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# Development of a new formulation for a Vitamin D3 spray

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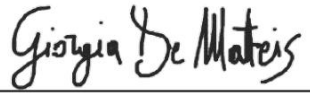

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# Acknowledgments

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In particular, I thank Prof. Zimmer, whose door was always open whenever I ran into a trouble spot or had a question about my research.

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Finally, I must thank Emiliano, because he supported me, inspired me and encouraged me continuously, never giving up on me. My profound gratitude to my parents for giving me the chance to follow my way.

Thank you.

# Abstract

Vitamin D3 is important for many human physiological functions and the currently marketing challenge aims to provide dosage forms, which are more tasty and manageable. This idea inspired the research for the master's thesis, which focuses on the development of a new formulation for vitamin D3 spray.

The dissolving medium, represented by a medium chain triglyceride oil, guarantees better solubility of the lipophilic vitamin as well as a successful oral absorption. Further an emulsifier reduces the surface tension in the buccal cavity, hence promoting the permeation of vitamin D3 through the membrane cells and attenuating the oil feeling in the mouth. The originality of this formulation is also related to the chosen orange flavouring agent, which confers a good taste.

The principal issue of this master thesis was the stability assessment of vitamin D3, due to possible decomposition caused by an incompatibility with excipients. To this purpose, HPLC tests were performed over six months to monitor the content of the API and register the potential formation of degradation products. Melting and crystallisation behaviour were investigated by differential scanning calorimetry, which was additionally used to analyse the oxidation kinetics. Further on the tested low viscosity ensured the functionality of the spray pump, while the measurement of density and refractive index provided the qualitative identification of the excipients of the formulation. Moreover, the atomization ability of the spray was assessed, by measuring the size distribution of sprayed drops.

Finally a predictive mathematical model was constructed by means of the software MODDE, in order to optimise the operating procedures during the manufacturing process.

# Kurzfassung

Vitamin D3 ist für viele physiologische Funktionen des Menschen wichtig. Die derzeitige Marketingherausforderung zielt darauf ab Dosierungsformen zu entwickeln, welche geschmacklich genießbarer und leicht dosierbar sind.

Diese Masterarbeit basiert auf dieser Herausforderung, wobei der Fokus auf der Entwicklung einer neuen Formulierung für ein Vitamin D3 Spray liegt. Das Trägermedium, ein Öl an mittelkettigen Triglyceriden, garantiert eine gute Löslichkeit des lipophilen Vitamins sowie eine orale Absorption. Der Zusatz eines Emulgators reduziert die Oberflächenspannung in der Mundhöhle und unterstützt somit die Permeation von Vitamin D3 durch die Zellenmembranen und vermindert zusätzlich das ölige Gefühl im Mund. Weiters verbessert ein Orangenaroma den Geschmack.

Die größte Herausforderung stellt die Stabilität des Vitamins D3 dar. Aus diesem Grund wurden über sechs Monate Gehaltsbestimmungen durch HPLC durchgeführt, um den Wirkstoffgehalt zu überwachen und die mögliche Zersetzung aufgrund von Inkompatibilitäten sowie Oxidation zu untersuchen. Sowohl das Schmelz- und Kristallisationsverhalten als auch die Oxidationskinetik wurden durch Differential-Scanning-Kalorimetrie analysiert.

Durch Prüfung der Viskosität der Spraylösung wurde die Funktionalität der Sprühpumpe geprüft, während die Messung der Dichte und des Brechungsindexes die qualitative Identifizierung der Hilfsstoffe der Formulierung erlaubt. Zusätzlich wurde die Zerstäubungsfähigkeit des Sprays bewertet, indem die Größenverteilung der versprühten Tropfen gemessen wurde.

Abschließen wurde im Rahmen der Arbeit ein prädiktives mathematisches Modell mit Hilfe der Software MODDE erarbeitet, um die Betriebsabläufe während des Herstellungsprozesses zu optimieren.

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# 1 Introduction

## 1.1 Aim of the thesis

The aim of this master's thesis was the development of a vitamin $D_3$ oil spray to be used for the treatment of subjects suffering from diseases or disorders associated with vitamin $D_3$ deficiency.

Vitamin D is known as a nutrient, which can promote the optimal intestinal absorption of calcium and thus a proper mineralisation of the skeleton, as well as the mobilisation of calcium from bone tissue when required [1].

A deficiency in vitamin D can mainly lead to debilitating bone diseases (e.g. *rickets*, *osteomalacia*), in adults, elders and kids. However, recently, it has been discovered that vitamin D can also act in other physiological functions, where the most relevant are the immune function stimulation, prevention of cancer and cardiovascular diseases.

