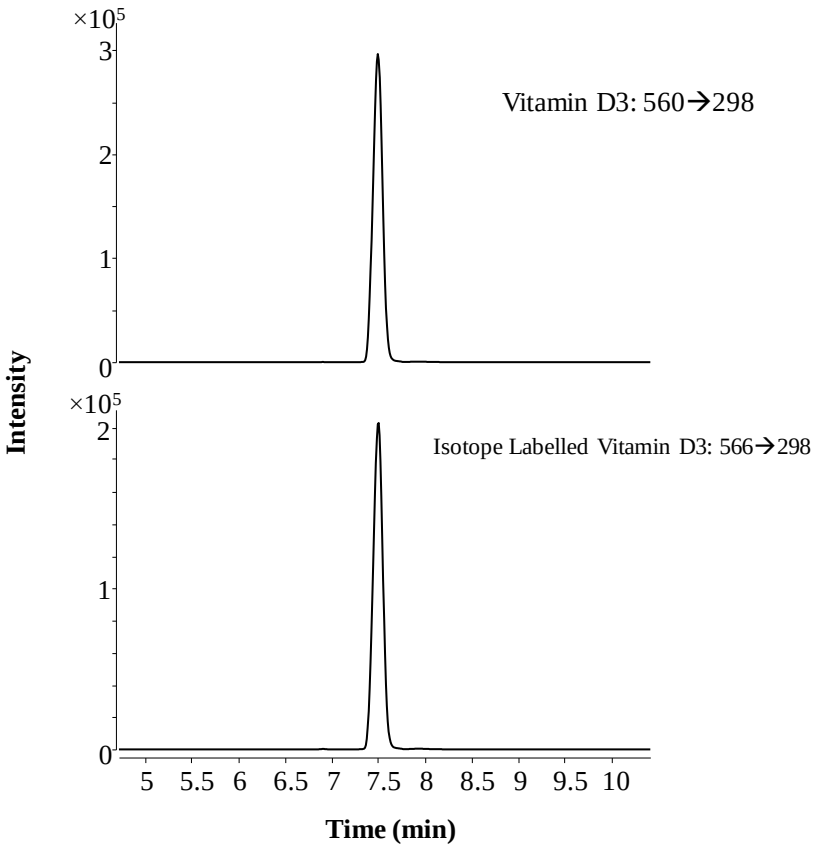


Figure 4.5. MRM chromatograms of quantifier of PTAD-derivatized vitamin D3 and its isotope labelled vitamin D3.

Vitamin D3 concentration of milk powders stored at different storage periods and temperatures were calculated based on the standard curve. Acceptable values for calibration linearity were obtained from the equation Y = 1.291X + 0.0859 and correlation coefficient $r^2 \geq 0.9944$. The accuracy of the developed method was assessed by analysing the standard vitamin D3 as reference material at five concentrations (2-100 ng/mL). Three replicates at each level were analysed on three different days to assess accuracy and precision with respect to standard curve. The accuracy and precision of method were 98.6-100.3 and 0.5-2.9%, respectively.

Vitamin D3 content of milk powders during storage, as influenced by processing and storage conditions is shown in Figure 4.6. As seen in Figure 4.6, heat treatment and storage temperature had a negative influence on the vitamin D3 content. In this respect, heat treatment resulted in lower vitamin D3 degradation for both samples stored at room temperature (RT) (B1) and 40°C (B2) compared to those without heat treatment (A1 & A2). However, upon storage, vitamin D3 content decreased in B2 and reached that of the sample without heat treatment (A2) (Figure 4.6). The rate of the vitamin D3 decline in A2 (sample without heat treatment stored at 40ºC) was the highest during 5 month-storage, and the rate increased for other samples after the fifth month. Significant differences ($p < 0.001$) were found in vitamin D3 decreasing rate in all samples during the storage period. The most severe loss of vitamin D3 observed was about 38% in 12 months.

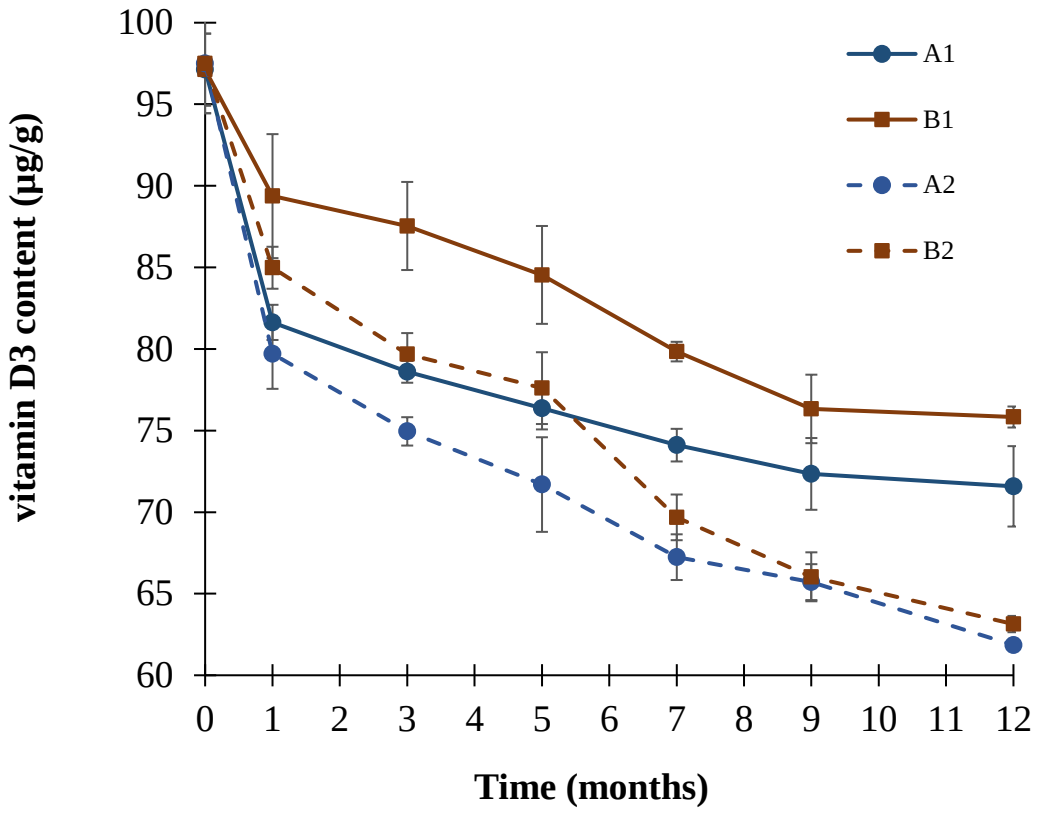


Figure 4.6. Vitamin D3 content of SWMPs during 12-month storage.

A1 & A2: samples without heat treatment at RT & 40ºC, respectively;

B1 & B2: samples with heat treatment at RT & 40ºC, respectively.

4.3.4. Correlation between lipid oxidation products and vitamin D3 loss

During 12-month storage, an inverse relationship was observed between lipid oxidation products and vitamin D3 content for simulated whole milk powders. Correlation among lipid oxidation parameters (TBARS and PV values) and vitamin D3 losses during the storage of milk powders is shown in Table 4.2. Good correlations were found between vitamin D3 degradation and TBARS concentration in all samples. Correlation of vitamin D3 losses to the other oxidation parameter, PV value, was closer in samples stored at room temperature than those stored at 40ºC.

Apart from that, significant correlations were not found between TBARS and PV values among all samples (Table 4.2). Unlike PV values, TBARS showed high correlation coefficients during 12-month storage. When TBARS concentrations were plotted against storage time, significant correlation coefficients were achieved: 0.96 for A1, 0.966 for A2, 0.964 for B1 and 0.961 for B2. However, good correlation coefficients between PV values and storage time were not found as PV value decreases due to the lipid oxidation progress to secondary oxidation products during storage time. The correlation coefficients of PV value during 12-month storage were 0.59 for A1, 0.36 for A2, 0.6 for B1 and 0.24 for B2.

Table 4.2. Correlation Coefficients between TBARS, PV and vitamin D3 loss in SWMPs during 12 months at different storage conditions.

SWMP Samples	Vitamin D3 loss & TBARS	Vitamin D3 loss & PV	PV & TBARS
A1	0.92	0.88	0.66
A2	0.98	0.71	0.65
B1	0.97	0.86	0.78
B2	0.99	0.59	0.53

A1 & A2: samples without heat treatment at RT & 40ºC, respectively
B1 & B2: samples with heat treatment at RT & 40ºC, respectively

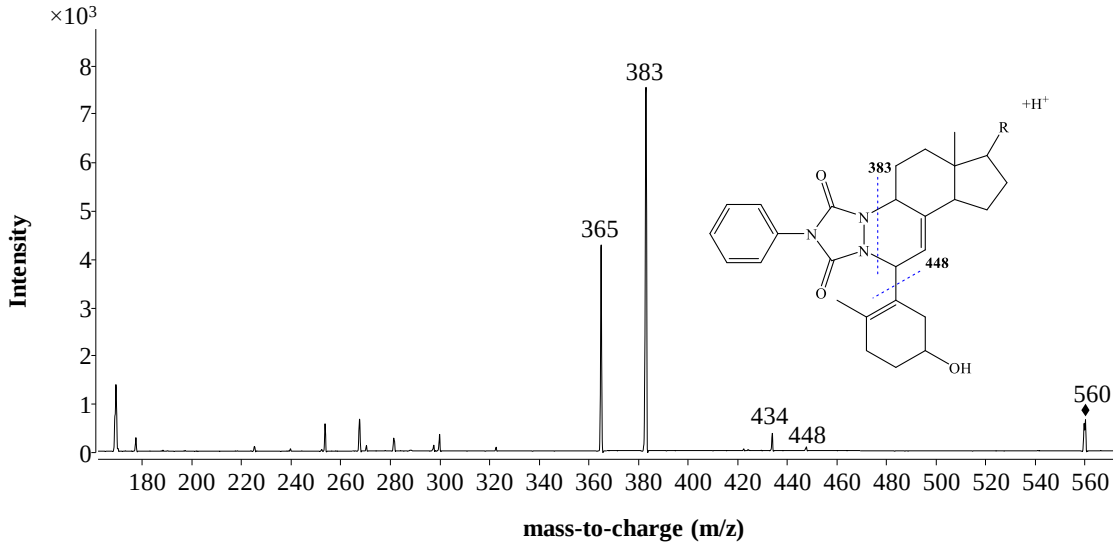
These findings indicated that the degradation of vitamin D3 is associated with the generation of secondary lipid oxidation products. These observations are consistent with those obtained for cholesterol oxidation products (COPs) during storage in previous studies (Angulo, Romera, Ramirez, & Gil, 1998; Chan et al., 1993; Monahan et al., 1992; Nielsen, Olsen, Duedahl, & Skibsted, 1995). The current results suggest that TBARS concentration could be a reliable index to monitor vitamin D3 degradation over the storage period of whole milk powder. The major advantage of TBARS values over PV is that it was observed to increase in a continuous and almost linear fashion, whereas the PV increased initially, and then declined.

4.3.5. Degradation studies of vitamin D3 in fresh and stored milk powders

Diels-Alder derivatization with PTAD is an approach to enhance the detection of sensitivity and selectivity in vitamin D3 and its metabolites using the mass spectrometer (Burild, Frandsen, Poulsen & Jakobsen, 2014, Lipkie, Janasch, Cooper, Hohman, Weaver & Ferruzzi, 2013). Thus, in this study, vitamin D3 derivatives were detected and quantified by mass spectrometry equipped with an ESI source. The PTAD derivatized vitamin D3 compounds are more readily ionized under ESI source conditions leading to a significant increase in signal to noise ratio and fewer isobaric matrix interferences, compared with underivatized vitamin D3 compounds. As mentioned in the previous section, vitamin D3 content was found to decrease in simulated whole milk powders during 12 months of storage. The degradation was higher in samples stored at 40ºC compared to those stored at room temperature.

According to previous studies, isomerization of vitamin D3 to previtamin D3 is a possible mechanism for vitamin D3 degradation (Mahmoodani, Perera, Fedrizzi, Abernethy & Chen, 2017; Temova & Roškar, 2016; Zhang et al., 2006). In terms of the mass spectra of PTAD-derivatized vitamin D3 and its previtamin isomer, different fragmentation patterns were reported (Abernethy, 2012, Mahmoodani at al., 2017). Abernethy (2012) suggested that previtamin D3 was derivatised by PTAD at the bicyclic end of the triene system and the formation of *m/z* 298 fragment was therefore not possible for previtamin D3-PTAD; therefore, the most intense product ions were fragments of *m/z* 383 and 365 for this compound (Figure 4.7 a). By monitoring the transition 560→383 in samples, previtamin D3 could also be observed at a retention time of about 7.8 min (Figure 4.7 b). As illustrated in Figure 4.7 b, when the MRM transition 560→383 was monitored, a small peak at a retention time of about 7.5 min was observed which showed that a small amount of vitamin D3 has generated a similar MRM to previtamin D3:PTAD.

a)



b)

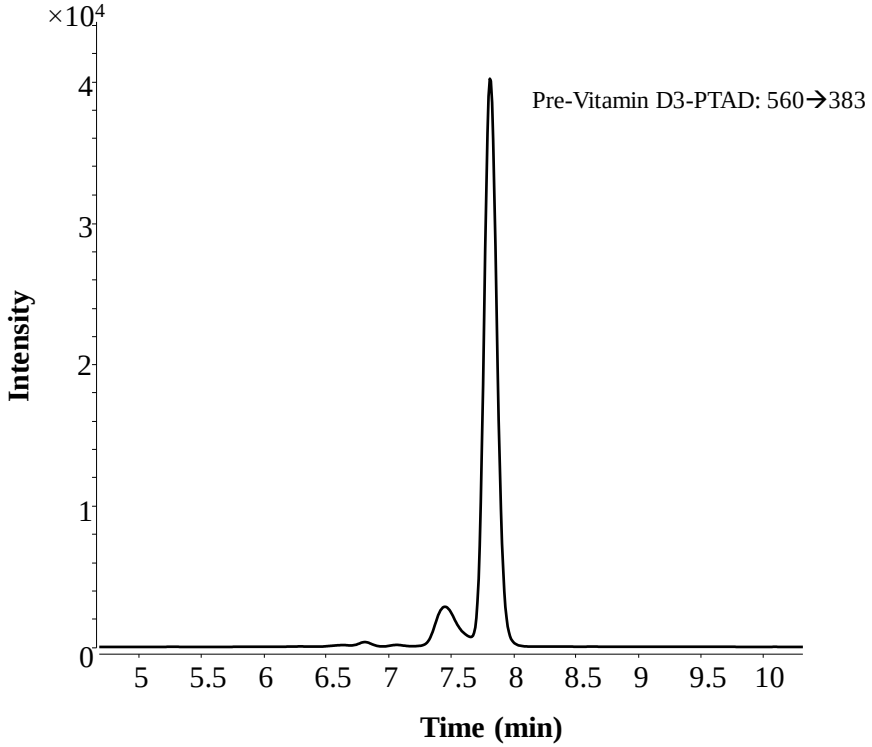


Figure 4.7. Product ion spectrum of PTAD-derivatized previtamin D3 at *m/z* 560 (**a**) and MRM chromatogram of PTAD-derivatized previtamin D3 at 560→383 (**b**).

Vitamin D, which appears to be rather stable towards atmospheric oxygen, could be susceptible to reactive oxygen species. Therefore, fatty acid lipoxidation, which produces reactive oxygen species, could be one of the ways of causing vitamin D degradation in fortified products containing fat (Charlton & Ewing, 2007; Riaz et al., 2009). Vitamin D3 degradation during storage of whole milk powder could be attributed to the oxidation of vitamin D3 and the generation of vitamin D3 oxidation products (VDOPs) mediated by lipid peroxidation reactions. This is an extrapolation to extend results of these simulated milk powders to other fortified milk powders. It will be expected that skim milk powder (SMP) might behave similarly, although the impact on sensory or generation of defect of oxidation is well known to be less significant in SMP.

Identification of VDOPs in natural foods is a challenge due to the low amount of their contents. Moreover, several difficulties may be involved in the analysis of these products, including lack of authentic standards and destruction of certain compounds and generation of others during extraction, separation and analysis. In order to isolate and identify VDOPs in whole milk powder, a preliminary study was carried out. Simple model samples including milk powder model fortified with high concentration of vitamin D3 and pure vitamin D3 solution were produced and analysed (discussed in Chapter 5). The extraction was carried out in dim light and rapidly without interruption as has been recommended for isolation of cholesterol oxidation products by previous studies (Angulo et al., 1998; Nourooz-Zadeh & Appelqvist, 1988).

Solid Phase Extraction (SPE) was used to extract and separate VDOPs and to concentrate them up to a level at which they could be measured. SPE was also employed as a clean-up step to remove interfering substances. According to the next Chapter, product ion scanning was carried out to generate fragment ion spectra and specific fragment characteristics were selected to analyse specific VDOPs using MRM with a triple quadrupole instrument (Table 4.3). The information obtained could be of help in the determination of the four proposed VDOPs in simulated whole milk powders after 12-month of storage. While, vitamin D3 and previtamin D3 were extracted in fraction 1 (F1), VDOPs were extracted in fractions 2 (F2) and 3 (F3). This is in alignment with the expected higher polar nature of VDOPs that include additional sites of oxygenation compared to vitamin D structures.

Table 4.3. Analysis parameters for determination of PTAD-derivatized vitamin D3 oxidation products (VDOPs).

Proposed VDOPs	Chemical formula $[M+H]^+$	Fraction	Precursor ion *(m/z)*	Product ions *(m/z)*	Collision Energy (eV)
EPX-D3[a]	$C_{35}H_{49}N_3O_4$	F2	576	298	25
Keto-EPX-D3[b]	$C_{35}H_{47}N_3O_5$	F2	590	298	25
OH-D3[c]	$C_{35}H_{49}N_3O_4$	F3	576	314	25
OH-EPX-D3[d]	$C_{35}H_{49}N_3O_5$	F3	592	298	25

[a] 7,8-epoxy-vitamin D3

[b] 1-keto-7,8-epoxy-vitamin D3

[c] 1-hydroxy-vitamin D3

[d] 1-hydroxy-7,8-epoxy-vitamin D3

The concentration of PTAD-derivatized previtamin D3 and vitamin D3 oxidation products (VDOPs) in fresh and stored model samples were measured using vitamin D3 as the standard and the results were expressed as vitamin D3 equivalent (VDE) per gram of sample. The previtamin D3 and VDOPs concentrations are illustrated in Figures 4.8 and 4.9, respectively.

As shown in Figure 4.8, freshly produced SWMP samples showed the lowest amount of previtamin D3. The stored samples at high temperature (40ºC) (A2 & B2) showed higher previtamin D3 concentrations compared to those stored at RT (A1 & B1). Apart from that, samples subjected to heat treatment showed lower previtamin D3 content (B1 & B2) than those without heat treatment at the same storage temperature (A1 & A2).

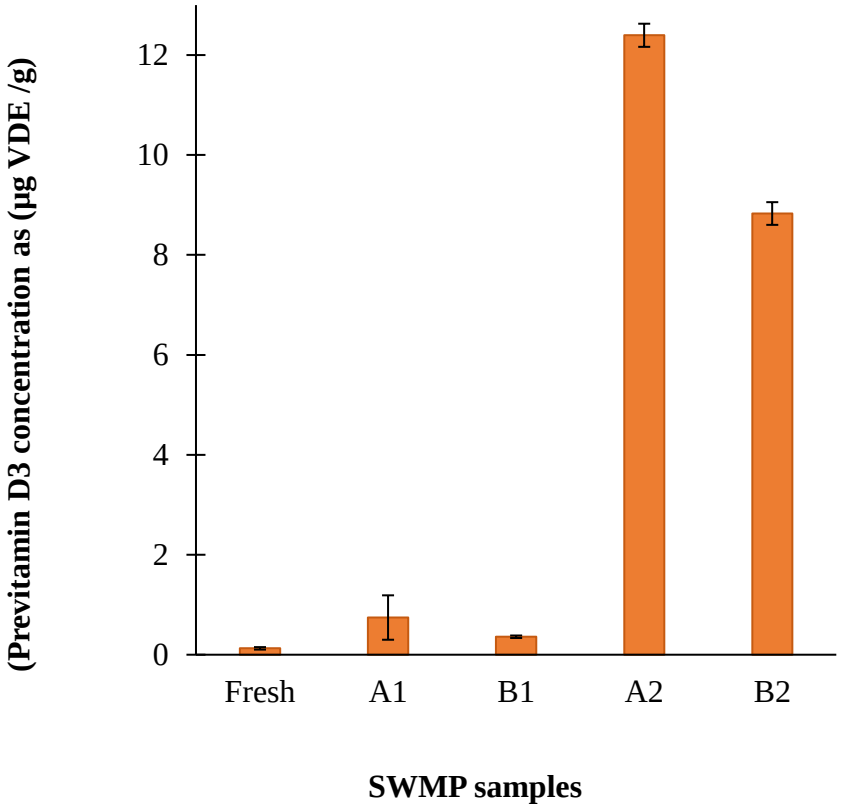


Figure 4.8. Previtamin D3 content in fresh and stored (12 months) SWMPs.
A1 & A2: samples without heat treatment stored at RT & 40ºC, respectively;
B1 & B2: samples with heat treatment stored at RT & 40ºC, respectively.

VDOPs concentrations followed a similar pattern as for previtamin D. While fresh samples showed the lowest content of VDOPs, the highest levels were found in the samples without heat treatment stored at 40ºC (A2) (Figure 4.9). Among stored samples, the sample with heat treatment stored at RT (B1), showed the lowest total VDOPs. It is worth noting that fresh samples did not contain any VDOPs with more than one oxygen.

Similar observations were reported in the case of cholesterol oxidation products (COPs) in previous studies. While no detectable levels of COPs were exhibited in fresh whole milk powder, several cholesterol oxides were indicated upon storage of milk powders (Angulo et al., 1998; Chan et al., 1993; Cluskey et al., 1997). They also showed increased levels of COPs in samples stored at higher temperatures. In terms of processing condition, Cluskey et al. (1997) reported that low temperature treatment resulted in greater levels of COPs in stored samples compared to those treated at high temperature.

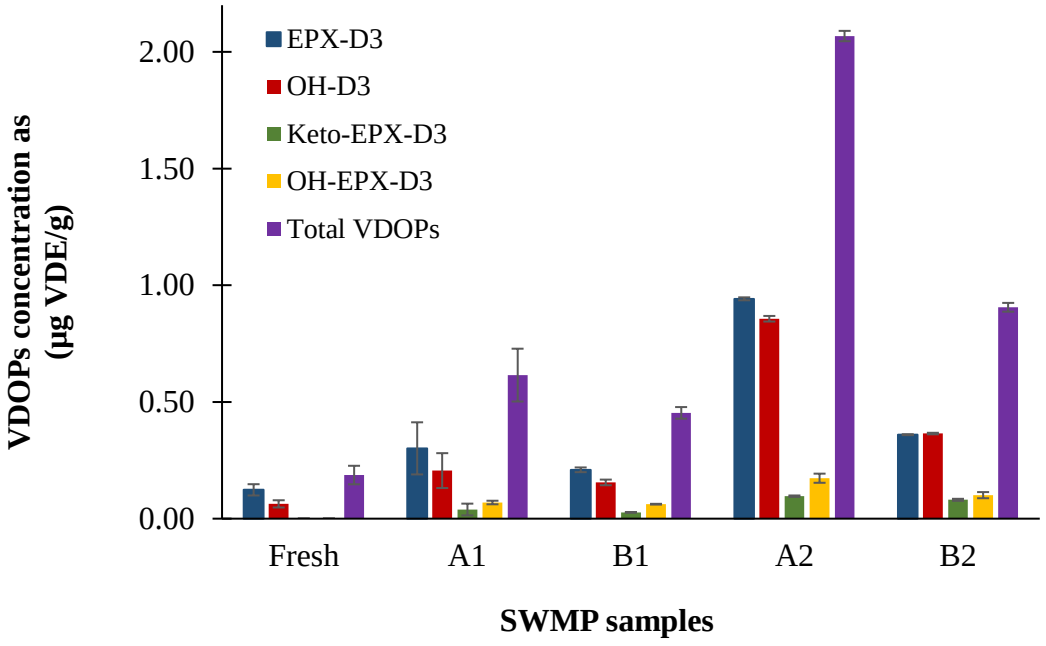


Figure 4.9. VDOPs concentrations in fresh and stored (12 months) SWMPs.
The four most abundant VDOPs and their PTAD-derivatized MRM transitions were as follow: EPX-D3 (7,8-epoxy-vitamin D3; 576→298), OH-D3 (1-hydroxy-vitamin D3; 576→314); Keto-EPX-D3 (1-keto-7,8-epoxy-vitamin D3; 590→298); OH-EPX-D3 (1-hydroxy-7,8-epoxy-vitamin D3; 592→298).
A1 & A2: samples without heat treatment at RT & 40ºC, respectively;
B1 & B2: samples with heat treatment at RT & 40ºC, respectively.

4.4. Conclusions

In simulated whole milk powders (SWMPs) stored up to 12 months, heat treatment and storage temperature showed an influence on lipid oxidation products. During the first 7-month of storage, primary lipid oxidation was dominant as evidenced by PV, while secondary lipid oxidation, characterized by TBARS measurement, dominated after 7-month of storage. Samples with heat treatment showed lower levels of lipid oxidation products compared to those without heat treatment. In addition, samples stored at higher storage temperature indicated higher levels of lipoxidation. In SWMPs, vitamin D3 content declined during storage and showed an inverse relationship with TBARS values, a secondary lipid oxidation indicator. Samples with higher levels of secondary lipid oxidation products showed the greatest vitamin D3 degradation rate.

For vitamin D3 and its related compounds analysis, the use of PTAD derivatization prior to MS detection improved the analytical possibilities and lead to higher sensitivity. This method was found that analysis of vitamin D3 was unaffected by the presence of previtamin D3. In addition, PTAD derivatization was a useful method for measurement of previtamin D3 by MS. Extraction and purification of VDOPs using aminopropyl-SPE cartridges eliminated a large extent of fatty material from the analytes. VDOPs' quantification by UHPLC-MS/MS using MRM was carried out for the most abundance VDOPs in stored SWMPs.

Based on the observations of this study, thermal isomerization accounts for the majority of the loss of vitamin D3. Then, thermal degradation could be emphasized as the predominant mechanism for vitamin D3 loss in stored SWMPs especially for samples stored at 40°C storage temperature. The result of this study shows that both lipid and vitamin D3 oxidation increased in all stored samples, but they could be minimized by exposure of milk to heat treatment prior to milk powder preparation and reduced storage temperature.

CHAPTER 5

Identification of vitamin D3 oxidation products using high-resolution and tandem mass spectrometry

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5.1. Introduction

Fortification is the practice of increasing the content of an essential micronutrient, i.e. vitamins and minerals in a food to improve health benefits and reduce dietary deficiencies within a population (Allen et al., 2006). In a successful fortification program, the stability of micronutrients added to a food is an important factor and determination of the actual level of the fortificant has always been a consideration. In terms of vitamin D3 fortification, the search for any degradation products in fortified food, most notably in dairy products, can result in the discovery of the mechanism of vitamin D3 degradation which can play an important role to minimize it.

Determination of vitamin D and its degradation products has been a challenge due to the low amount of their contents even in vitamin D enriched products. Any degradation products will be less abundant since there may be several breakdown products and isomers of each breakdown product (Japelt & Jakobsen, 2013; Lipkie et al., 2013). Therefore, any method to extract vitamin D3 compounds has to ensure that both non-polar and polar breakdown products are captured and they are not altered by the extraction process. Previous studies have demonstrated the extraction and determination of vitamin D metabolites such as 25-hydroxyvitamin D in biological samples (Aronov, Hall, Dettmer, Stephensen, & Hammock, 2008; Lipkie et al., 2013). However, the information on compounds related to vitamin D degradation products in foods is limited. In food systems, vitamin D can comprise a large class of isomerization products under various conditions, which were characterised in Chapter 3. These isomers have the same molecular formula, and structural differences among them are primarily related to the variation of the double bonds across the triene system (Mahmoodani et al., 2017).

Another form of vitamin D degradation, oxidation of vitamin D, was reported by limited number of studies (Jin et al., 2004; King & Min, 2002; Min & Boff, 2002b; Yamamoto, Imae, & Yamada, 1990). Jin et al. (2004) reported a self-initiated autoxidation of isotachysterol, an isomer of vitamin D, under atmospheric oxygen. Most of the reported vitamin D oxidation products are structurally similar to vitamin D except for having one or more additional oxygen atoms. In fact, it has proven difficult to differentiate among these structurally similar unknown compounds with classical analytical methods (Adamec et al., 2011; Kobold, 2012). Therefore, considering the ever-increasing rise in interest in these compounds, a more sophisticated method for the identification and confirmation of these products is clearly needed.

Nuclear magnetic resonance (NMR) spectroscopy has proven useful in the characterisation of the structures of unknown compounds. However, NMR could be applied only to reasonably pure compounds and it might be insufficient for the characterisation of structurally related compounds in a complex matrix without purification (Elipe, 2003; Lee, Kim, Liu, Oh, & Lee, 2005). In contrast, liquid chromatography (LC) is rather well suited for the analysis of complex mixtures and reversed-phase liquid chromatography (RP-HPLC) methods are widely used for the separation of vitamin D and its metabolites (Clarke, Rindgen, Korfmacher, & Cox, 2001; Elipe, 2003; Wu, 2000).

HPLC methods with UV detection have limitations in distinguishing compounds with very similar structures as they often exhibit similar UV absorption characteristics. Therefore, LC-MS has been the more extensively applied hyphenated technique (Lee et al., 2005). Mass spectrometry (MS) can determine the analyte's elemental composition as well as provide structural information, such as accurate mass-to-charge ratio (*m/z*), isotope abundance and fragmentation patterns (Kind & Fiehn, 2007; Vaniya & Fiehn, 2015).

Tandem mass spectrometry (MS/MS) alone has some limitations such as being unable to explain all fragmentation pathways even when structures are known, while multistage MS^n analysis can link product ions to specific precursor ions to generate fragmentation pathways. MS^n analysis can select the product ions of the initial fragmentation step and subject them to another fragmentation reaction, which can reveal additional information about the dependencies between the fragments. The resulting fragment ions can again be selected as precursor ions for further fragmentation (Vaniya & Fiehn, 2015). Previous studies successfully used MS^n to characterise structurally fragmented ions and fragmentation mechanisms of flavonoids, oligosaccharides and sugar nucleotides (Fabre, Rustan, de Hoffmann, & Quetin-Leclercq, 2001; Shi, He, Song, Qu, & Cheng, 2007; Waridel et al., 2001). Despite the growing popularity of versatile ion trap instruments, analysis of MS^n spectra remains difficult due to the lack of generic software tools. Recently, acquisition of MS^n data and development of tools for structure elucidation and spectra annotations have been considered. *In silico* prediction tools can be used to generate virtual MS^2 and MS^n spectral libraries in complex matrices (Gerlich & Neumann, 2013; Neumann & Böcker, 2010).

The objective of this study was to extract VDOPs in simulated whole milk powder. For identification of unknown VDOPs, some experiments with a high-resolution MS/MS instrumentation using a Q-Exactive Orbitrap mass spectrometer were carried out. Further, MS^n spectra were generated by an ion trap mass spectrometer. Data were organized and stored by Mass Frontier software to show the fragmentation pathways of VDOPs by mass spectral trees. PTAD derivatization was also used to enhance the sensitivity and selectivity of mass spectrometry analysis. Finally, PTAD-derivatized VDOPs were separated and identified using UHPLC-MRM-MS/MS.

5.2. Material and methods

5.2.1. Chemicals

Vitamin D3 (cholecalciferol, 99%), 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), formic acid and pyrogallol were obtained from Sigma-Aldrich (Auckland, New Zealand). LC grade methanol, ethanol, acetone, acetonitrile, isooctane were obtained from Scharlab, S.L (Barcelona, Spain). ACS reagent grade of ethyl acetate, diethyl ether and hexane were obtained from ECP (Auckland, New Zealand). Vanillin (98%) was obtained from AK Scientific (CA, USA). Copper (Cu) cube (1 cm^3) was obtained from Delta Educational (Auckland, New Zealand). Sodium caseinate, whey protein and anhydrous milk fat (AMF) were supplied by Fonterra Co-operative Group Ltd. (Palmerston North, New Zealand).

5.2.2. Sample preparation

Simulated whole milk powder (SWMP) was prepared as according to the process described in Chapter 4 (section 4.2.2) with some differences. Simulated whole milk was fortified with high concentration of vitamin D3 standard solution in ethanol (10 mg/g), and freeze-dried powders were stored for 12 month at 40°C. In addition, 10 mL of vitamin D3 standard solution in isooctane (10 mg/mL) was oxidized under air in the presence of a copper cube for 5 hours to generate vitamin D3 oxidation products in a sample without fat matrices.

5.2.3. Vitamin D3 oxidation products (VDOPs) extraction, separation and derivatization

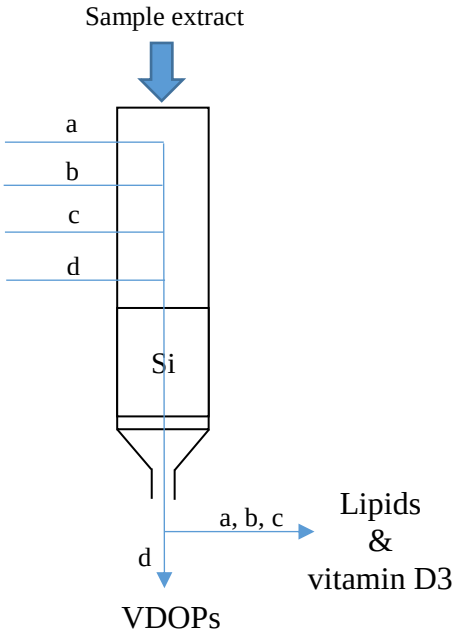
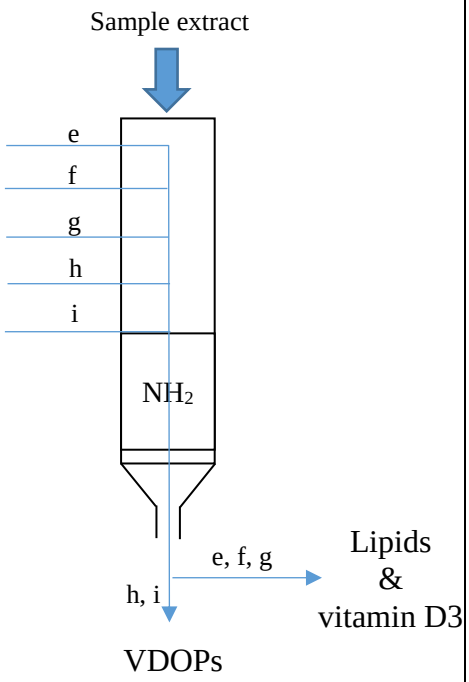
The extraction of VDOPs in samples of oxidized vitamin D3 solution and stored SWMPs involved the following steps: liquid-liquid extraction (LLE) (only for SWMP), solid phase extraction (SPE), derivatization using PTAD, chromatographic separation, and detection by MS. For liquid-liquid extraction of simulated whole milk powder, the method of Abernethy (2012) with some modifications was used. To 1 g of milk powder, 5 mL of water was added in a centrifuge tube and the sample was suspended by vortex mixing and allowed to stand for 10 min. To the centrifuge tube, 25 mL of methanolic pyrogallol solution (1%, w/v), 10 mL of isooctane and 5 mL of water were added. The tubes were capped, vigorously mixed and then centrifuged for 5 min at 2000×g. An aliquot of the upper layer (5 mL) was transferred to a test tube and dried under nitrogen. The sample of vitamin D3 standard solution, which was oxidized under air, was also dried under

nitrogen. Dried samples were dissolved in 3 mL hexane for solid phase extraction (SPE) as a clean-up step.

SPE optimization was carried out to achieve a selective SPE extraction that eliminates interfering compounds to the greatest extent possible without loss of analytes (Rose-Sallin et al., 1995; Trenerry et al., 2011). Based on the previous studies on cholesterol oxidation products, extraction in fat-containing food, silica (Si-) and aminopropyl (NH2-) SPE cartridges were suitable for lipid-rich products such as milk powders (Calderón-Santiago, Peralbo-Molina, Priego-Capote, de Castro, & Dolores, 2012; Nourooz-Zadeh & Appelqvist, 1988; Rose-Sallin at al., 1995; Ulberth & Rössler, 1998). In the present study, since the analysed matrix is oily, normal-phase cartridges including silica and aminopropyl were used for a clean-up step in the VDOPs extraction process. In addition to the sorbent material, the solvent types and ratios were optimized using hexane, ethyl acetate, diethyl ether and acetone as elution solvents (Table 5.1).

Selective SPE was performed on a Bond Elut (2 g) aminopropyl (NH_2) SPE cartridge (Agilent, CA, USA) under the optimized conditions. The cartridge was conditioned with hexane (5 mL) prior to sample loading. The cartridge was washed subsequently with hexane (10 mL) and hexane/ethyl acetate 90:10 (v/v) (10 mL). Fraction 1 (F1), vitamin D3, was eluted using hexane/ethyl acetate 80:20 (v/v) (10 mL). Finally, fractions 2 and 3 (F2 and F3), VDOPs, were eluted with hexane/ethyl acetate 60:40 (v/v) (10 mL) and acetone (10 mL), respectively. To derivatize vitamin D3 and its oxidation products, fractions were dried under nitrogen and dissolved in 1 mL ethyl acetate containing 0.5 mg/mL PTAD reagent and kept at room temperature for 1 hour. Finally, all fractions were dried under nitrogen and dissolved in methanol for further analysis.

Table 5.1. Solid Phase Extraction (SPE) optimization

SPE cartridge	Mobile phase	Fractions
Si	a) 10 mL hexane/diethyl ether (95:5) b) 25 mL hexane/diethyl ether (90:10) c) 15 mL hexane/diethyl ether (80:20) d) 10 mL acetone	
NH_2	e) 10 mL hexane f) 10 mL hexane/ethyl acetate (90:10) g) 10 mL hexane/ethyl acetate (80:20) h) 10 mL hexane/ethyl acetate (60:40) i) 10 mL acetone	

5.2.4. Thin Layer Chromatography (TLC)

A control of the fractions eluted from the aminopropyl and silica phase cartridges was performed using TLC (Ulberth & Rössler, 1998). Kieselgel F254 plates 20 × 20 cm, 0.2 mm thickness (Merck, Darmstadt, Germany) were developed with hexane/diethyl ether (70:30 v/v). Vitamin D3 and pre-isomer vitamin D3 were revealed by spraying with a freshly prepared solution of 3% vanillin in ethanol containing 1% H_2SO_4 followed by heating for 5 min with warm air (heat gun) until spots appeared. This spray reagent is a general reagent for steroids and it was used for detection of cholesterol oxidation products in previous studies (Ulberth & Rössler, 1998; Rose-Sallin et al., 1995).

5.2.5. Liquid chromatography mass spectrometry analysis

5.2.5.1. High-resolution mass spectrometry

Ultra High Performance Liquid Chromatography (Accela 1250 UHPLC) instrumentation with high resolution MS/MS using the Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Auckland, New Zealand) equipped with an Ion Max electrospray ionization source (ESI) in positive mode was used. Profile positive MS scans were acquired, with Full MS in the mass range of 200 – 2000 *m/z* using the Orbitrap mass spectrometer with mass resolution of 70,000 (FWHM at *m/z* 200). The Top 5 positive MS^2 were acquired with mass resolution of 17,500 (FWHM at *m/z* 200). The source settings were: Sheath gas flow rate, 50 arbitrary units, auxiliary gas flow rate, 13 arbitrary units, spray voltage 4.00 kV, capillary temperature 263ºC and heater temperature 425ºC. Mass calibration was performed before each analytical batch using an instrument manufacturer defined calibration mixture.

5.2.5.2. MS^n analysis

Multistage mass spectra were obtained using a linear ion trap (LTQ) mass spectrometer, equipped with a survey HPLC system (Thermo Fisher Scientific, CA, USA). The MS operating parameters were as follow: Source voltage, 4.6 kV; sheath gas flow rate, 15 arbitrary units; auxiliary gas flow rate, 5 arbitrary units; capillary voltage and capillary temperature, 47 V and 275 ºC, respectively. The normalized collision energy was set to 30% and the activation Q was 0.25 for collision-induced dissociation (CID) mass spectra. For stepwise fragmentation experiments, Data

Dependent Ion Tree was chosen using a maximum Breadth of 5 and Depth of 4. In this mode, the acquisition software probed the MS spectra to select the most intense parent ions for MS^n analysis. The method allowed for the fragmentation of the five highest peaks of the MS^2, MS^3 and MS^4 spectra for a subsequent scan.

5.2.5.3. Quadrupole mass spectrometry

Multiple reaction monitoring (MRM) analyses were carried out using 6460C Triple Quad Mass Spectrometer system (Agilent, USA) equipped with an electrospray ionization (Agilent Jet Stream ESI) source in positive mode. Analyst software (MassHunter, version B.07.00, USA) was used for instrument control and data processing.

5.2.5.4. Liquid chromatography

Chromatographic separation of compounds of interest was similar to the method explained in section 4.2.5.3.

5.2.5.5. Linear Ion Trap Quadrupole LC-MS/MS mass spectrometer

VDOPs were also analysed in real stored whole milk powders which were submitted to a commercial dairy manufacturer's laboratory using the method of Gill et al. 2015 which was described in the section 4.2.5.

A Shimadzu Ultra High Performance Liquid Chromatography comprising a CBM20A controller, SIL30AC auto-sampler, two LC30AD pumps and a CTO20AC column oven at 35 °C was used. The LC column used was Phenomenex Kinetex 50 × 2.1 mm, 2.6-µm core-shell reverse-phase C18. The mobile phases were (A) 0.2% ammonium format in water and (B) 100% methanol. The gradient started from 75% B and moved to 100% B in 3.3 min and held until 4.8 min, then returned to the initial conditions by 4.9 min and held until 5.5 min. The flow rate was 0.6 mL/min and the injection volume was 5 µL. Detection was carried out using an ABSciex QTRAP 6500 mass spectrometer in MRM mode, using positive ESI at 300 ºC.

5.2.5.6. Data analysis

The Xcalibur data system (version 3.0, Thermo Fisher Scientific, CA, USA) was used for data processing of both LTQ-ion trap and Orbitrap mass spectrometry. Based on the accurate mass, the elemental composition of the compounds of interest was calculated using the elemental composition tool within the Xcalibur software with 10 ppm mass tolerance. Parameter settings for the proposed elemental compositions for all the compounds detected were carbon (C: 0–40), hydrogen (H: 0–50), and oxygen (O: 0–4), while the PTAD derivatives of these compounds contained nitrogen (N: 0–3) as well. The double-bond equivalent (DBE) parameter, which was set (-1 to +100), was used as an indicator of the stability (degree of conjugation) and likelihood of the calculated elemental composition.

All library building and searching operations were also conducted using Mass Frontier 7.0 software (HighChem, LLC). Mass Frontier software used candidate compound structures as input and generated spectra which were compared to the measured spectra. This software generates fragments based on rule-based predictions, produces mass spectral trees and calculates fragmentation pathways.

5.3. Results and discussion

5.3.1. Extraction of VDOPs from simulated whole milk powder

Liquid-liquid extraction (LLE) followed by a solid phase extraction (SPE) is the main method for the extraction of cholesterol oxidation products (COPs) (Georgiou & Kapnissi-Christodoulou, 2012; Guardiola, Codony, Rafecas, & Boatella, 1995; Nielsen et al., 1995; Ulberth & Rössler, 1998). Many studies preferred saponification of the fat-containing food as the principal enrichment and clean-up procedure (Calderón-Santiago, Peralbo-Molina, Priego-Capote, de Castro, & Dolores, 2012; Rose-Sallin et al., 1995; Sander, Addis, Park, & Smith, 1989). However, artifact formation was observed during saponification in the extraction process of COPs (Dionisi et al., 1998; Park, Guardiola, Park, & Addis, 1996).

Solid phase extraction using different cartridges such as silica, florisil, aminopropyl or reversed-phase cartridges has been proposed as an alternative to saponification for the isolation and enrichment of COPs by several studies (Chen & Chen, 1994; Guardiola et al., 1995; Hwang & Maerker, 1993; Johnson, 1996; Lai, Gray, & Zabik, 1995; Penazzi et al., 1995; Sallin, Baumann, Bütikofer, & Sieber, 1993). Therefore, in the present study, LLE extraction without saponification was used to prevent additional degradation of VDOPs or formation of artifacts during saponification.

After solvent extraction, vitamin D3 and its oxidation products were purified using solid phase aminopropyl and silica extraction cartridges which allowed the removal of vitamin D3 and nonpolar compounds. In order to develop a method for efficient separation of vitamin D3 and its oxidation products from fat matrices, solid phase extraction using different cartridges and solvents were used (Table 5.1). Figure 5.1 shows TLC separation of vitamin D3 and previtamin D3 purified with Si- and NH_2- phase extraction cartridges. As seen in this figure, the aminopropyl (NH_2) cartridge showed separation of vitamin D3 and its pre-isomer from VDOPs and lipid matrices. However, a relatively small spot for vitamin D3 and no spot for previtamin D3 was seen by using silica (Si) cartridge. Moreover, among different solvents employed, a mixture of hexane/ethyl acetate was useful to separate vitamin D3 from other components.

After PTAD reagent was added to the fractions, the sample solutions had a pink color; however, this color disappeared during the incubation period (Figure 5.2). As shown in Figure 5.2, F1 showed the fastest apparent decolourization in the first 5 minutes, while pink colour has remained in F2 and F3 after 1 hour of derivatization.

By using the aminopropyl cartridge, F1 showed the fastest apparent decolorization compared with the F1 separated by silica cartridge (Figure 5.3). As it is illustrated in Figure 5.3, F1 purified by NH_2 cartridge contained vitamin D3 compared to that purified using Si cartridge. This additional observation revealed the suitability of aminopropyl (NH_2) cartridge compared to the silica (Si) one for vitamin D3 and its related compounds separation.

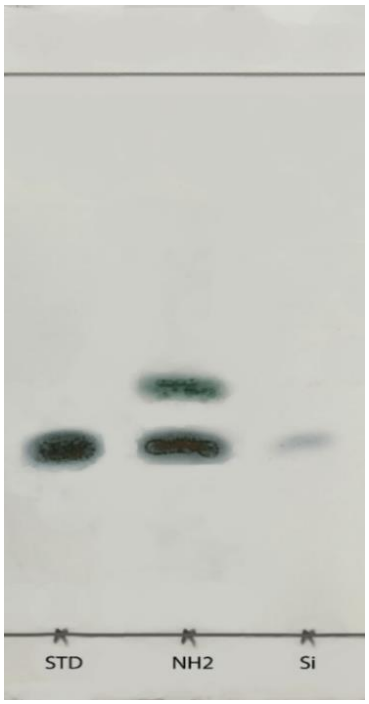


Figure 5.1. TLC separation of vitamin D3 and previtamin D3 purified with aminopropyl (NH_2) and silica (Si) phase extraction cartridges. (STD) standard solution of vitamin D3; (NH_2) F1 purified using aminopropyl cartridge; (Si) F1 purified using silica cartridge.

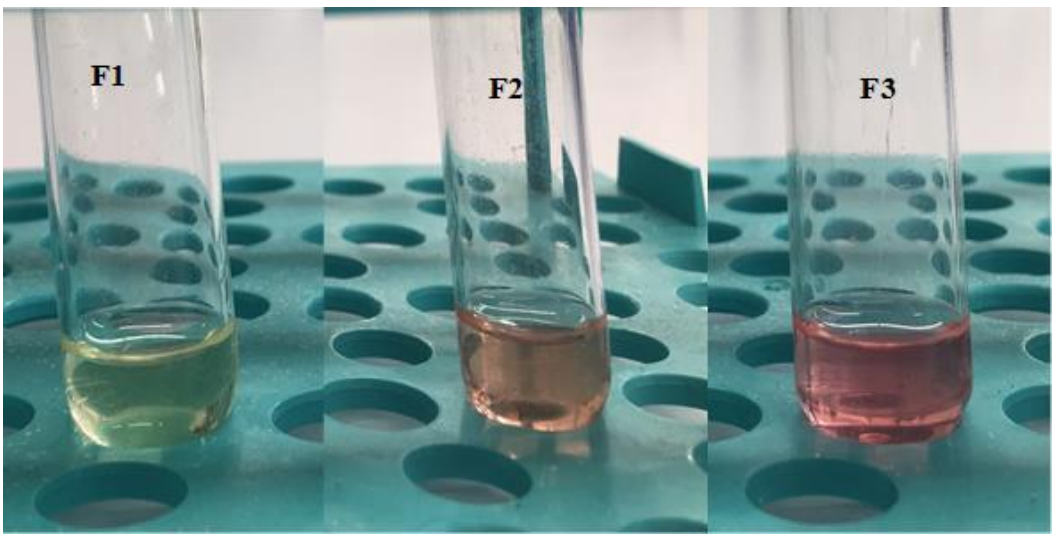


Figure 5.2. Colour changes after PTAD derivatization of fractions containing vitamin D3 (F1) and VDOPs (F2 and F3).

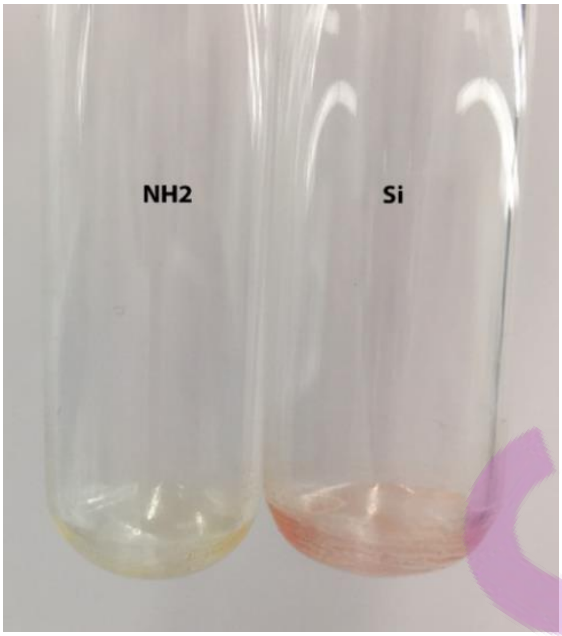


Figure 5.3. PTAD derivatization of fraction 1 (F1) purified with aminopropyl (NH_2) and silica (Si) phase extraction cartridges.

5.3.2. Mass spectrometry analysis of vitamin D3 oxidation products

5.3.2.1. Introduction

It is believed that hydroperoxides derived from oxidation of unsaturated fatty acids play a significant role to facilitate cholesterol oxidation (Guardiola, 2002; Iuliano, 2011). In this study, the hypothesis was that vitamin D3 might have the same oxidation process as influenced by lipoxidation reactions of unsaturated fats. Vitamin D3 **(1)** autoxidation can start at an allylic carbon, such as C-1, by abstraction of hydrogen following the addition of an oxygen molecule forming 1-hydroxy-vitamin D3 **(2)**. In addition, 1-keto-vitamin D3 **(3)** might be formed by dehydration of 1-hydroxy-vitamin D3 in the presence of radicals. In addition, the hydroperoxides derived from oxidation of unsaturated fatty acids may play a significant role to facilitate vitamin D3 oxidation at double bonds to form vitamin D3 epoxy compounds, such as 7,8-epoxy-vitamin D3 **(4)**, 1-hydroxy-7,8-epoxy-vitamin D3 **(5)** and 1-keto-7,8-epoxy-vitamin D3 **(6)**. The proposed formations of vitamin D3 oxidation products (VDOPs) are illustrated in Figure 5.4.

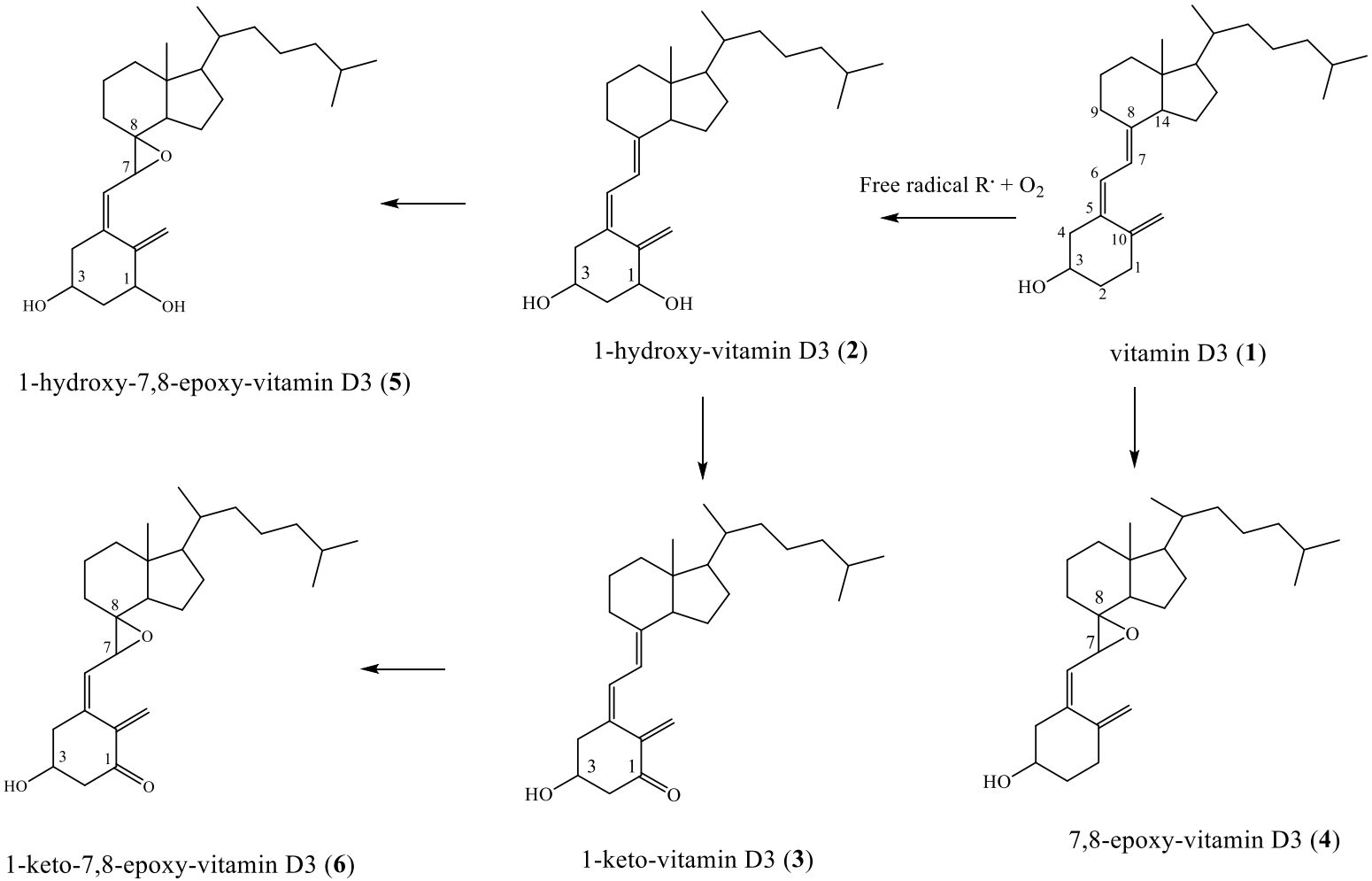


Figure 5.4. Proposed formation of vitamin D3 oxidation products.

After VDOPs extraction, analytes were identified using various mass spectrometry systems. The analysis of VDOPs were divided into several parts including, the generation of possible fragments to interpret the fragmentation pathways of unknown VDOPs using Mass Frontier software; the identification of VDOPs using MS^2 and MS^n analysis with a Q-Exactive Orbitrap and a LTQ-ion trap mass spectrometry, respectively. Structural elucidation was further aided by calculating elemental compositions by exact mass measurements. Finally, PTAD-derivatized VDOPs were separated and verified using the MRM in a quadrupole LC-MS/MS. This analysis was used to quantify VDOPs in stored simulated whole milk powders as discussed in Chapter 4.

At the first stage, vitamin D3, its oxidation products and their PTAD derivatives were chosen for theoretical fragmentation generation using Mass Frontier Software. The fragmentation patterns established for VDOPs and their PTAD-derivatized were used to identify these compounds as discussed in the following sections.

5.3.2.2. Accurate mass screening

Accurate mass analysis of target compounds using the Q-Exactive Orbitrap mass spectrometer was possible due the high mass resolution potential of this instrument. The ability to determine the *m/z* of an ion within 5 ppm allows the determination of a unique elemental composition based on the mass defect of the constituent atoms from theoretical calculation. The ability to closely match the theoretical mass with the observed mass greatly increases the reliability of identification.

Tables 5.2 and 5.3 show the results of proposed VDOPs and their PTAD-derivatized analogues, including the accurate mass of their protonated molecules, the most probable elemental composition, the double-bond equivalent (DBE) and the mass tolerance in ppm. Since milk is a complex matrix and the identification of low abundance VDOPs might be affected by isobaric interferences, a pure solution of vitamin D3 was oxidized to identify VDOPs in a simple matrix.

Table 5.2. Results of MS/MS screening of vitamin D3 and its oxidation products obtained by Q-Exactive Orbitrap.

Compound	Accurate mass of $[M+H]^+$	Chemical formula of $[M+H]^+$ ions	DBE[a]	Mass error (ppm)
VD3[b]	385.34750[g]	$C_{27}H_{45}O$	5.5	2.84
	367.33664[h]	$C_{27}H_{43}$	6.5	3.05
	259.20634[h]	$C_{19}H_{31}$	5.5	2.69
	259.24450[h]	$C_{18}H_{27}O$	5.5	3.01
	241.19676[h]	$C_{18}H_{25}$	6.5	2.78
OH-D3[c]	401.34255[g]	$C_{27}H_{45}O_2$	5.5	2.84
	383.33173[h]	$C_{27}H_{43}O$	6.5	2.31
	365.32118[h]	$C_{27}H_{41}$	7.5	2.47
EPX-D3[d]	401.34371[g]	$C_{27}H_{45}O_2$	5.5	5.73
	383.33289[h]	$C_{27}H_{43}O$	6.5	5.34
	365.32229[h]	$C_{27}H_{41}$	7.5	5.50
Keto-EPX-D3[e]	415.32302[g]	$C_{27}H_{43}O_3$	6.5	5.65
	397.31246[h]	$C_{27}H_{41}O_2$	7.5	5.92
	379.30169[h]	$C_{27}H_{39}O$	8.5	5.66
	361.29107[h]	$C_{27}H_{37}$	9.5	5.79
OH-EPX-D3[f]	417.33869[g]	$C_{27}H_{45}O_3$	5.5	5.67
	399.32811[h]	$C_{27}H_{43}O_2$	6.5	5.89
	381.31738[h]	$C_{27}H_{41}O$	7.5	5.73
	363.30669[h]	$C_{27}H_{39}$	8.5	5.67

[a] double-bond equivalent parameter; each ring or a double bond increases the DBE value by 0.5.

[b] Vitamin D3

[c] 1-hydroxy-vitamin D3

[d] 7,8-epoxy-vitamin D3

[e] 1-keto-7,8-epoxy-vitamin D3

[f] 1-hydroxy-7,8-epoxy-vitamin D3

[g] Precursor ions

[h] Product ions

Table 5.3. Results of MS/MS screening of PTAD-derivatized vitamin D3 and its oxidation products obtained by Q-Exactive Orbitrap.

Compound	Accurate mass of $[M+H]^+$	Chemical formula of $[M+H]^+$ ions	DBE[a]	Mass error (ppm)
VD3[b]	560.38672[g]	$C_{35}H_{50}O_3N_3$	12.5	3.66
	298.12106[h]	$C_{16}H_{16}O_3N_3$	10.5	2.45
	280.11067[h]	$C_{16}H_{14}O_2N_3$	11.5	2.23
OH-D3[c]	576.38135[g]	$C_{35}H_{50}O_4N_3$	12.5	2.63
	314.11636[h]	$C_{16}H_{16}O_4N_3$	10.5	1.29
	296.10562[h]	$C_{16}H_{14}O_3N_3$	11.5	1.83
EPX-D3[d]	576.38453[g]	$C_{35}H_{50}O_4N_3$	12.5	3.58
	298.12096[h]	$C_{16}H_{16}O_3N_3$	10.5	3.16
	280.11067[h]	$C_{16}H_{14}O_2N_3$	11.5	2.77
Keto-EPX-D3[e]	590.36182[g]	$C_{35}H_{48}O_5N_3$	13.5	5.03
	298.12109[h]	$C_{16}H_{16}O_3N_3$	10.5	3.99
	280.11050[h]	$C_{16}H_{14}O_2N_3$	11.5	3.98
	413.30710[h]	$C_{27}H_{41}O_3$	7.5	5.27
	395.29754[h]	$C_{27}H_{39}O_2$	8.5	4.99
	377.28747[h]	$C_{27}H_{37}O$	9.5	3.59
OH-EPX-D3[f]	592.37708[g]	$C_{35}H_{50}O_5N_3$	12.5	4.35
	298.12137[h]	$C_{16}H_{16}O_3N_3$	10.5	3.76
	280.11060[h]	$C_{16}H_{14}O_2N_3$	11.5	4.55
	415.32740[h]	$C_{27}H_{43}O_3$	6.5	3.48
	397.31477[h]	$C_{27}H_{41}O_2$	7.5	3.88
	379.30282[h]	$C_{27}H_{39}O$	8.5	3.50

[a] double-bond equivalent parameter; each ring or a double bond increases the DBE value by 0.5.

[b] Vitamin D3:PTAD

[c] 1-hydroxy-vitamin D3:PTAD

[d] 7,8-epoxy-vitamin D3:PTAD

[e] 1-keto-7,8-epoxy-vitamin D3:PTAD

[f] 1-hydroxy-7,8-epoxy-vitamin D3:PTAD

[g] Precursor ions

[h] Product ions

Analysis of VDOPs with Orbitrap allowed assigning the elemental formulas to both the precursor ions and product ions; however, the MS/MS prediction alone was not sufficient for a successful assignment of a suitable structure to a molecular formula. Additional computational approaches were also required in this step and the use of fragment libraries with accurate mass information was likely to improve the match between measured data and candidate structures. Mass Frontier software was used for this purpose as discussed in the next section.

5.3.2.3. Determination of fragmentation patterns

While MS/MS is the dominant technique for interpreting fragmentation patterns to find structural information, using MS/MS alone has intrinsic limitations because product ions found in the MS/MS spectrum may be derived from intermediary ions instead of being produced directly from the precursor ion. Therefore, many fragment ions in MS/MS cannot be explained through fragmentation pathways even if structures are known (Kind & Fiehn, 2010; Rudewicz & Straub, 1986; Vaniya & Fiehn, 2015). In order to obtain maximum structural information, samples were analysed in both MS/MS (MS^2) and MS^n mode in this study. MS^n measurements were performed to gain information on fragment ions generated in the linear ion trap. Since the ion trap lacked capabilities to do high mass accuracy, the Q-Exactive Orbitrap with a reasonable high mass accuracy was used to associate elemental compositions for VDOPs as described in the previous section.

Data structures, which are defined by graph theory to organize and store data including the fragmentation process of an analyte of interest and/or MS^n spectra generated by an ion trap mass spectrometer, are referred as trees. Typically, the graphs are called fragmentation trees, if these show the fragmentation pathways of a compound. In contrast, mass spectral trees refer to the sequential stages and relationships of mass spectral acquisition in MS^n processes (Böcker & Rasche, 2008; Hufsky, Scheubert, & Böcker, 2014; Sheldon, Mistrik, & Croley, 2009). Therefore, MS^n trees can reveal both the dependency of precursor ion/product ion and product ion/product ion within the same MS^n stage or between different MS^n stages. For both fragmentation and mass spectral trees, computational methods are required to organize specific information such as an implication of the fragmentation relationship between precursor ions and product ions. Since it was not possible to acquire reference mass spectra for VDOPs and their PTAD derivatives,

in silico prediction tools such as Mass Frontier were used to generate much larger virtual MS^2 and MS^n spectral libraries.

The MS^2 and MS^n spectra of vitamin D3 and its oxidation products were illustrated in Figures 5.5, 5.7, 5.9 and 5.11 (a & b). In addition, generation of fragmentation trees from MS^n data and using the Mass Frontier software are shown for vitamin D3 and its oxidation products in Figures 5.6, 5.8, 5.10 and 5.12.

After solid phase extraction, fraction 1 (F1) of sample showed a molecular ion with *m/z* 385.3477 which represented the presence of vitamin D3 (Figure 5.5 a). As shown in the MS^2 spectrum of vitamin D3 (Figure 5.5 b), the most significant product ions were fragments *m/z* 367 (a dehydrated vitamin D3 ion) and *m/z* 259. As for *m/z* 259, a structure without any oxygen atom was reported by previous studies (Adamec et al., 2011; Trenerry et al., 2011). However, Mass Frontier software suggested a structure containing one atom of oxygen was possible for fragment *m/z* 259 (Figure 5.6). The elemental formula of both structures were also suggested by accurate mass analysis for *m/z* 259 (Table 5.2). In order to confirm the proposed structure of *m/z* 259, MS^3 on the ion trap was used to re-fragment this fragment ion, which could only lose water and generate the dehydrated fragment with *m/z* 241 from a hydroxylated precursor (Figure 5.5 b, MS^3:259). The formation of the dehydrated fragment form *m/z* 259 confirmed that the proposed structure of this fragment created by Mass Frontier was correct (Figure 5.6). In addition, the fragment *m/z* 367 (a dehydrated vitamin D3 ion) did not generate the fragment *m/z* 259 after re-fragmentation reasonable given the lack of oxygen in the precursor, while the fragment *m/z* 241 was generated instead (Figure 5.5 b, MS^3:367). Another fragment with *m/z* 273 was also picked up by the ion trap for re-fragmentation which showed fragment *m/z* 255 $[273\text{-}H_2O]^+$ (Figure 5.5 b, MS^3:273).

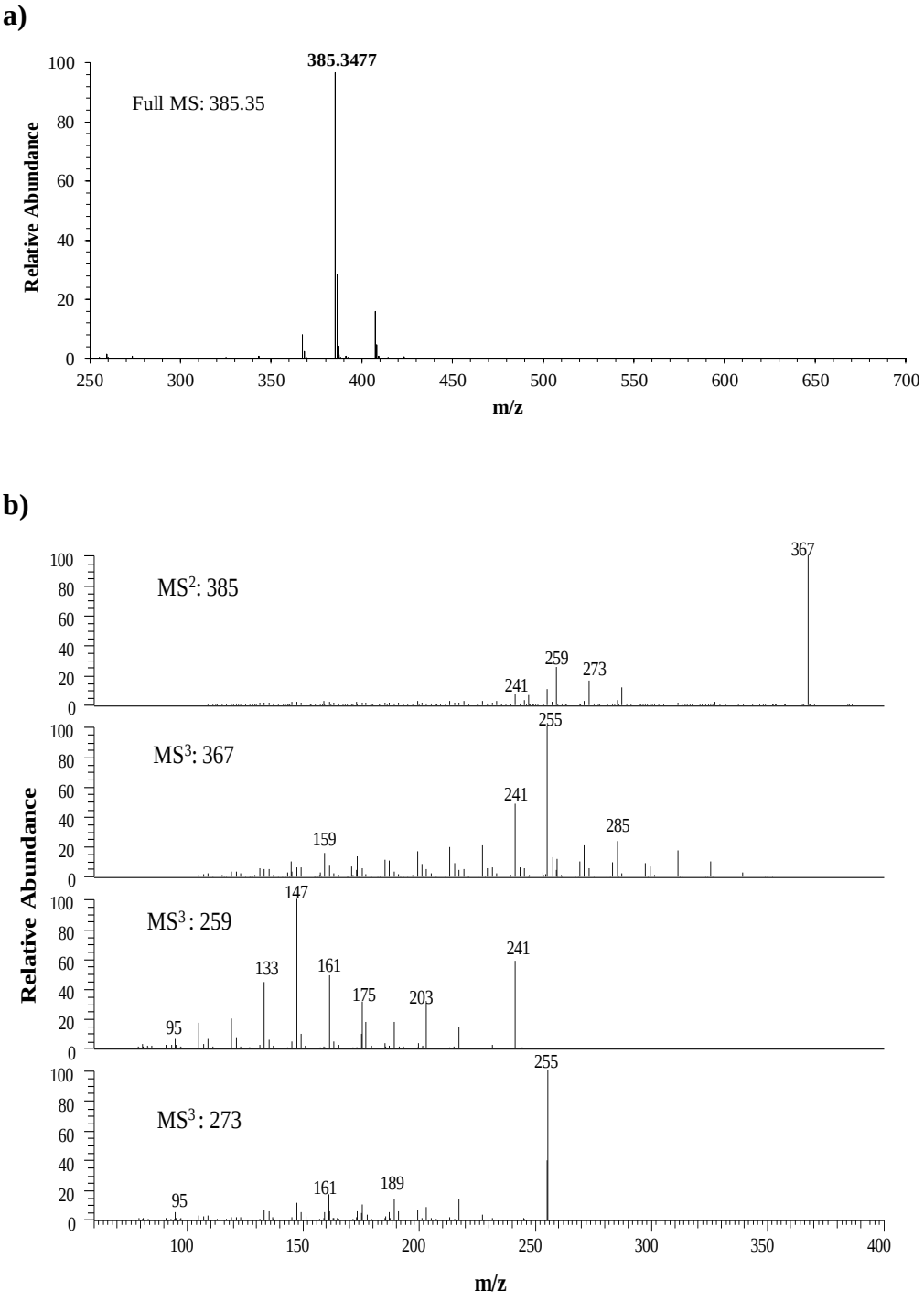


Figure 5.5. Full scan spectrum **(a)** and MS^n spectra of the indicated fragment ions **(b)** of vitamin D3 + H^+.

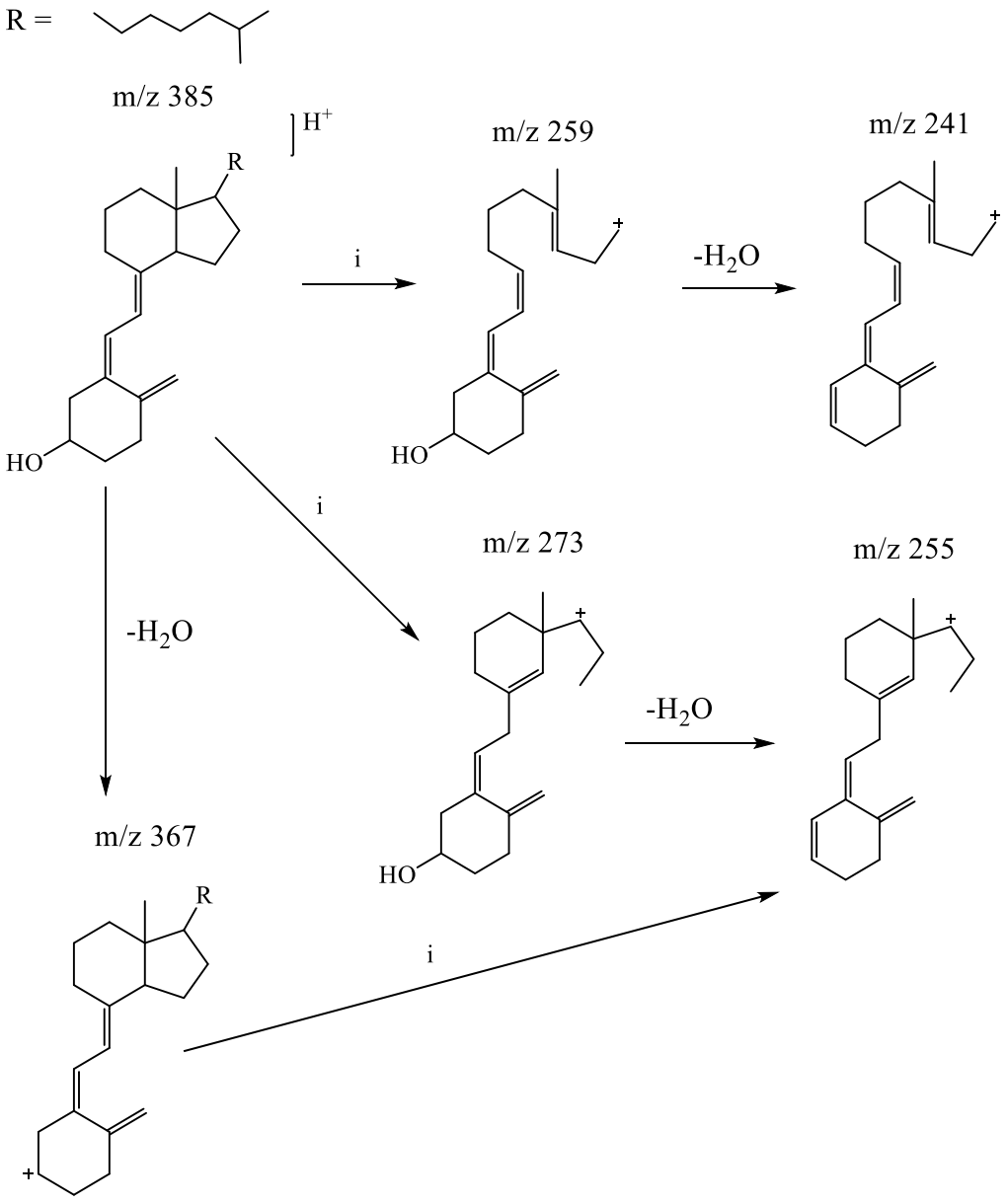


Figure 5.6. Proposed fragmentation tree of vitamin D3. ‘i’ indicates inductive cleavage; The ‘-’ symbols and the molecules above the arrows represent the neutral loss of these molecules.

Fractions 2 and 3 (F2 and F3) of the oxidized solution of vitamin D3 after solid phase extraction showed molecules with accurate mass of *m/z* 401.3426 in the Q-Exactive Orbitrap mass analysis, which represented the formation of VDOPs with one extra oxygen (Figure 5.7 a). Both fractions showed similar chemical formulas related to these VDOPs and their product ions (Table 5.2). MS^n spectra of both fractions were also similar and are illustrated in Figure 5.7 b. The MS^2 spectrum of the compounds with a precursor ion of*m/z* 401 $[M+H]^+$ showed product ions with *m/z* 383 $[M+H–H_2O]^+$ and 365 $[M+H–2H_2O]^+$ as a result of the loss of one and two water molecules, respectively. In addition, these compounds showed product ions with *m/z* 271 and 253, however, they had 2 hydrogen atoms less than those found in vitamin D3 mass spectra (Figure 5.5 b, MS^2:385 and Figure 5.7 b, MS^3:383). This is because these fragments resulted from a fragment with *m/z* 383 (a dehydrated ion of *m/z* 401) instead of the precursor ion with *m/z* 385 (as would be the case for vitamin D3). Collectively, this data can confirm the presence of VDOPs with one additional oxygen.

The proposed structures and fragmentation trees of these VDOPs with an additional oxygen, 7,8-epoxy-vitamin D3 and 1-hydroxy-vitamin D3, are illustrated in Figure 5.8 a and b. Since both VDOPs had similar fragmentation patterns and no $[M+H-16]^+$ fragment ion was observed in the case of 7,8-epoxy-vitamin D3, identification of these compounds without using any commercial standards or a method to distinguish between them was impossible.

After the loss of one hydroxyl, if the molecules were fragmented to provide fragments between the two proposed sites of oxygenation, then it could be possible to differentiate 7,8-epoxy-vitamin D3 and 1-hydroxy-vitamin D3. However, the mode of fragmentation was dominated by fragmentation in the side chain, which would not allow differentiation of the location of either site of oxygenation. In this study, derivatization with PTAD was proposed as a method to distinguish between these VDOPs which were detailed later in this section.

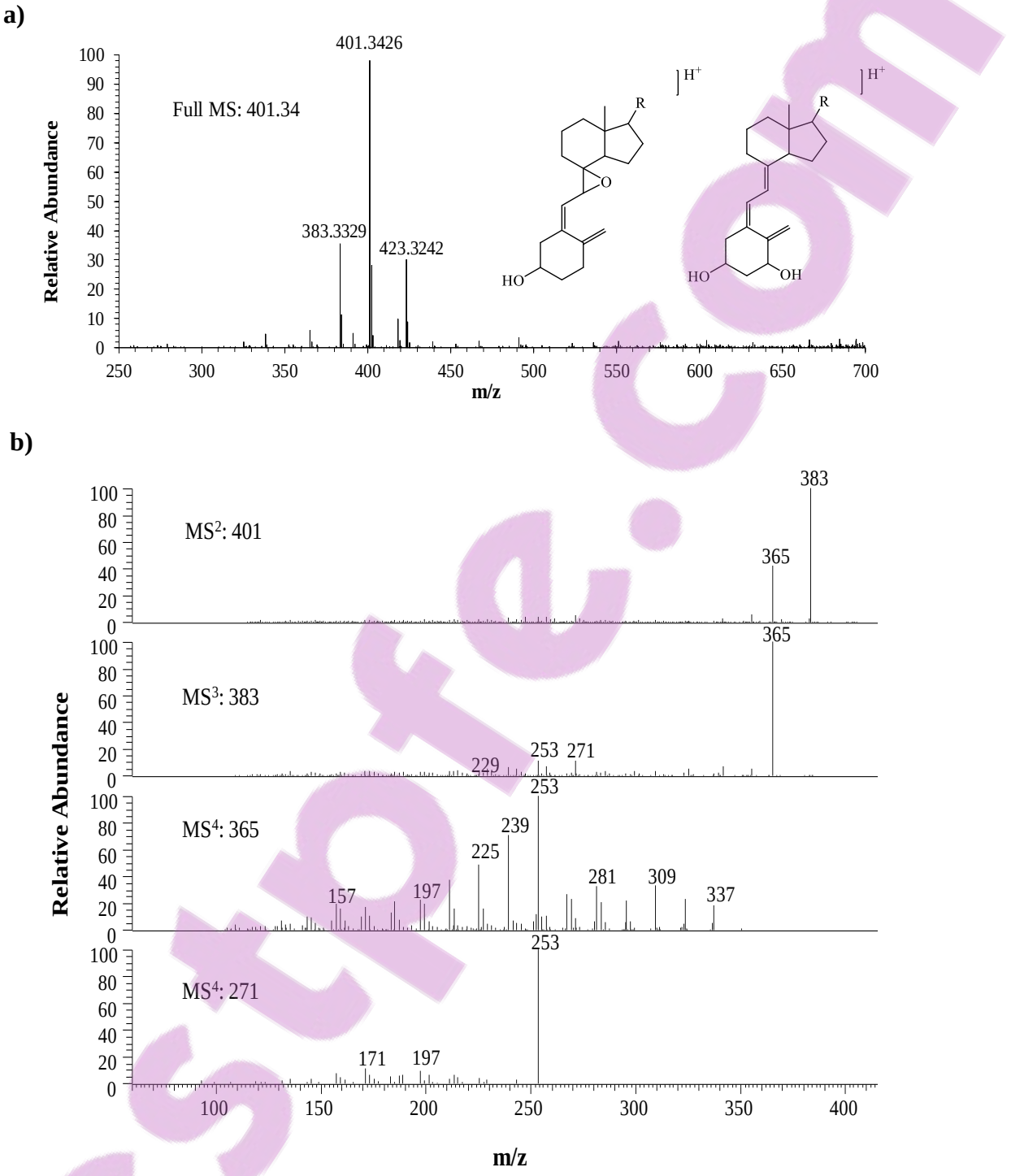


Figure 5.7. Full scan spectrum **(a)** and MS^n spectra **(b)** of VDOPs with one additional oxygen atom.

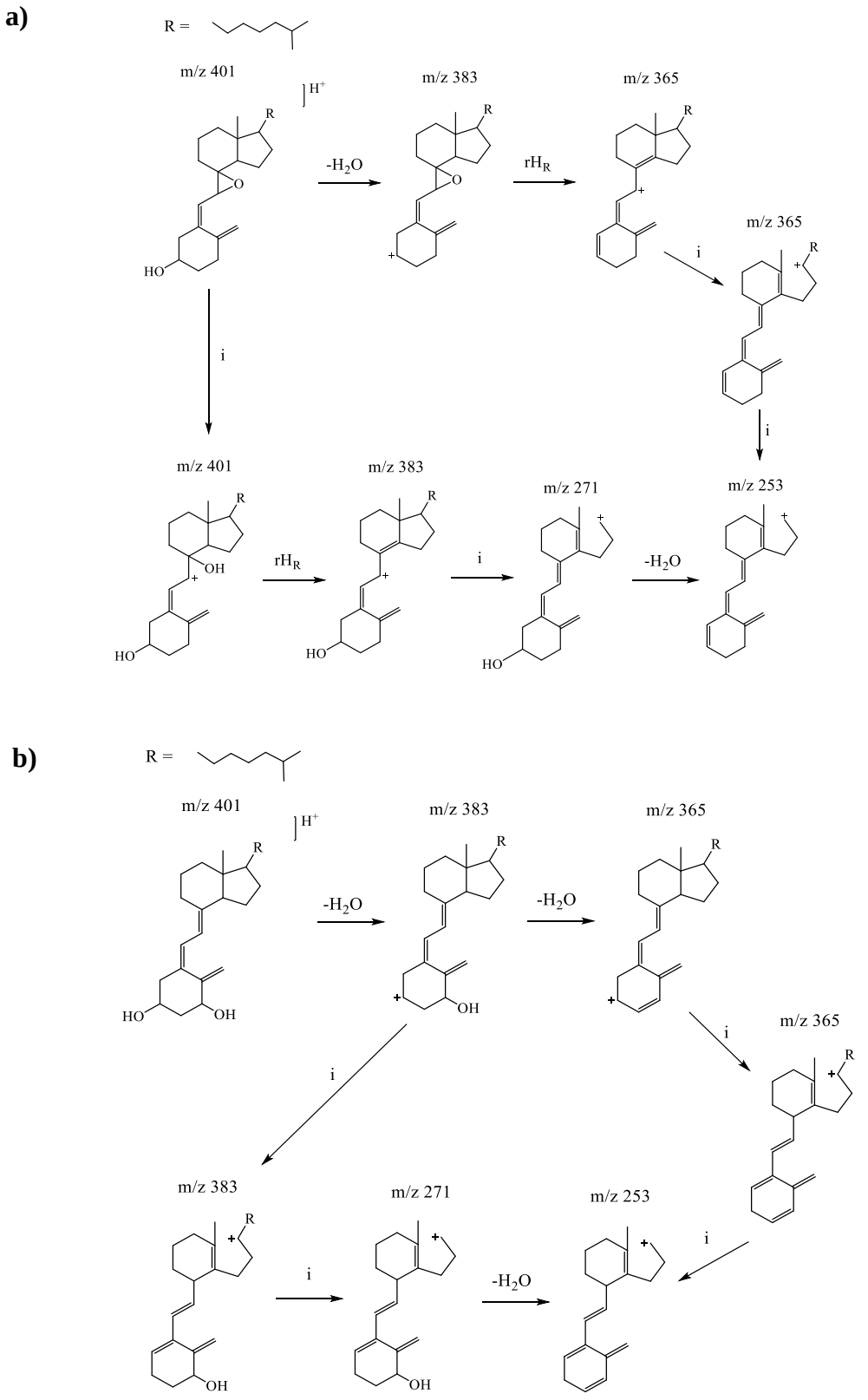


Figure 5.8. Proposed fragmentation trees of VDOPs with *m/z* 401 (7,8-epoxy-vitamin D3) **(a)** and (1-hydroxy-vitamin D3) **(b)**. 'i' indicates inductive cleavage; 'rH_R' stands for the charge-remote rearrangement.

Fractions 2 and 3 (F2 and F3) also showed compounds with accurate masses of *m/z* 415.3226 and 417.3383, respectively. The full scan and MS^n spectra of these compounds are shown in Figures 5.9 (a and b) and 5.11 (a and b).

The MS^2 spectrum of VDOP with two additional oxygen atoms with *m/z* 415 $[M+H]^+$ showed the fragments with *m/z* 397 $[M+H–H_2O]^+$, 379 $[M+H–2H_2O]^+$, and 361 $[M+H–3H_2O]^+$ (Figure 5.9 b). The MS^3 of fragments with *m/z* 397 gave the fragment ions *m/z* 379 and 361 which resulted from the loss of one and two water molecules, respectively. The fragmentation tree of VDOP with *m/z* 415 is illustrated in Figure 5.10. This figure shows the fragments by losing water molecules and epoxides. The fragments which was obtained by losing epoxides, *m/z* 399 $[M+H–O]^+$ and 383 $[M+H–2O]^+$, and fragment with *m/z* 365 $[383–H_2O]^+$, was suggested by Mass Frontier and observed in very low intensities by MS^n analysis.

Additionally, another type of VDOP with two oxygen atoms but a different precursor ion was obtained with *m/z* 417 $[M+H]^+$ (Figure 5.11 a). MS^2, MS^3 and MS^4 spectra of this compound gave the product ions with *m/z* 399 $[M+H–H_2O]^+$, 381 $[M+H– 2H_2O]^+$ and 363 $[M+H–3H_2O]^+$ (Figure 5.11 b). Unlike the compound with *m/z* 415, any fragments by loss of an oxygen atom (-16) were not seen for VDOP with *m/z* 417. Then, it was indicated that while the compound with *m/z* 415 represented a ketone (1-keto-7,8-epoxy-vitamin D3), VDOP with *m/z* 417 had a hydroxyl moiety (1-hydroxy-7,8-epoxy-vitamin D3) as a functional group. Since the quality of the mass spectra is reduced with each additional fragmentation reaction, analysis is usually limited to a few fragmentation reactions beyond MS^2.

The structure and fragmentation trees of the VDOP with *m/z* 417 is displayed in Figure 5.12. While the fragmentation tree of 1-keto-7,8-epoxy-vitamin D3 demonstrated the loss of both water and oxygen moieties (Figure 5.10), only loss of water was observed in the fragmentation tree of 1-hydroxy-7,8-epoxy-vitamin D3 (Figure 5.12).

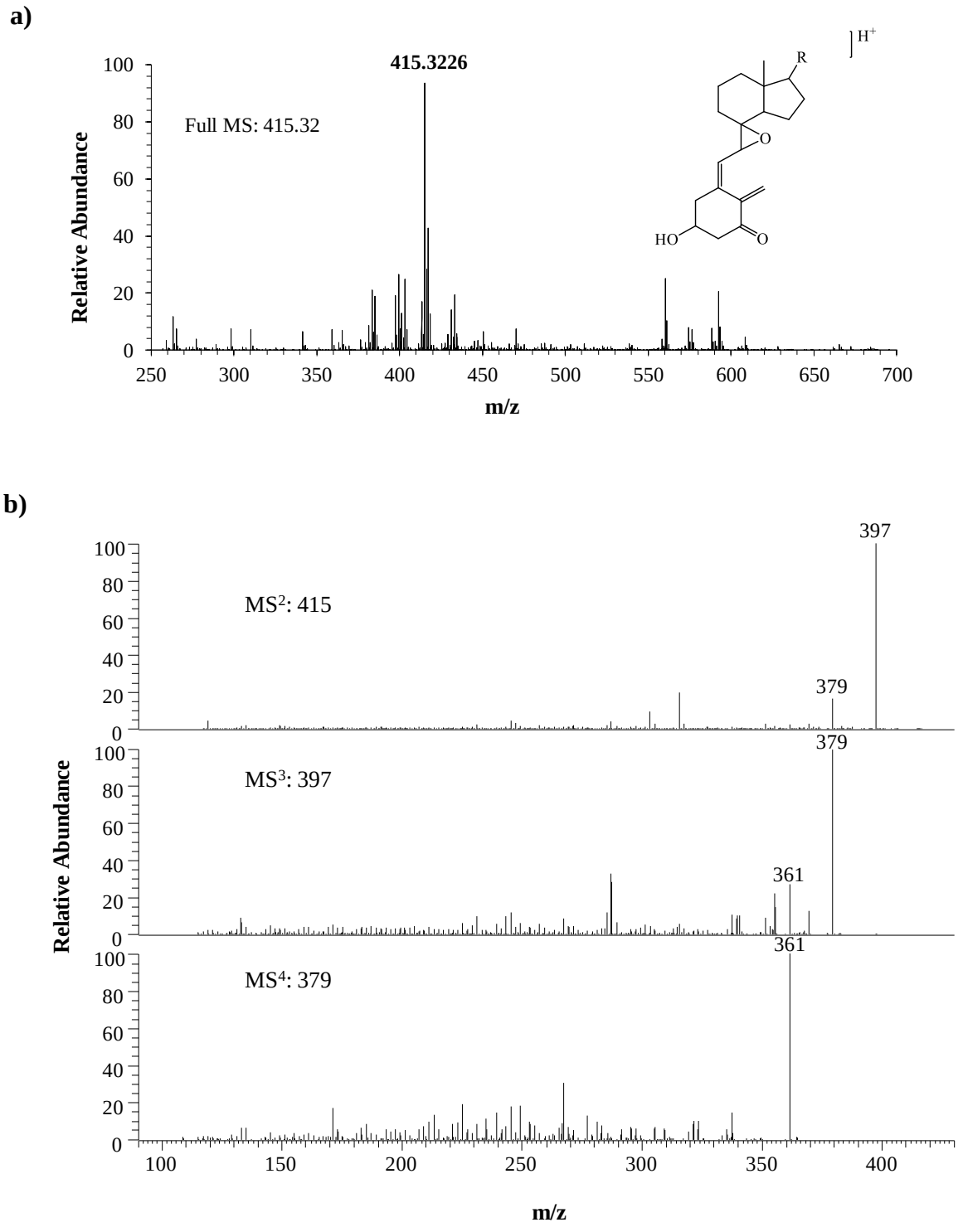


Figure 5.9. Full scan spectrum **(a)** and MS^n spectra **(b)** of VDOP with two additional oxygen atoms.

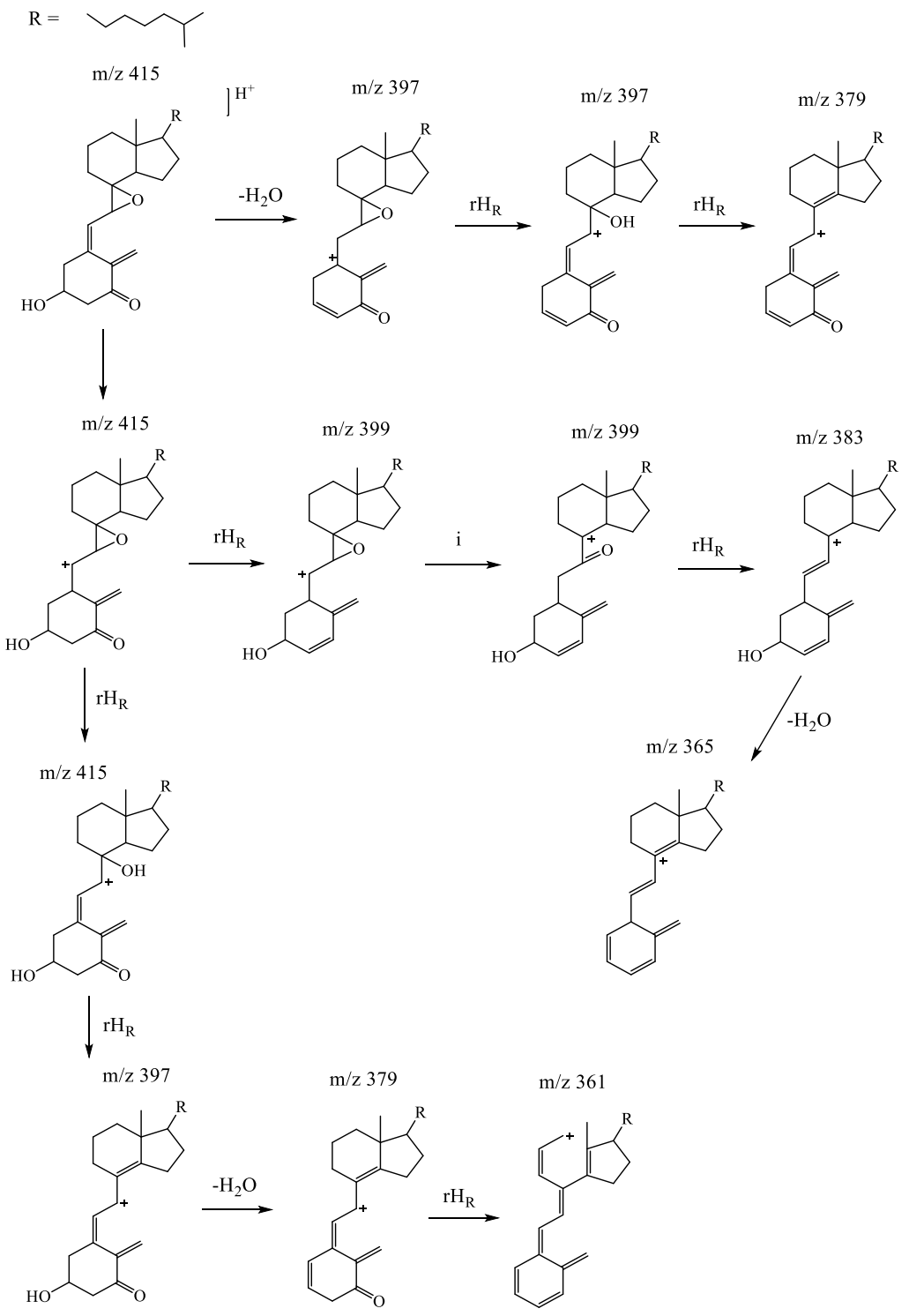


Figure 5.10. Proposed fragmentation tree of VDOP with *m/z* 415 (1-keto-7,8-epoxy-vitamin D3). 'rH_R' stands for the charge-remote rearrangement; 'i' indicates inductive cleavage; The '-' symbols and the molecules above the arrows represent the neutral loss of these molecules.

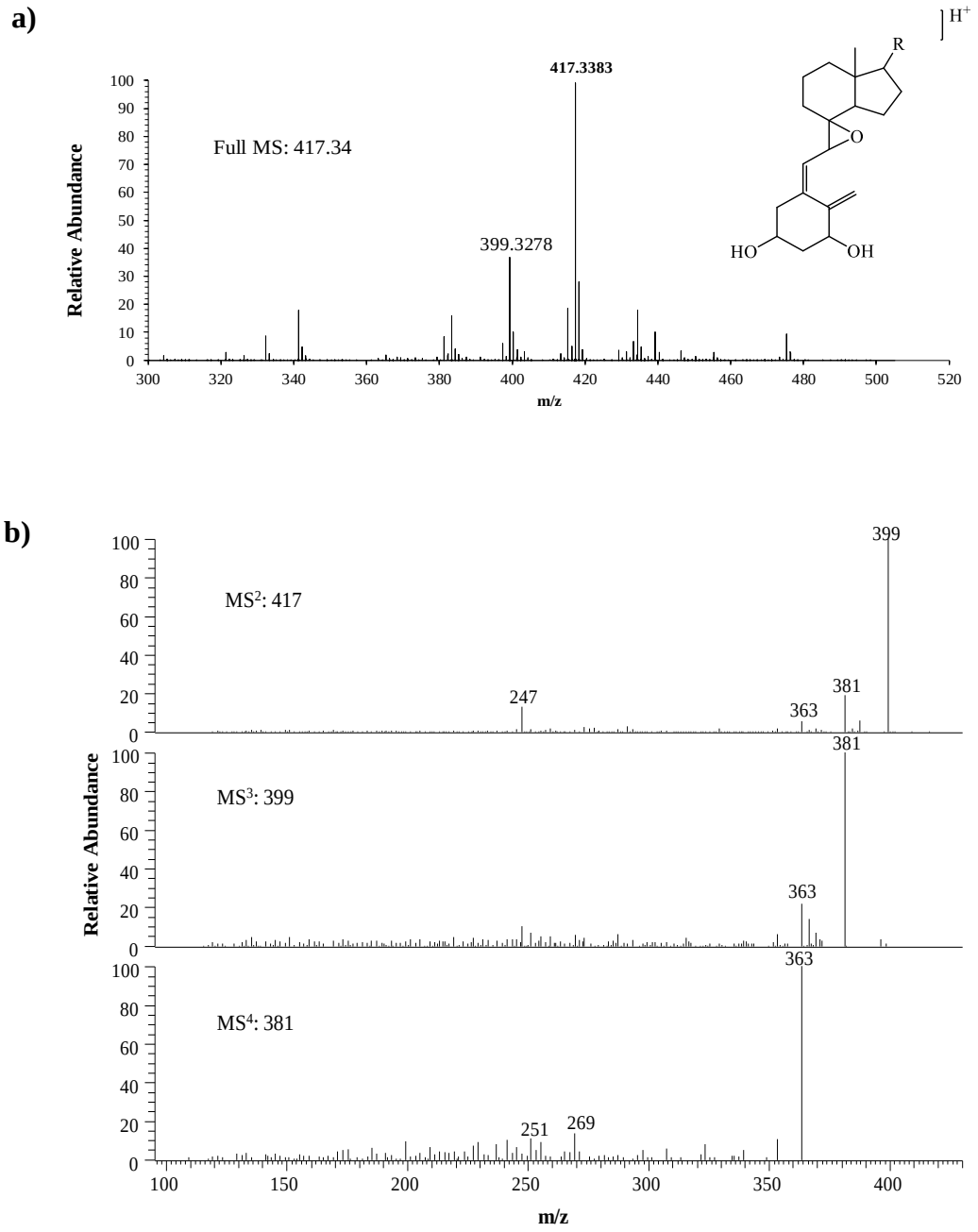


Figure 5.11. Full scan spectrum **(a)** and MS^n spectra **(b)** of VDOP with two additional oxygen atoms.

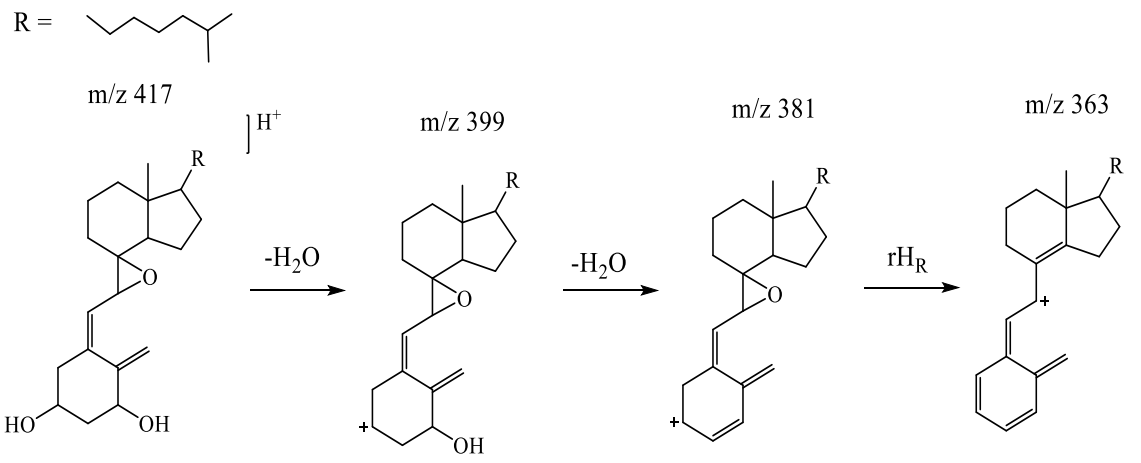


Figure 5.12. Proposed fragmentation tree of VDOP with *m/z* 417 (1-hydroxy-7,8-epoxy-vitamin D3). 'rH_R' stands for the charge-remote rearrangement; The '-' symbols and the molecules above the arrows represent the neutral loss of these molecules.

In order to detect the low abundant vitamin D3 oxidation products in complex matrices, more sensitive analyses were required. The sensitivity of vitamin D3 and its oxidation products' detection can be significantly improved using Diels–Alder derivatization with PTAD. Additionally, a further increase of the ionization efficiency of vitamin D metabolites were achieved after PTAD derivatization as previous studies reported (Aronov et al., 2008; Burild, et al., 2014; Higashi, Miura, Kitahori, & Shimada, 1999). Derivatization with PTAD also shifted the *m/z* ratios of the precursor ions to higher molecular weight, thus reducing interfering low *m/z* background ions. Underivatized vitamin D3 compounds showed a large number of product ions, due to the availability of low-energy fragmentation pathways. On the other hand, the product ion spectrum of PTAD-derivatized vitamin D3 and its oxidation products exhibited only one or two major fragment ions, which was beneficial for sensitive MRM analysis in UHPLC-MS/MS.

It is clear that VDOPs, a class compound with very similar structures, follow similar fragmentation pathways. PTAD derivatization was used to not only increase the sensitivity of these compounds for mass spectrometry, but also to distinguish between VDOPs with similar molecular weight due to the different fragmentation patterns after derivatization. This is another advantage of using

derivatization before analysis as previously discussed for vitamin D3 isomerization products in Chapter 3.

Apart from that, the lack of commercially available standards of VDOPs created the need of other methods for their confirmation, and PTAD derivatization could be a way to confirm VDOPs formation. Thus, in this study, the application of Diels–Alder derivatization was carried out for the identification of VDOPs. Diels–Alder reaction is a selective reaction between dienes and dienophile compounds and the triene system is preferred for this reaction because it has two possible dienes available. Then, VDOPs with the intact triene system could be possible candidates for PTAD-derivatization.

The full scan and MS^n spectra of PTAD-derivatized vitamin D3 and VDOPs, and their fragmentation trees are illustrated in Figures 5.13 - 5.22. As it is shown in the MS^2 spectrum of vitamin D3:PTAD adduct with *m/z* 560.3866 (Figure 5.13 b), the most intense product ions were fragments *m/z* 298 and its dehydrated fragment (*m/z* 280). The fragmentation tree of the vitamin D3:PTAD adduct showed the formation of these two product ions as specified in Figure 5.14. These fragment ions and their structures were previously reported for vitamin D3:PTAD adduct by Abernethy, (2012).

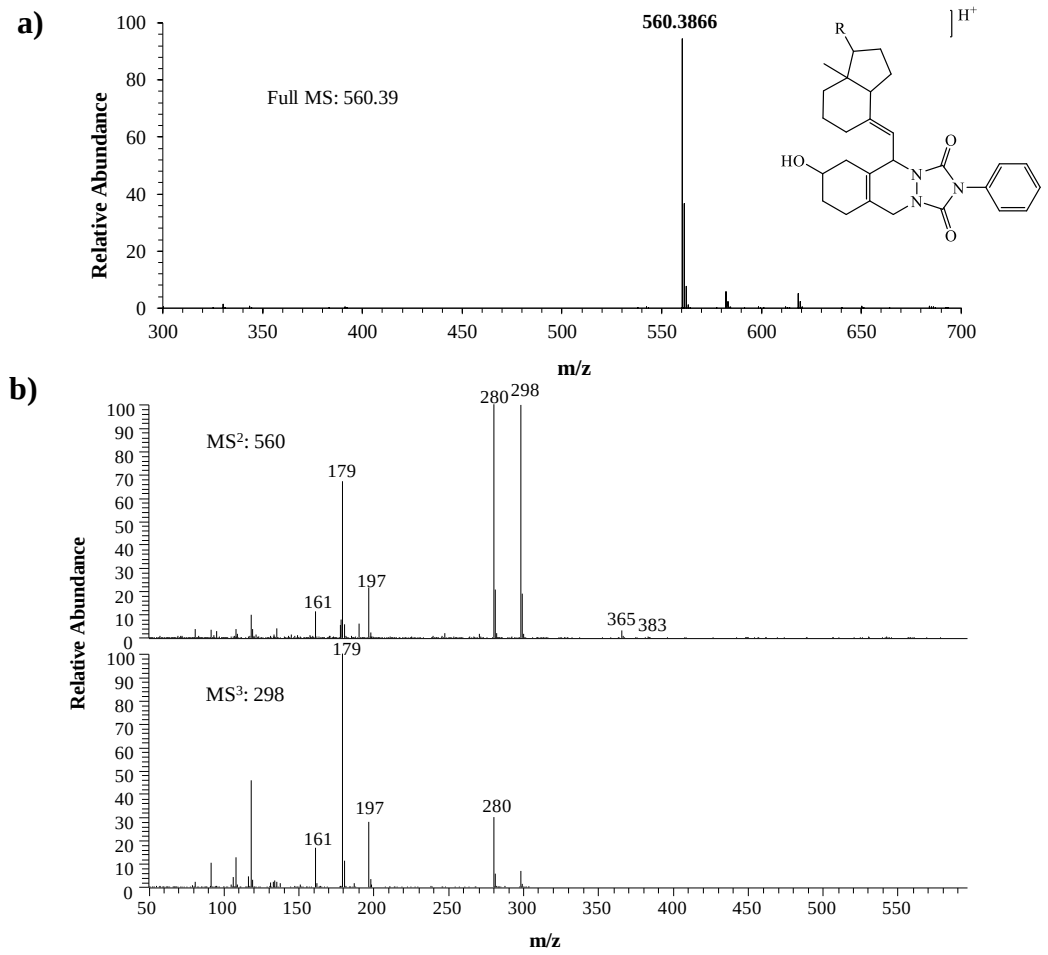


Figure 5.13. Full scan spectrum **(a)** and MS^n spectra **(b)** of vitamin D3:PTAD adduct.

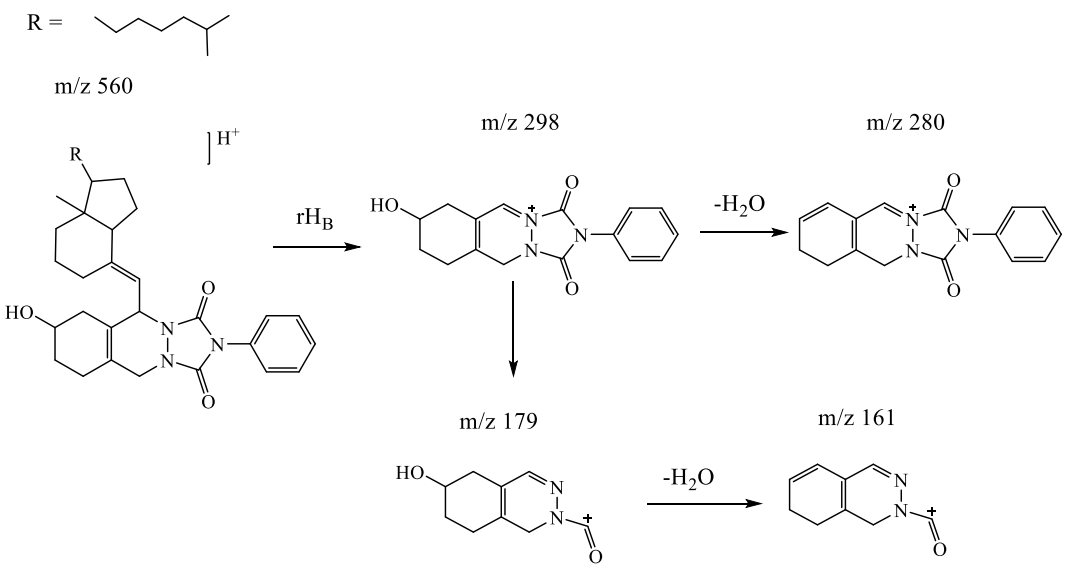


Figure 5.14. Proposed fragmentation tree of vitamin D3:PTAD adduct. 'rH_B' stands for the α, β-charge-site rearrangement.

As discussed earlier, both fractions 2 and 3 (F2 and F3) in sample extracts gave VDOPs with one extra oxygen with similar molecular formulas and product ions after MS analysis (Figure 5.7). In fact, MS spectra yielded insufficient data to distinguish between VDOPs with *m/z* 401 because no specific diagnostic ions were present to differentiate these compounds. In contrast, PTAD-derivatives of these VDOPs showed different fragmentation patterns and helped with their identification. While MS^2 spectra of VDOPs with *m/z* 401 showed similar product ions, different MS^2 spectra were achieved for PTAD-derivatives of these compound (*m/z* 576.3813) (Figures 5.15 b and 5.17 b).

While MS^2 spectrum of F2 showed product ions with *m/z* 298 and 280 (Figure 5.15 b), F3 displayed a fragment with *m/z* 314 as the most significant product ion (Figure 5.17 b). The fragmentation trees of these two VDOPs are illustrated in Figure 5.16 & 5.18. Different mass spectra and fragmentation pathways demonstrated different types of VDOPs with one extra oxygen were present in F2 (7,8-epoxy-vitamin D3:PTAD) and F3 (1-hydroxy-vitamin D3:PTAD).

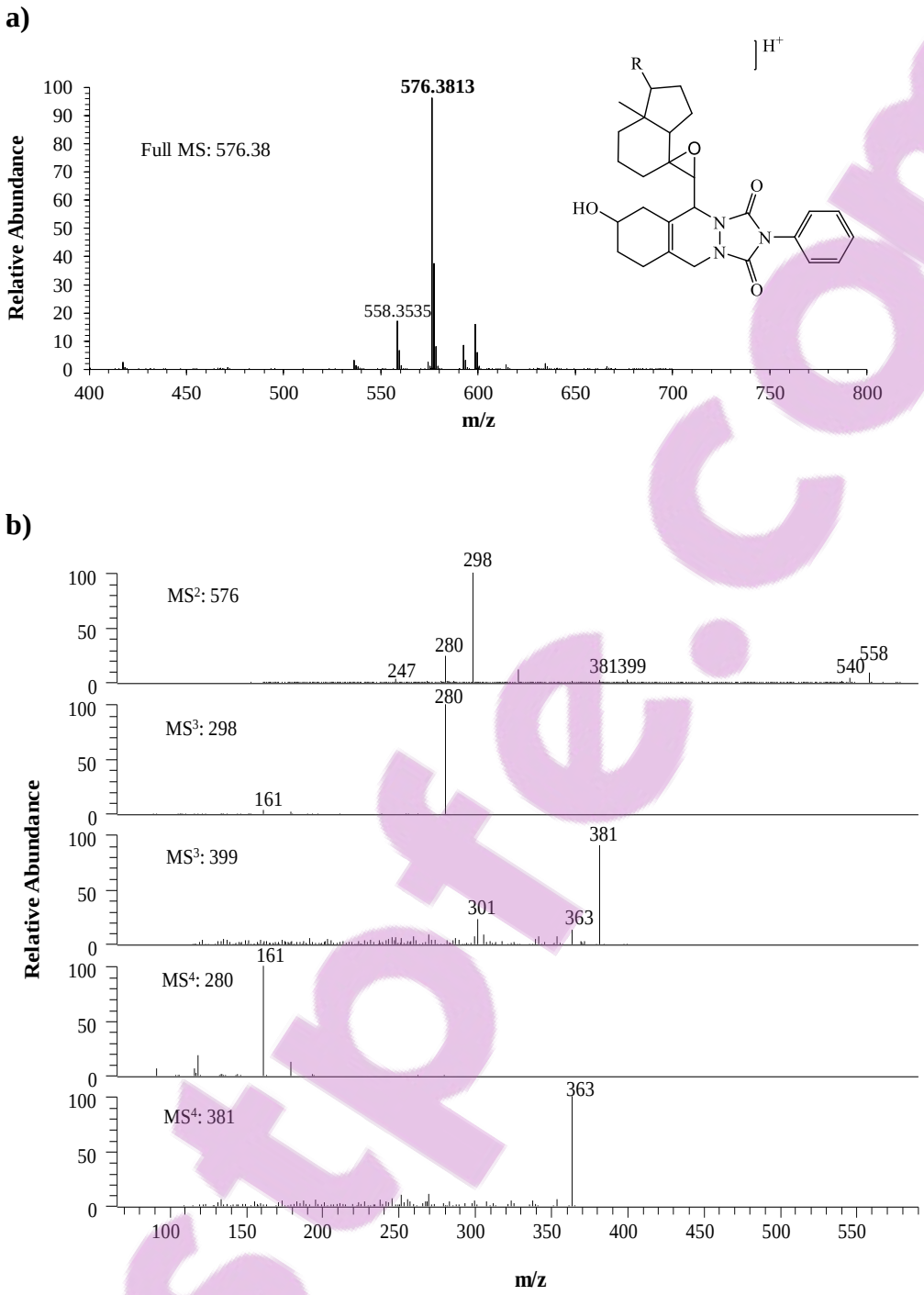


Figure 5.15. Full scan spectrum **(a)** and MS^n spectra **(b)** of PTAD-derivatized VDOP with one additional oxygen atom.

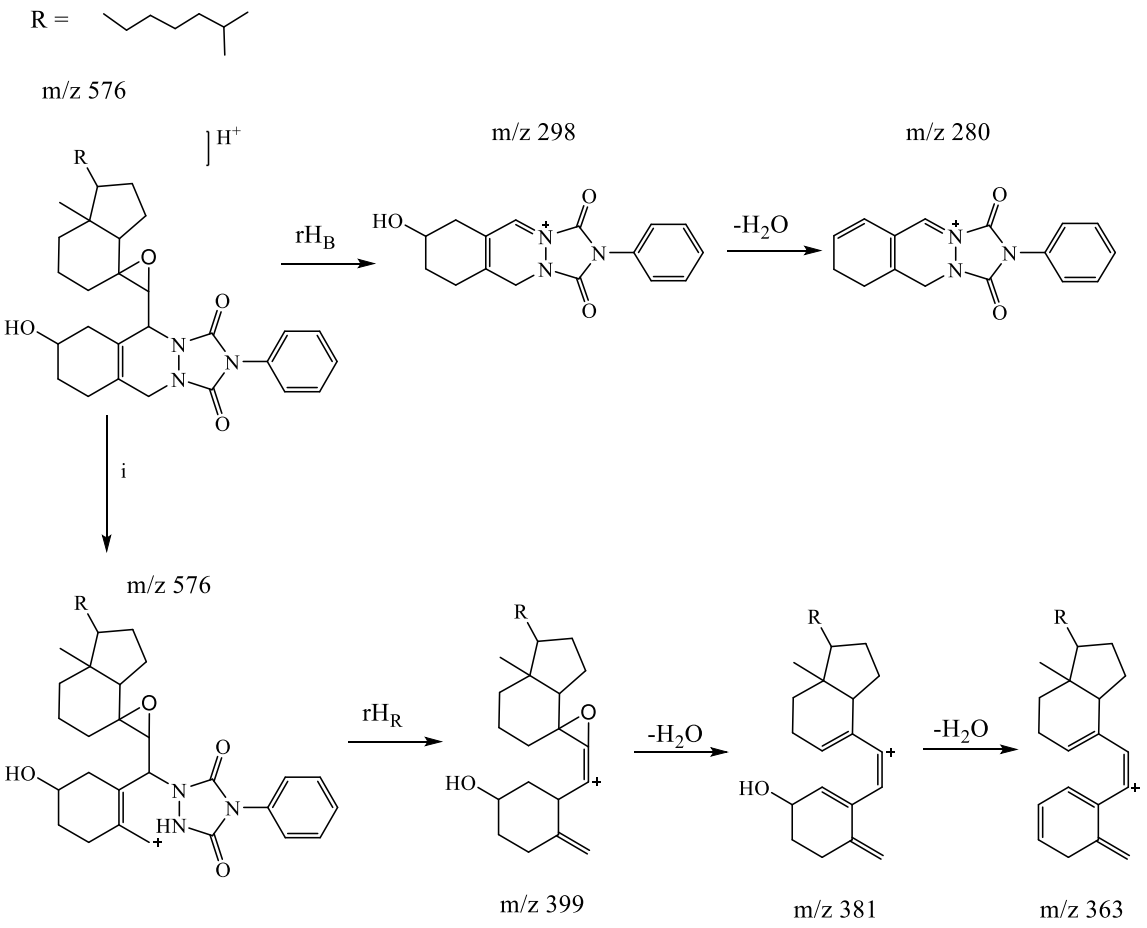


Figure 5.16. Proposed fragmentation tree of PTAD-derivatized VDOP with *m/z* 576 (7,8-epoxy-vitamin D3:PTAD). 'rH_R' stands for the charge-remote rearrangement; 'i' indicates inductive cleavage; 'rH_B' stands for the α, β-charge-site rearrangement; The '-' symbols and the molecules above the arrows represent the neutral loss of these molecules.

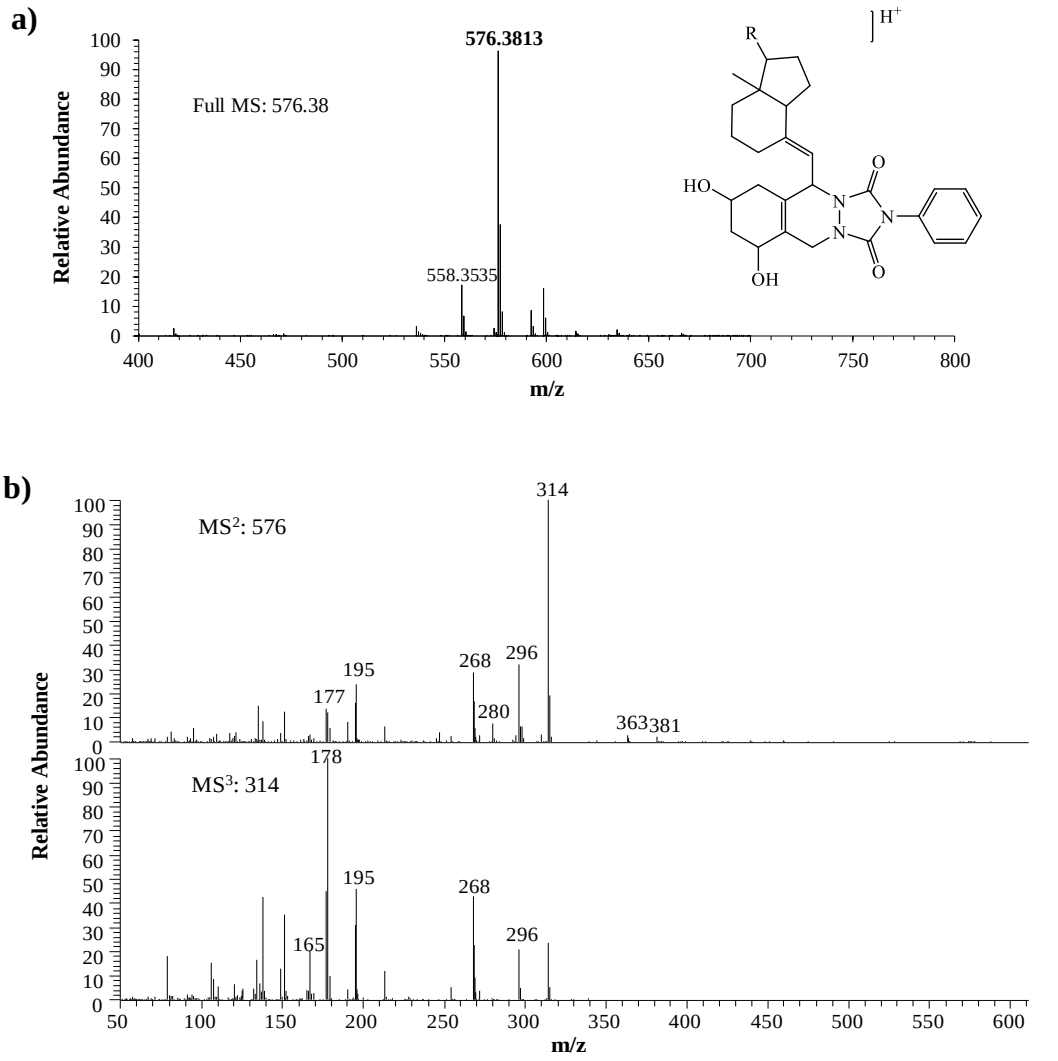


Figure 5.17. Full scan spectrum **(a)** and MS^n spectra **(b)** of PTAD-derivatized VDOP with one additional oxygen.

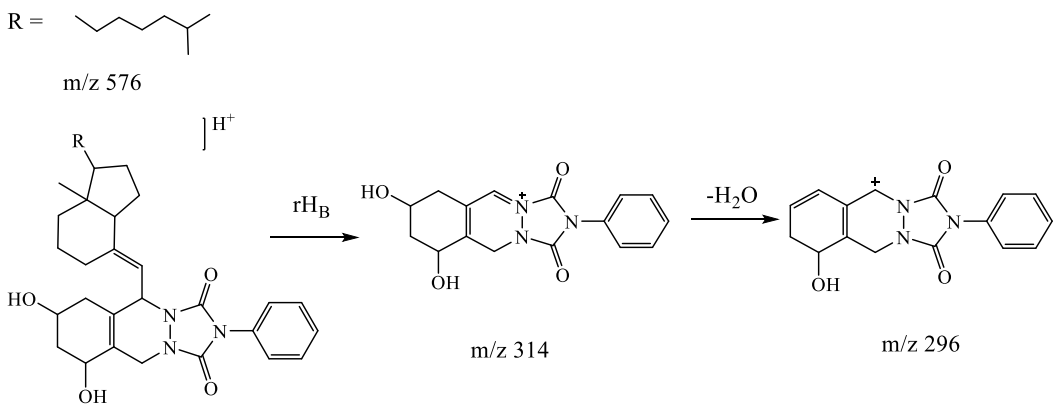
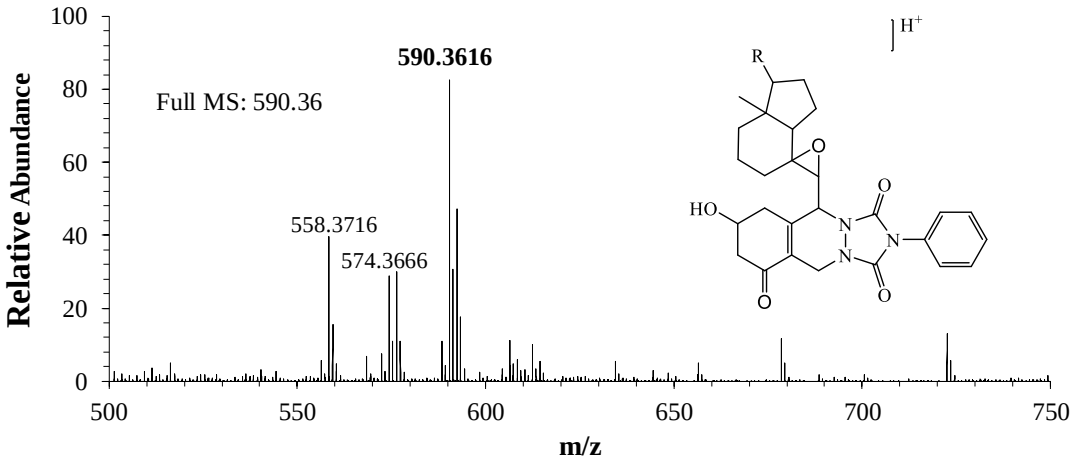


Figure 5.18. Proposed fragmentation tree of PTAD-derivatized VDOP with *m/z* 576 (1-hydroxy-vitamin D3:PTAD). 'rH_B' stands for the α, β-charge-site rearrangement.

The full scan and MS^2 spectra of PTAD-derivatized vitamin D3 plus two more oxygen atoms with *m/z* 590.3616 and 592.3771 are shown in Figures 5.19 (a and b) and 5.21 (a and b), respectively. Both VDOPs gave fragment ions *m/z* 298 and 280 as the most significant product ions. However, these VDOPs can be distinguished by the product ions which were generated from the loss of PTAD(2H). As shown in Figure 5.19 (b), the compound with *m/z* 590 $[M+H]^+$ showed the fragments *m/z* 413 $[M+H–PTAD(2H)]^+$, 395 $[M+H–PTAD(2H)–H_2O]^+$ and 377 $[M+H–PTAD(2H)–2H_2O]^+$. However, the compound with *m/z* 592 $[M+H]^+$ displayed the same fragments with two mass units larger (415 $[M+H–PTAD(2H)]^+$, 397 $[M+H–PTAD(2H)–H_2O]^+$ and 379 $[M+H–PTAD(2H)–2H_2O]^+$) (Figure 5.21 b). The two mass units difference can confirm the formation of different types of VDOPs with two oxygen atoms (*m/z* 415 and 417).

Fragmentation trees of PTAD-derivatives of these VDOPs are illustrated in Figures 5.20 and 5.22. While VDOP with *m/z* 590 showed two fragments as a result of the loss of the oxygen atom (*m/z* 379 $[M+H–PTAD(2H)–H_2O–O]^+$ and 363 $[M+H–PTAD(2H)–H_2O–2O]^+$, respectively), the compound with *m/z* 592 gave only one fragment by losing the oxygen atom (*m/z* 363 $[M+H–PTAD(2H)–2H_2O–O]^+$). It can be concluded that while VDOP with *m/z* 590 was 1-keto-7,8-epoxy-vitamin D3:PTAD, the another VDOP (*m/z* 592) can be 1-hydroxy-7,8-epoxy-vitamin D3:PTAD (Figures 5.20 and 5.22).

a)



b)

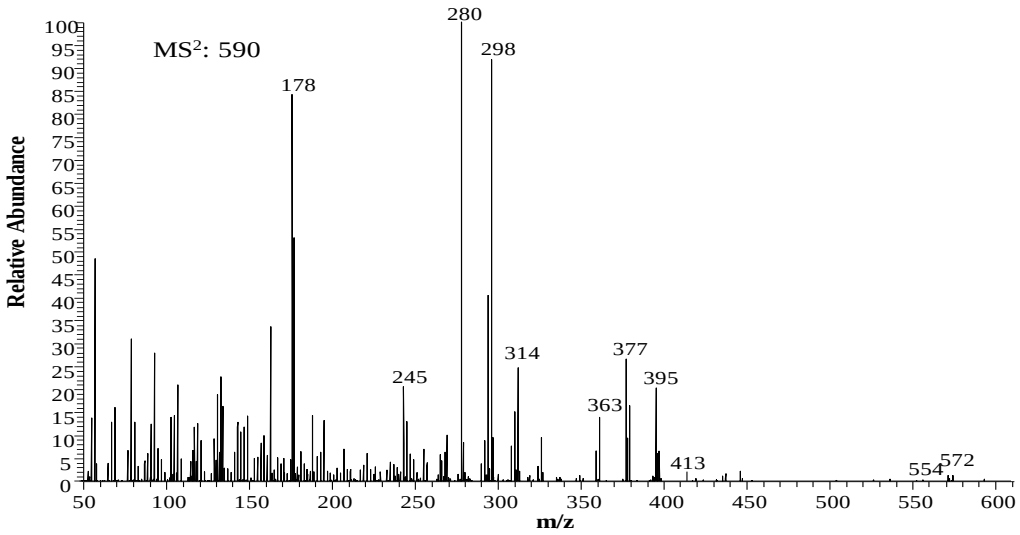


Figure 5.19. Full scan **(a)** and MS^2 **(b)** spectra of PTAD-derivatized VDOP with two additional oxygen atoms.

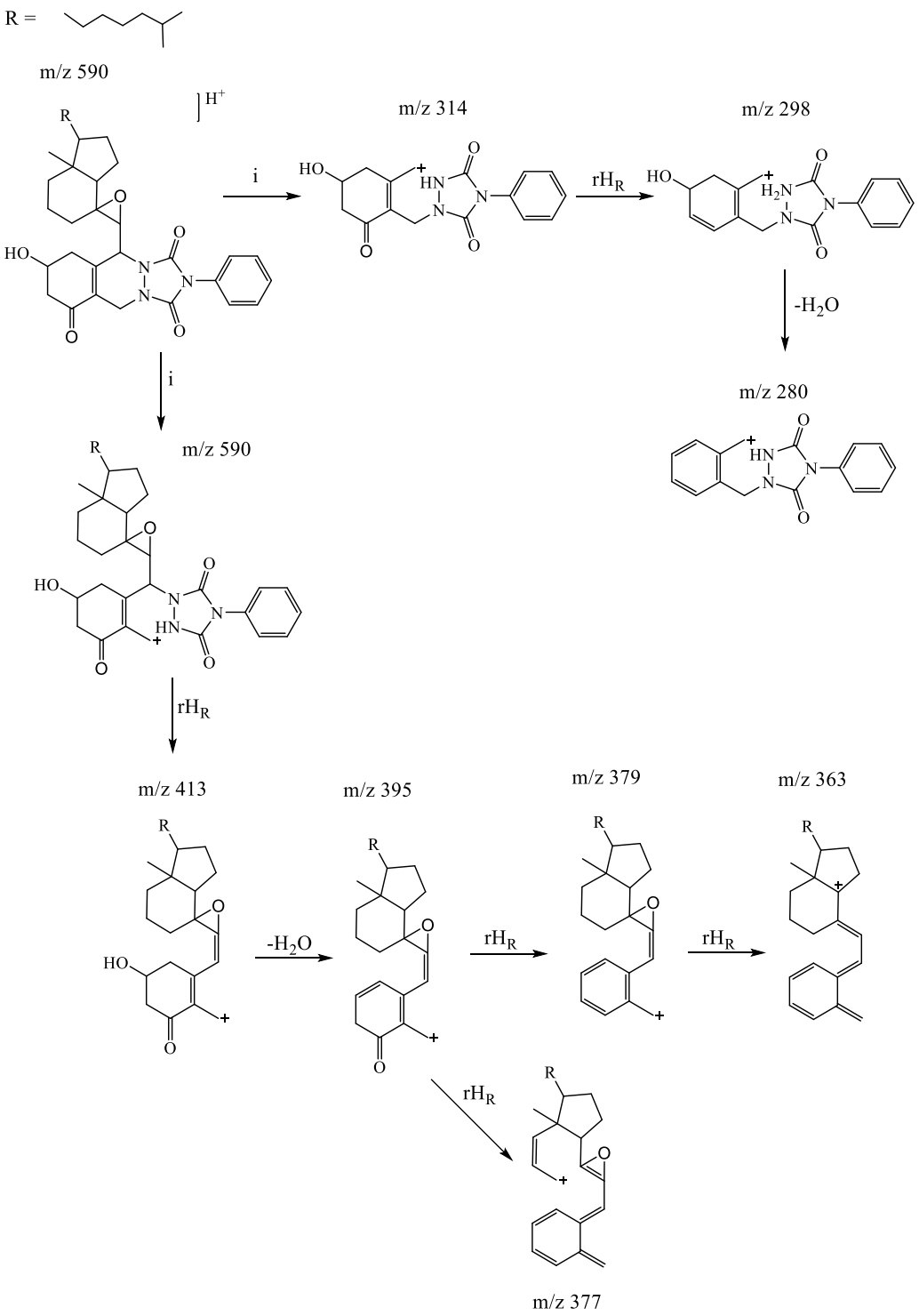


Figure 5.20. Proposed fragmentation tree of PTAD-derivatized VDOP with *m/z* 590 (1-keto-7,8-epoxy-vitamin D3:PTAD). 'rH_R' stands for the charge-remote rearrangement; 'i' indicates inductive cleavage; The '-' symbols and the molecules above the arrows represent the neutral loss of these molecules.

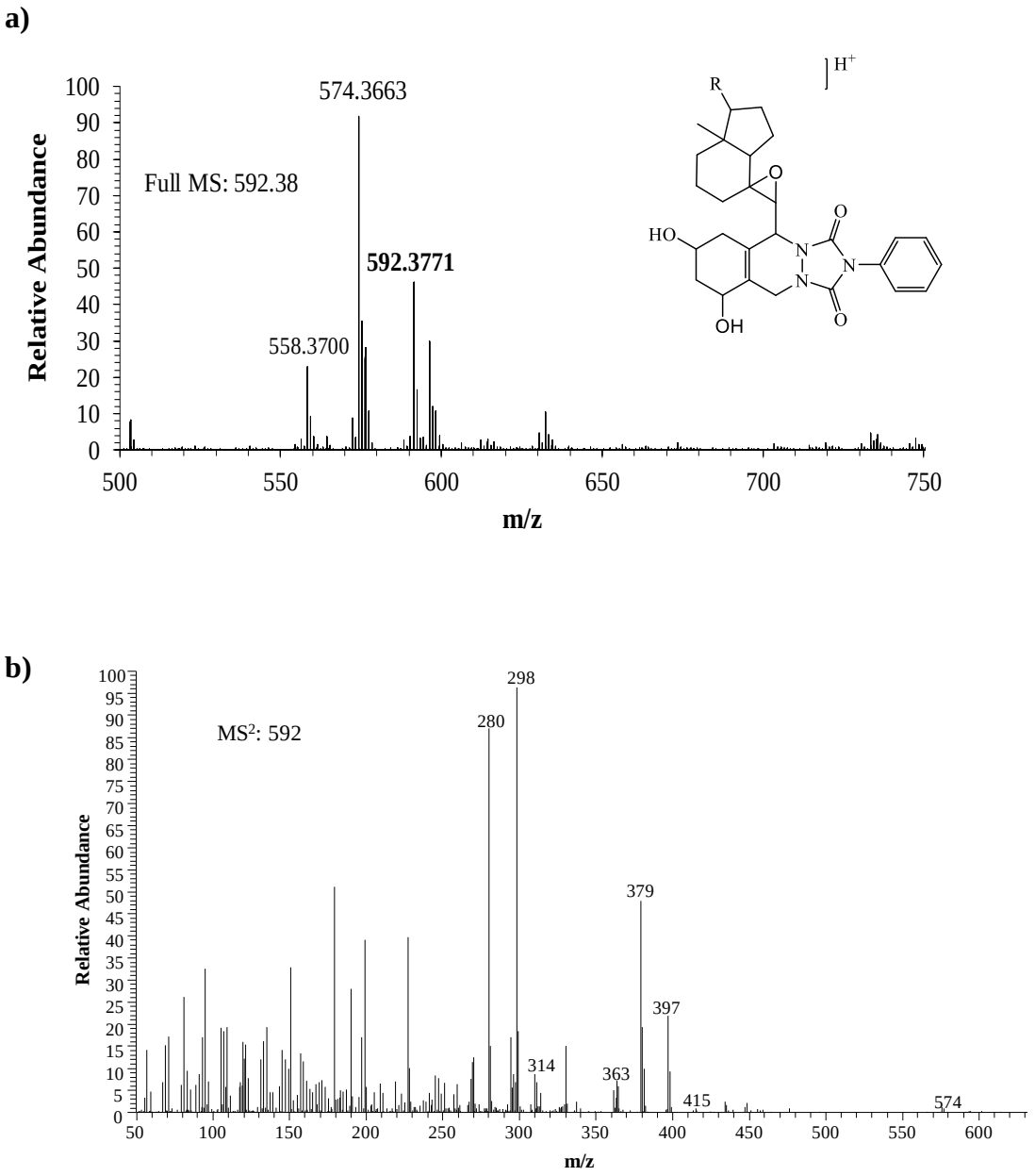


Figure 5.21. Full scan **(a)** and MS^2 **(b)** spectra of PTAD-derivatized VDOP with two additional oxygen atoms.

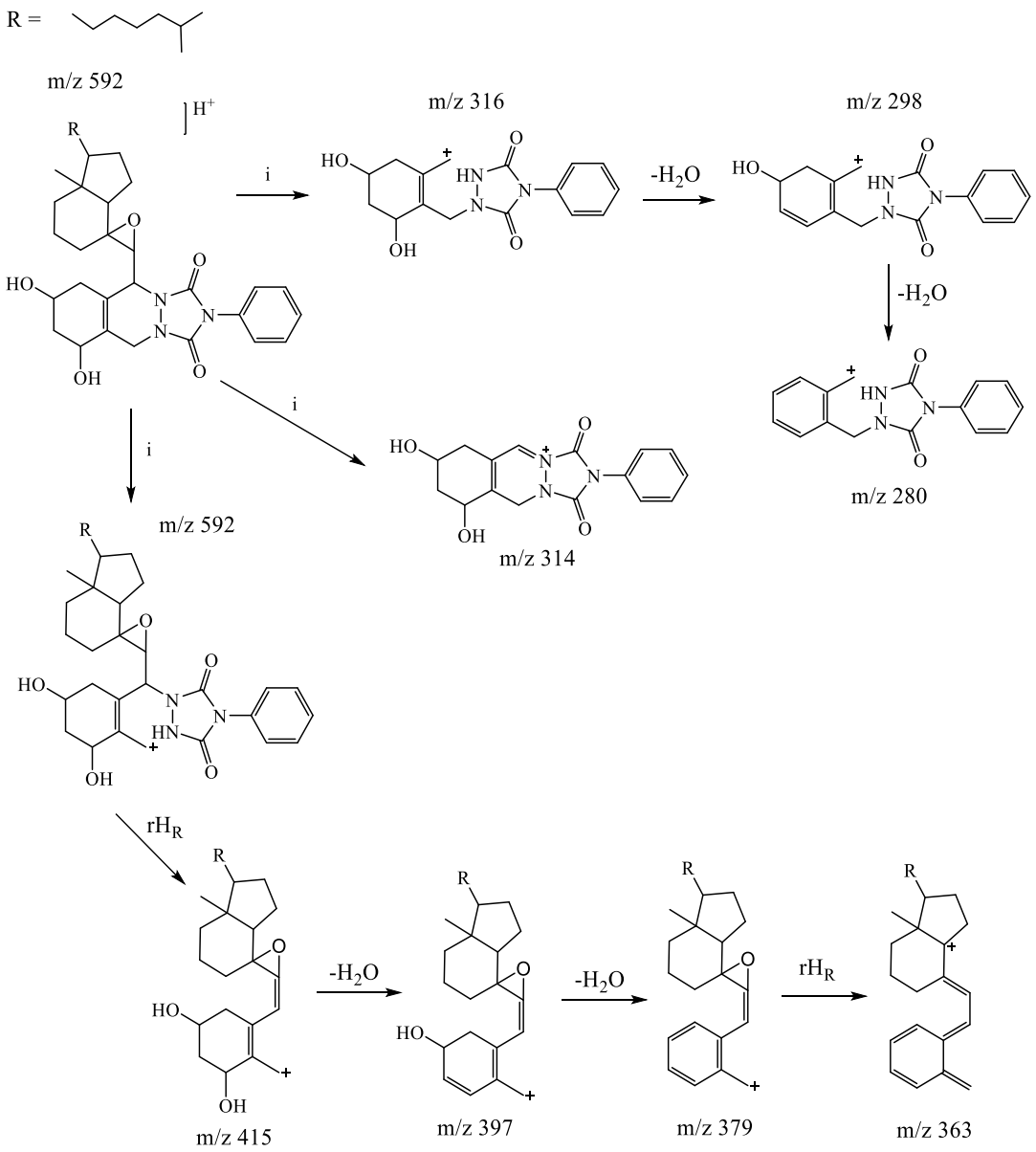


Figure 5.22. Proposed fragmentation tree of PTAD-derivatized VDOP with *m/z* 592 (1-hydroxy-7,8-epoxy-vitamin D3:PTAD). 'rH_R' stands for the charge-remote rearrangement; 'i' indicates inductive cleavage; The '-' symbols and the molecules above the arrows represent the neutral loss of these molecules.

5.3.2.4. MRM analysis of PTAD-derivatized VDOPs using UHPLC-QQQ-MS/MS

Multiple reaction monitoring (MRM), also known as selected reaction monitoring (SRM), is a targeted MS approach accessible on triple quadrupole (QQQ) mass instruments (Gallien, Duriez, & Domon, 2011; Picotti & Aebersold, 2012). In this technique, the precursor ions are selected in the first quadrupole and undergo fragmentation through CID dissociation in the second quadrupole to generate product ions. The pair of *m/z* values for a given precursor and product ion is referred to as a MRM transition and the success of MRM analysis relies on the selection of suitable targets and their optimal MRM transitions. Monitoring of specific transition for each analyte yields a superior signal-to-noise ratio with significantly higher sensitivity and selectivity.

Target VDOPs and their potential MRM transitions were selected based on the data obtained from Q-Exactive Orbitrap and ion trap mass spectrometry analysis. Mass spectrometry conditions for MRM analysis of PTAD-derivatized vitamin D3 and its oxidation products are displayed in Table 5.4. MRM parameters were optimized using commercial standards of vitamin D3 and 1-hydroxy-vitamin D3.

Vitamin D3 and its oxidation products were extracted from stored simulated whole milk powder (SWMP). The extraction method was based on the liquid-liquid extraction followed by the solid phase extraction as explained in section 5.2.3. Separated fractions (F1, F2 and F3) were derivatized with PTAD and identified by UHPLC-MRM-MS/MS using the parameters explained in Table 5.4. A simple matrix of oxidized solution of vitamin D3 was also analysed with the same method to verify VDOPs formation. The MRM chromatograms of PTAD-derivatized vitamin D3 and its oxidation products extracted from the oxidized solution of vitamin D3 and stored SWMP are shown in Figures 5.23 - 5.27. Both samples gave a peak at retention time of about 7.5 min for vitamin D3:PTAD adduct (Figure 5.23 and Table 5.4). This is the same retention time found for PTAD-derivatized vitamin D3 standard as discussed in Chapter 4.

Table 5.4. Mass spectrometry conditions for MRM analysis of PTAD-derivatized vitamin D3 and its oxidation products.

PTAD derivative	Fraction	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision Energy (eV)	Quantifying MRM transition (*m/z*)	Retention time (min)
D3[a]	F1	560	298 280	25	560 → 298	7.5
EXP-D3[b]	F2	576	298 280	25	576 → 298	6.6 & 7.2
Keto-Exp-D3[c]	F2	590	314 298 280 395 377	25	590 → 298	6.4
OH-D3[d]	F3	576	314 296	25	576 → 314	6.6 & 6.8
OH-EPX-D3[e]	F3	592	298 280 397 379	25	592 → 298	5.9

[a] vitamin D3

[b] 7,8-epoxy-vitamin D3

[c] 1-keto-7,8-epoxy-vitamin D3;

[d] 1-hydroxy-vitamin D3

[e] 1-hydroxy-7,8-epoxy-vitamin D3

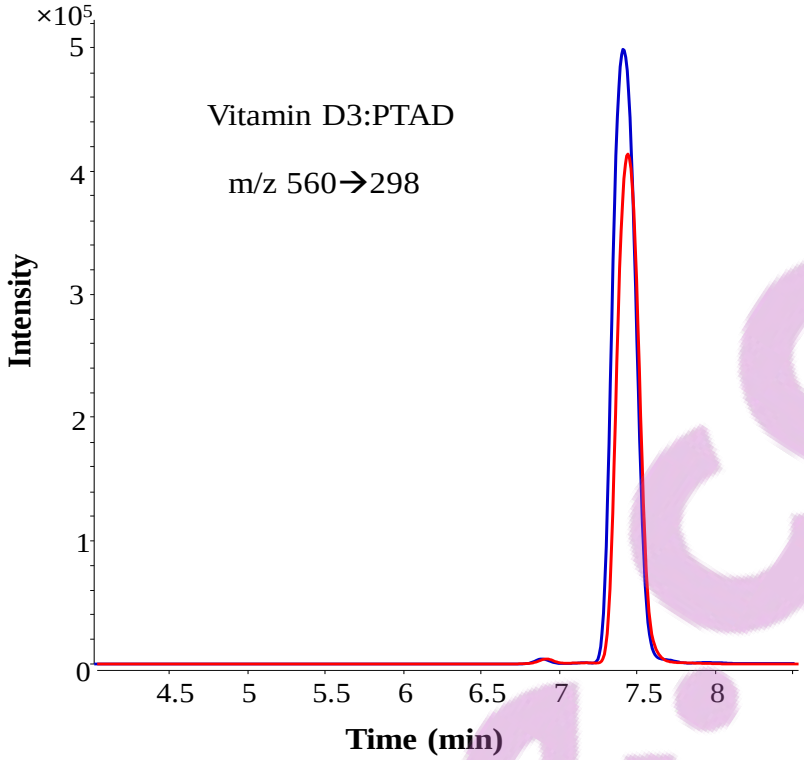


Figure 5.23. MRM chromatograms of PTAD-derivatized vitamin D3 at *m/z* 560→298 in F1 of the oxidized vitamin D3 solution (blue) and stored SWMP (red).

Fractions 2 and 3 (F2 and F3) of samples were analysed for MRM transition *m/z* 576→298 and 576→314. While F2 only showed peaks for the transition *m/z* 576→298, two peaks were detected in F3 when the transition *m/z* 576→314 was monitored (Figures 5.24 and 5.25). As it is shown in Figure 5.24, F2 showed two peaks for 7,8-epoxy-vitamin D3:PTAD at retention times of about 6.9 and 7.2 min. Both samples of vitamin D3 solution and SWMP showed similar retention times for this compound which confirmed the presence of this VDOP in both simple and more complex samples. When the transition *m/z* 576→314 was monitored, 1-hydroxy-vitamin D3:PTAD was eluted at the retention times of about 6.6 and 6.8 min in F3 (Figure 5.25). In addition, the commercial standard of PTAD-derivatized 1-hydroxy-vitamin D3 was analysed which showed similar peaks with similar retention times (Figure 5.25). According to previous studies, derivatization of vitamin D metabolites with PTAD produces two epimers, 6S and 6R, because the reagent reacts with the S-*cis*-diene moiety from both the α- and β-sides (Aronov et al., 2008; Burild et al., 2014). As a result, two peaks were separated for each VDOP in the MRM ion chromatograms provided that the two epimers are fully separated during the chromatography of these compounds.

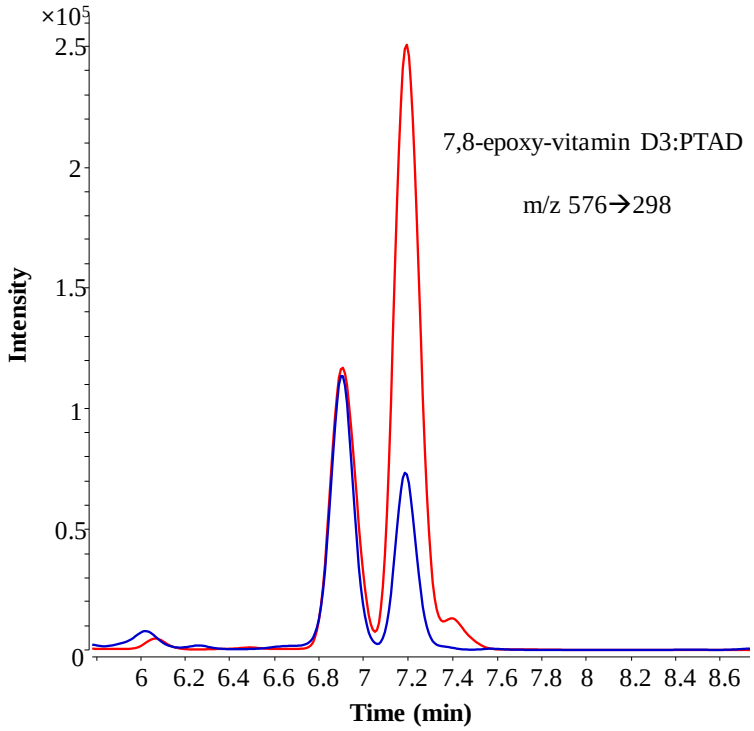


Figure 5.24. MRM chromatograms of PTAD-derivatized 7,8-epoxy-vitamin D3 at *m/z* 576→298 in F2 of the oxidized vitamin D3 solution (blue) and stored SWMP (red).

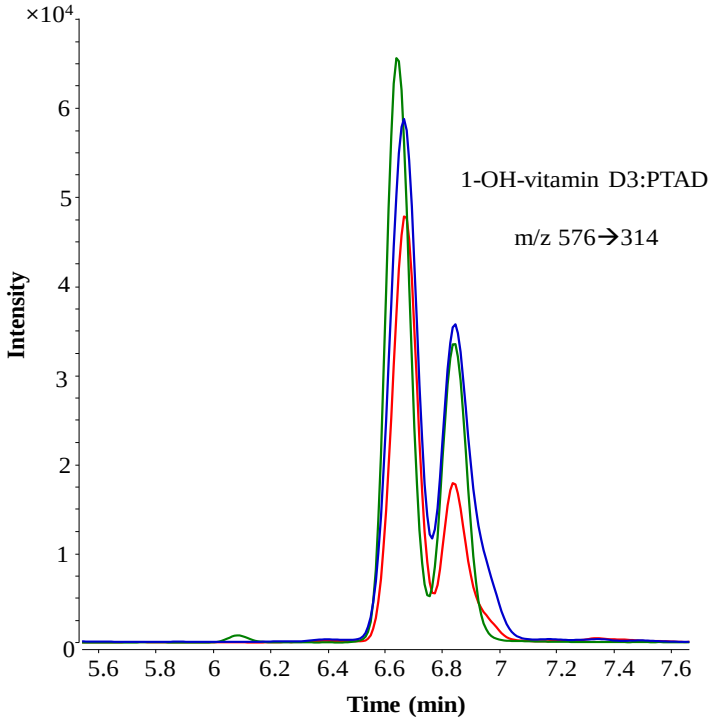


Figure 5.25. MRM chromatograms of PTAD-derivatized 1-hydroxy-vitamin D3 at *m/z* 576→314 in F3 of the oxidized vitamin D3 solution (blue), stored SWMP (red) and commercial standard of 1-hydroxy-vitamin D3 (green).

The MRM transition *m/z* 590→298 and 592→298 were monitored in F2 and F3 for the detection of 1-keto-7,8-epoxy-vitamin D3:PTAD and 1-hydroxy-7,8-epoxy-vitamin D3:PTAD, respectively. PTAD-derivatized 1-keto-7,8-epoxy-vitamin D3 was observed in F2 at a retention time of about 6.4 min (Figure 5.26), while F3 eluted 1-hydroxy-7,8-epoxy-vitamin D3:PTAD at a retention time of about 5.9 min (Figure 5.27). Both of these VDOPs were available in the oxidized solution of vitamin D3 and stored SWMP.

Apart from that, SWMP was also prepared without vitamin D3 addition and stored in the same conditions. When the MRM transitions of VDOPs were monitored for this sample, no peaks were observed. Therefore, it could be concluded that the observed peaks were related to vitamin D3 compounds.

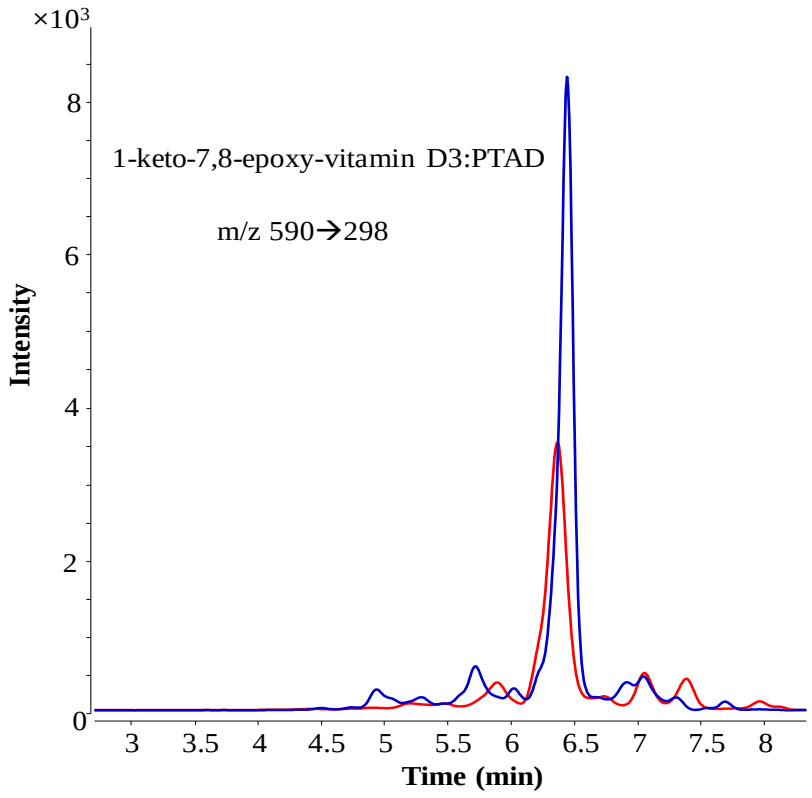


Figure 5.26. MRM chromatograms of PTAD-derivatized 1-keto-7,8-epoxy-vitamin D3 at *m/z* 590→298 in F2 of the oxidized vitamin D3 solution (blue) and stored SWMP (red).

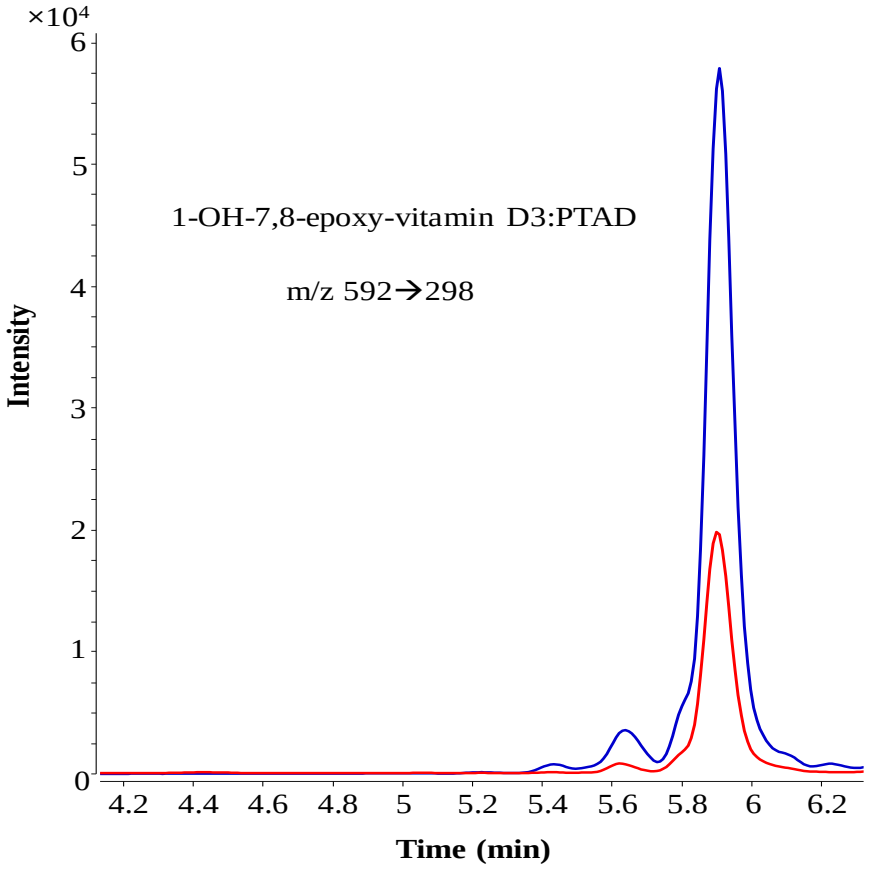


Figure 5.27. MRM chromatograms of PTAD-derivatized 1-hydroxy-7,8-epoxy-vitamin D3 at *m/z* 592→298 in F3 of the oxidized vitamin D3 solution (blue) and stored SWMP (red).

5.3.3. Quantification of VDOPs in stored whole milk powder provided by Fonterra

Stored whole milk powders (around 2 years storage period) were collected from different range of plants, Te Rapa (TR D4) and Kauri (K2) for VDOPs analysis. The batch number of collected samples were IZ28 and FZ28 (Te Rapa), and HZ07 and FZ26 (Kauri). VDOPs in stored whole milk powder were extracted and derivatized based on the method of Gill et al, (2015) as discussed in section 4.2.5 for vitamin D3 quantification. The analyses were based on the direct saponification and extraction of lipophilic components in isooctane. An aliquot of PTAD was added to a portion of isooctane layer to derivatize vitamin D3 and its oxidation products.

Target MRM transitions, which were obtained for VDOPs identification in SWMP, were used to quantify these compounds in real whole milk powder samples. VDOPs in stored whole milk powder samples were measured using vitamin D3 as the standard and the results were expressed as vitamin D3 equivalent per 100 gram of sample (µg VDE/100 g) using below formula:

$$Y = \frac{PA\ (VDOP)}{\text{PA (VD)}} \times \text{Cons (VD)}$$

Where Y is VDOPs concentration expressed as vitamin D3 equivalent, PA(VDOP) is the peak area of vitamin D3 oxidation product in sample, PA(VD) is the peak area of vitamin D3 in sample and Cons (VD) is the concentration of vitamin D3 in sample. Concentration of VDOPs in different batches of stored whole milk powders are shown in Table 5.5. Among all MRM transitions which were monitored, transition *m/z* 576→298 was not observed in real samples. In addition, the transition *m/z* 592→314 was observed and showed a high concentration especially in sample FZ28. Since the storage time of these samples was one year more than SWMP, the VDOPs with one oxygen might be degraded to compounds with two more oxygen atoms. Moreover, the analysis of VDOPs in Waitoa laboratory was based on hot saponification which could result in the degradation of VDOPs and formation of the artefacts. Apart from that, the relationship between vitamin D3 and total VDOPs contents are illustrated in Figure 5.28. Generally, samples with higher content of vitamin D3 showed lower concentration of VDOPs. More studies on identification of VDOPs in real whole milk powders are needed to obtain better results especially in terms of chromatography of these compounds.

Table 5.5. Determination of PTAD-derivatized vitamin D3 oxidation products in real stored whole milk powders

Sample (batch No.)	MRM transition (*m/z*)	VDOP concentration (µg VDE/100 g)
IZ28	576 → 314	0.09
	590 → 298	0.03
	592 → 298	0.07
	592 → 314	0.47
FZ28	576 → 314	0.16
	590 → 298	0.04
	592 → 298	0.03
	592 → 314	2.65
HZ07	576 → 314	0.14
	590 → 298	0.03
	592 → 298	0.03
	592 → 314	0.72
FZ26	576 → 314	0.07
	590 → 298	0.04
	592 → 298	0.03
	592 → 314	0.42

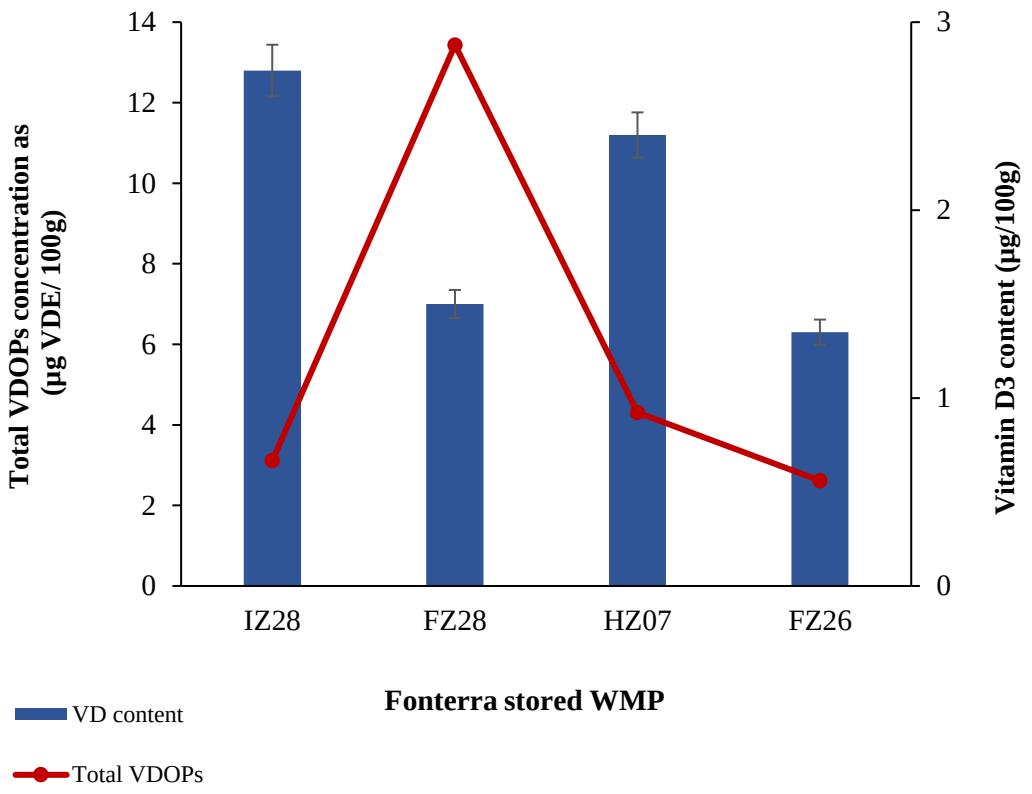


Figure 5.28. Vitamin D3 content and total VDOPs concentration in real whole milk powders.

5.4. Conclusions

The present method for the isolation of VDOPs was adapted based on the extraction of cholesterol oxidation products in dairy products. Liquid-liquid extraction without saponification was used to minimize the risk of artifacts generation. Further, solid phase extraction was applied as a clean-up step to separate different classes of lipid and vitamin D3 from VDOPs. Aminopropyl-SPE cartridges eliminated a large extent of fatty material from the analytes and allowed a reliable separation of vitamin D3 and its oxidation products. Different mass spectrometry analysis schemes have been developed and optimized to analyse the unknown VDOPs. The sensitivity of VDOPs' detection was significantly improved using Diels–Alder derivatization with PTAD.

The lack of standardized mass spectra libraries and limited publicly-available data with respect to authentic MS^n spectra limit LC-MS/MS fragmentation studies of unidentified vitamin D3 compounds. The bottleneck of the compounds identification could not be overcome without mass spectra prediction tools. Fragmentation trees and MS^n spectral trees can give reliable methods to identify vitamin D3 unknown compounds such as VDOPs. Methods based on high accurate MS^2 and MS^n analysis have been developed for the identification and determination of vitamin D3 and its oxidation products.

In this Chapter, Mass Frontier software was used to calculate fragmentation pathways of VDOPs which was helpful in the annotation of compounds detected using ion trap mass spectrometry. The precursor-product ion relationships observed in MS^n spectra not only allowed to efficiently identify VDOPs, it also allowed to assign elemental formula and structure to each relevant fragment ion. This new method could help in comparing MS^n data and in the annotation and identification of known and unknown vitamin D3 compounds.

Finally, the MS/MS step was carried out by MRM which enabled us to identify these compounds with high selectivity at very low concentrations of a few parts per billion. For the first time, oxidized vitamin D3 products have been detected in real fortified whole milk powder, and this provides compelling support that the mode of degradation of vitamin D is in fact oxidation. The high selectivity and sensitivity obtained demonstrated the suitability of the proposed method for the determination of these compounds in more complex matrices such as food systems.

CHAPTER 6

Conclusions and Future work

6.1. General conclusions

The main aim of this thesis was to elucidate the mechanism of vitamin D3 degradation in fortified milk powders. To achieve this goal, the research project was developed in three parts; isomerization studies of vitamin D3, monitoring of vitamin D3 stability in simulated whole milk powders (SWMPs) as influenced by processing and storage conditions, and identification of vitamin D3 oxidation products in the stored SWMPs. Below is a brief summary of the main findings from this study:

1) Isomerisation studies of vitamin D3 using HPLC-DAD, UHPLC-MS/MS and chemical derivatization.

Vitamin D3 is well known to isomerize under various conditions which resulted in the generation of its isomerization products. In Chapter 3, the effects of different physicochemical stresses upon vitamin D3 were studied with the aim of creating markers of degradation pathways in situ. In this step, vitamin D3 isomerization products were generated, and analytical methods were developed for their identification.

In this respect, the effects of temperature, iodine, acidic conditions and oxidation on the isomerization of vitamin D3 were studied. Results have shown that vitamin D3 thermally and reversibly transforms to previtamin D3 isomer. In the presence of iodine, *cis/trans* isomerization of both vitamin D3 and previtamin D3 takes place to form *trans*-vitamin D3 and tachysterol, respectively. Isotachysterol, another isomer of vitamin D3, was formed under acidic conditions and its autoxidation was reported at room temperature after two days.

Vitamin D3 and its isomerization products were extracted and analysed using HPLC-DAD and UHPLC-MS/MS. These compounds were identified by their specific UV spectra which were compared with UV spectra of vitamin D2 and its isomers reported by previous studies. Product ion spectra of vitamin D3 and its isomerization products were also acquired using a liquid chromatography/triple-quadrupole mass spectrometry (UHPLC-QQQ-MS/MS) instrument. Vitamin D3 isomers showed similar product ion spectra and fragmentation patterns to that of vitamin D3, revealing the generation of similar structured products.

Vitamin D3 isomerization products were also identified using a combination of Diels–Alder derivatization using a dienophile and UHPLC-QQQ-MS/MS. Diels–Alder derivatization with PTAD was used to not only increase the ionization efficiency of compounds of interest for mass spectrometry analysis, but also to distinguish between these compounds. While vitamin D3, previtamin D3, *trans*-vitamin D3 and tachysterol showed different reactivity with the dienophile PTAD, isotachysterol did not show any reaction. Moreover, unlike the intact vitamin D3 and its isomers which showed similar mass spectra, PTAD derivatized vitamin D3 gave different mass spectrum pattern compared to its isomers after derivatization. This finding was useful to distinguish and identify PTAD derivatized vitamin D3 isomers such as previtamin D3 in the next section of this study.

2) Lipid oxidation and vitamin D3 degradation in simulated whole milk powders (SWMPs) as influenced by processing and storage.

In this study, the main hypothesis was investigating vitamin D3 degradation in simulated whole milk powder during storage time. Previous studies reported the relationship between cholesterol and lipid oxidation in whole milk powders. They reported the effects of heat treatment, and storage temperature and time on the generation of cholesterol oxidation products (COPs).

In Chapter 4, long-term storage trials conducted to monitor vitamin D3 stability in SWMPs are reported. Since food products are fortified with vitamin D3 at low concentrations in fortification practices, identification of vitamin D3 and its degradation products is a challenge due to their low concentrations in the samples. In addition, vitamin D3 compounds have similar structure to other compounds in milk powder such as steroids, cholesterols and plant sterols which make their identification more challenging. Therefore, in this study, model milk powders with simple matrices were fortified with higher concentration of vitamin D3 compared to what is commonly used in a fortification practice.

The work included investigating the influence of processing and storage conditions on lipid oxidation and vitamin D3 degradation in SWMPs. Samples under two processing conditions, with and without heat treatment, were stored for 12 months at two storage temperatures (20 and 40°C). Primary and secondary lipid oxidation products were monitored in stored samples by analysing

Peroxide Value (PV) and Thiobarbituric Acid Reactive Substances (TBARS), respectively. Lipid oxidation was found to increase in stored SWMPs with higher levels in samples stored at 40ºC than those stored at room temperature (RT, 20ºC). In addition, samples with heat treatment showed the lower levels of lipid oxidation products compared to those without heat treatment, which was attributed to the denaturation of whey proteins and consequently increased levels of free sulfhydryl groups. Similar observations were reported on the lipid oxidation of whole milk powder prepared by heat-treated milk by several authors.

Vitamin D3 content of SWMPs during storage period was analysed using UHPLC-QQQ-MS/MS after PTAD derivatization. A rapid method for vitamin D3 extraction based on the direct saponification of the milk powder followed by the PTAD derivatization of vitamin D3 was carried out. Vitamin D3 content was decreased in SWMPs during the storage period which showed lower levels in samples stored at higher temperature. In addition, the heat treated samples retained higher levels of vitamin D3 content for both samples stored at RT and 40ºC compared to those without heat treatment.

Based on the observation of 12-month storage, an inverse relationship was observed between lipid oxidation products and vitamin D3 content for SWMPs. Significant correlations were found between vitamin D3 decreasing rates and secondary lipid oxidation products quantified by TBARS concentration in all samples. These findings indicated that the degradation of vitamin D3 was associated with the generation of secondary lipid oxidation products. These observations were also consistent with those obtained for cholesterol oxidation products reported in previous studies. In this research, vitamin D3 degradation in SWMPs during storage period was attributed to the oxidation of vitamin D3 mediated by lipid oxidation. The next step was to identify vitamin D3 oxidation products (VDOPs) in the stored SWMPs.

The results reported in Chapter 4 showed that heat treatment prior to drying had an effect on vitamin D3 degradation during storage period, in the milk powders produced. The effects of storage time and temperature of samples on vitamin D3 degradation were also observed. Based on this observation, it can be concluded that pasteurization temperature of milk prior to the fortification can have influence on the stability of vitamin D3 during storage period.

In addition, storage of vitamin D fortified products at low temperatures can result in a decease rate of vitamin D3 degradation.

3) Identification of vitamin D3 oxidation products using high-resolution and tandem mass spectrometry

As mentioned earlier, identification of vitamin D3 degradation products such as VDOPs in natural foods is a challenge due to the low amount of their contents and their possible transformation to other compounds during analysis. Moreover, lack of availability of commercial standards for VDOPs makes their analysis more difficult, especially in complex matrices like milk powders.

In order to identify VDOPs in SWMPs, an extraction method was optimized to separate these compounds form vitamin D3 and the lipid matrices. The extraction was based on a liquid-liquid extraction (LLE) followed by a solid phase extraction (SPE) and derivatization using PTAD. Since saponification was not used in the extraction process, SPE was the important step for enrichment and clean-up procedure. SPE with the aminopropyl cartridge not only allowed the removal of vitamin D3 compounds from lipid matrices, but also affected the separation of vitamin D3 from its oxidation products. While vitamin D3 was eluted in fraction 1 (F1), VDOPs were extracted in fractions 2 and 3 (F2 and F3). VDOPs extracts with and without PTAD derivatization were identified using LC-MS^n techniques.

Chapter 5 focuses on the analysis of VDOPs using LC-MS and identification of their fragmentation rules by incorporating MS data with *in silico* calculated MS fragmentation pathways. With the growth of combinatorial libraries and mass spectral prediction tools, which can overcome the bottleneck of identification of these compounds, mass spectrometry has become an important analytical technique. In this study, Mass Frontier software which generates fragments, produces mass spectral trees and calculates fragmentation pathways was used as the prediction tool.

The identification of VDOPs were carried out using MS^2 and MS^n analysis with a Q-Exactive Orbitrap and a LTQ-ion trap mass spectrometry, respectively. High accurate full scan and MS^2 analysis were conducted to assign the elemental formulas to both the precursor ions and

product ions of each VDOPs which were calculated using the Xcalibur software. The full scan mass and MS^2 spectra of VDOPs in F2 and F3 of samples showed the molecular ions with *m/z* 401, 415 and 417, which represented VDOPs with one and two additional oxygen atoms. After PTAD derivatization, the compounds with *m/z* 576, 590 and 592 were observed which corresponded to the PTAD adducts of VDOPs.

Since MS^2 spectrum may contain fragment ions which were derived from intermediary ions instead of the precursor ion, MS^2 analysis has limitations to explain all product ions. Therefore, multi stage mass spectrometry (MS^n) analysis was applied to gain the maximum structural information and MS^n spectra that could help to propose plausible pathways for unknown compounds and their fragmentations. By incorporating MS data with calculated MS fragmentation pathways, fragmentation trees and MS^n spectral trees were developed, which provided reliable methods to identify unknown VDOPs.

Finally, multiple reaction monitoring (MRM), which is a targeted MS approach on triple quadrupole (QQQ) mass instruments, was used to quantify VDOPs after PTAD derivatization. The MRM transitions of PTAD derivatized VDOPs were selected based on the data obtained from Q-Exactive Orbitrap and ion trap mass spectrometry analysis. As mentioned above, derivatization with a dienophile helped to increase the sensitivity of vitamin D3 and its related compounds by enhancing the ionization efficiency of mass spectrometry. In terms of VDOPs, which have very similar structures to vitamin D3 and follow similar fragmentation pathways, PTAD derivatization also helped to distinguish between these compounds due to different fragmentation patterns after derivatization. High resolution peaks with reproducibility were obtained for VDOPs extracted from SWMPs using UHPC-QQQ-MS/MS. In addition, target MRM transitions, which were obtained from VDOPs identification in SWMPs, were used to quantify these products in stored real whole milk powders provided by Fonterra. These compounds were observed in real whole milk powders which were stored around two years period. Generally, samples with lower vitamin D3 content showed higher levels of total VDOPs concentrations in real whole milk powders.

This project was a successful contribution to food science, as up until now, researchers have known that vitamin D is unstable in milk powders, but the molecular fate of its degradation products were completely unknown. Additionally, the project was very complex in terms of unknown variety of possible outcomes, volume and complexity of data. Identification and quantification of vitamin D3 degradation products in food systems are complex and require extensive studies. Further work in this area is discussed in the next section.

6.2. Future work

In order to elucidate the mechanism of vitamin D3 degradation, preliminary studies were conducted and model samples of milk powders with simple matrices were analysed. In this respect, an immediate aim of the future studies would be the studies on degradation of vitamin D3 in various stressed formulations and different food systems from simple to more complex matrices. For example, in the case of fat containing foods, it would be interesting to investigate whether there is any reactivity between triglycerides and fatty acids with vitamin D3 and/or its degradation products. The identification of esters of vitamin D3 and its degradation products formed by a transesterification with triglycerides would be interesting. To do so, model samples with the mixture of pure vitamin D3 and fatty acids can be prepared to synthesis and purify fatty acid esters of vitamin D3. These compounds can be analysed by LC/MS and NMR with the aim of creating reference data to analyse these compounds in more complex matrices.

For the first time, oxidized vitamin D3 products have been detected in fortified whole milk powders with a time consuming and an expensive extraction method. In order to develop the laboratory routine analysis for VDOPs quantification, more studies are needed to make cheaper and faster methods with a high degree of reliability. In this case, the synthesis of VDOPs would be of interest to generate pure compounds to use as standards for their analysis. To isolate these compounds, the purification method can be improved using multi-step purification with different solid phase extraction cartridges. The pure fractions could be analysed using UHPLC combined with MS and NMR.

It would be also of a great interest to investigate the mechanism of vitamin D3 degradation in the presence of psychrophilic microorganisms. In milk processing, pasteurisation eliminates most of the bacteria, so they normally would not be a serious problem. However, psychrophilic bacteria leave behind heat stable enzymes, including lipases, proteases, esterase that are active in products after pasteurisation and also in dry products. These enzymes continue to be active well into the shelf life causing changes on milk components including vitamin D3 stability. Thus, the initial bacterial population may indirectly dictate the stability and shelf life of vitamin D3 in final products. Residual lipid-active enzymes activity in dried milk powders would also be of great interest in the degradation of lipid and their oxidation. A suggestion is that future studies can establish a correlation between the psychrophilic bacteria and the heat-stable lipid-active enzymes they produce and vitamin D3 degradation.

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Appendices

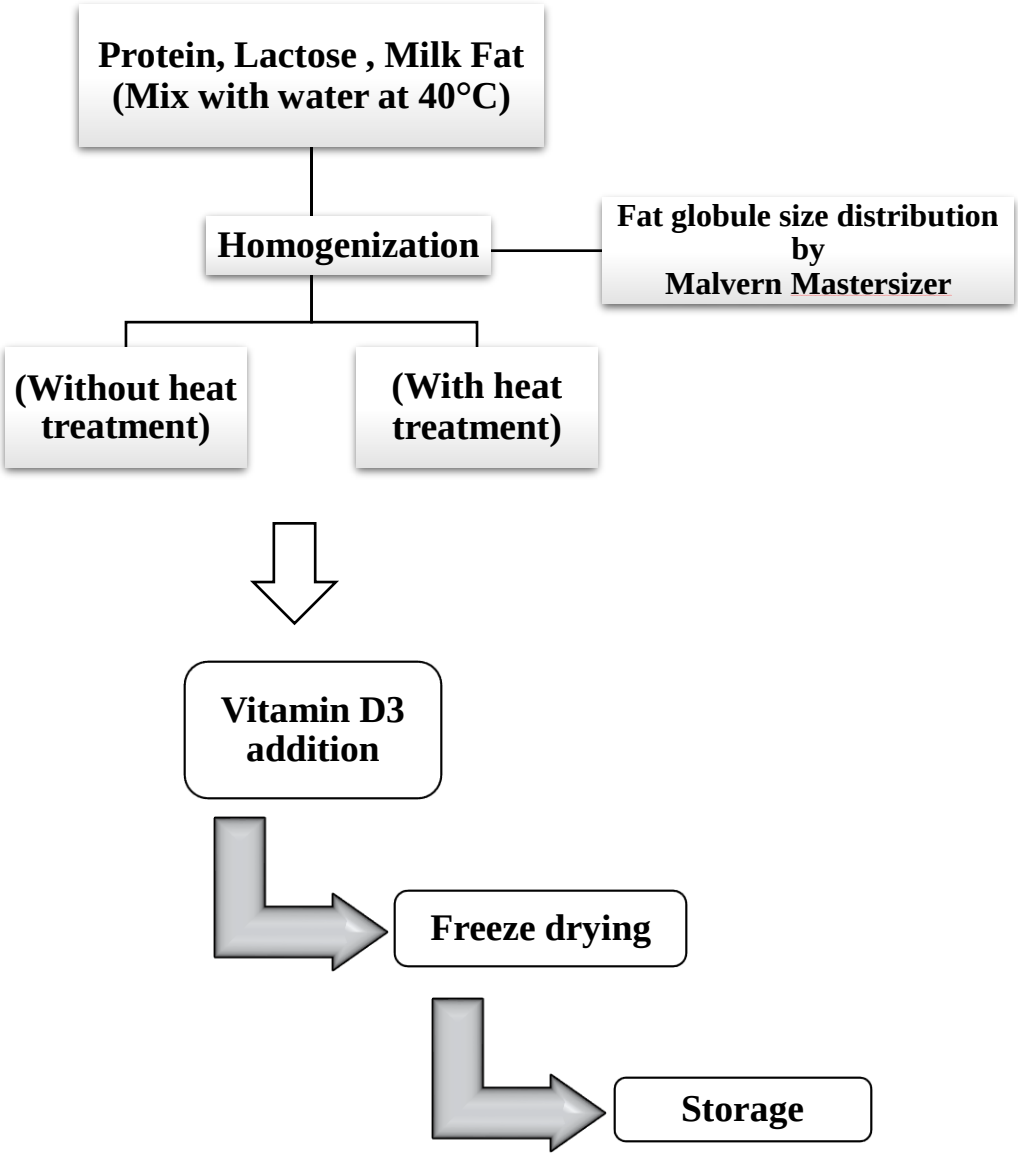
Appendix 4.1. Composition of milk components. (Chapter 4, section 4.2.1)

Chemical composition	**AMF** [a]	**Sodium caseinate**	**WPI** [b]	**Lactose**
Protein (g/100g)	<0.01	92.7	93.9	0.2
Carbohydrate (g/100g)	<0.01	0.2	0.4	99.5
Moisture (g/100g)	<0.1	4.3	4.7	_
Ash	_	3.64	1.5	_
Total fat (g/100g)	99.9	0.7	0.3	0
Fatty Acids (g/100g)				
Saturated fatty acid	66.2	_	_	_
Mono unsaturated fatty acid	20.2	_	_	_
Ploy unsaturated fatty acid	1.3	_	_	_

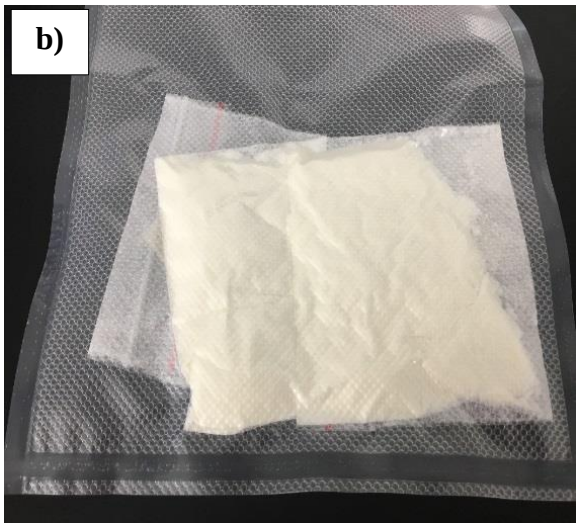
[a] Anhydrous Milk Fat

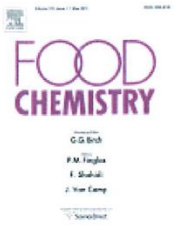
[b] Whey Protein Isolate

Appendix 4.2. Simulated whole milk powder (SWMP) preparation procedure (Chapter 4, section 4.2.2)



Appendix 4.3. Final freeze-dried simulated whole milk powder (SWMP) products (Chapter 4, section 4.2.2)





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