Given the importance of vitamin D for health, the interest in the optimisation of supplementation strategies has increased, in order to contrast the diffusion of diseases related to vitamin D deficiency, which especially affects industrialised countries [1].

Up to now, formulations based on vitamin $D_3$ in the form of oily drops, have been considered more suitable as dosage forms than the solid ones, e.g. tablets, soft or hard capsules. However, the taste and the handiness of administration can make oily drops rather hateful, because of the oil feeling in the mouth and the absence of appetising taste.

In addition to this, there is often the difficulty to keep adherence to the therapy, for possible mistakes during the release of drops in the buccal cavity, which compromise the constant daily intake of the drug.

As aspired by the company Fresenius Kabi Austria GmbH in Linz, this master thesis focused on the overcoming of the mentioned issues by developing an oily spray formulation with a suitable flavouring agent and emulsifier, able to allow a better palatability of the dosage form.

In the course of the thesis work, the formulation was defined as concerns the type of excipients to be employed and the relative concentrations. Afterwards, HPLC analysis was conducted for a period of six months, in order to determine the content of vitamin $D_3$ in the formulation during the storage time.

Considering the sensitivity of vitamin $D_3$ to light, temperatures and oxygen, the formulation was stressed at high temperatures under oxygen and sunlight action and then examined by HPLC, to evaluate and estimate the stability at extreme conditions, which can eventually occur during administration phase and the steps of the manufacturing process.

Melting and crystallisation behaviour were investigated by means of the differential scanning calorimetry (DSC) with the purpose to understand the physical behaviour of lipophilic excipients, flavouring agent and whole formulation. Moreover, DSC oxidation stability tests were additionally performed, with the purpose to investigate the oxidation kinetics, involving the developed spray, and compare it to Oleovit™, an already marketed oily vitamin $D_3$ reference solution.

Since the viscosity is considered a relevant factor to guarantee the spray uniformity and the good functionality of the spray pump, the rheology behaviour of the spray was tested. The formulation was then characterised according to its qualitative attributes, by executing density and refractive index measurements.

Furthermore, firstly the surface tension analysis and secondly the drop size analysis of the sprayed oil was useful in assessing the atomization ability of the spray, correlated to the percentage of drops, which manages to reach the respiratory system thus reducing the amount absorbed by the buccal cavity and the therapeutic effect.

Finally, the data resulting from the stability and stress tests were collected for the development of a mathematical model, constructed by means of software MODDE, which allows predicting the variability of vitamin $D_3$ amount by changing the input parameters, regarding the raw materials as well as the process.

## 1.2 Calcium homeostasis

Calcium is mostly known as principal mineral involved in the bone mineralisation. However, it is also necessary for the proper functioning of intracellular and extracellular processes, including muscle contraction, nerve impulse conduction, membrane and cytoskeletal functions, hormone release, regulation of several enzymes and blood coagulation [2]. The homeostasis of calcium has to be controlled and maintained in order to detect diseases related to abnormalities of serum concentration of calcium and regulating organ systems, as parathyroid gland, skeleton, kidney, gastro-intestinal (GI) tract, skin [2].

The calcium in the body exists in two principal forms:

- as *inorganic salt* (e.g. calcium carbonate) together with the phosphate, it contributes to support bones and teeth, creates the mineral reserves in the bones then available for the body [3];
- as circulating *ionic form*, through which it helps during the blood clotting process, hormones secretion, muscle contraction and neuronal activation mechanism, enzymatic reactions as "cofactor" [3].

The total content of adult organism calcium is about 1kg [2], and its distribution is shown in the table below.

| **Calcium distribution in the body** | |
|---|---|
| 99% | In the bones and teeth in the form of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ and calcium carbonate ($CaCO_3$) [2] |
| 1% | Blood calcium (total plasma calcium = 10mgs/100mls) [2]:<br>• inactive and non-diffusible (3.5 mgs) bound to proteins (For albumin 2.8 mgs and globulin 0.7mgs) [2];<br>• active and diffusible (6.5 mgs): ionized $Ca^{2+}$ (5.3 mgs); complexed (1.2 mgs) to phosphate, bicarbonate, citrate [2] |
| | Intracellular calcium ( the endoplasmic reticulum and mitochondria) [2] |

Table 1: Overview of calcium distribution in the human body

Together with the calcium, also other minerals such as phosphate (P) and magnesium (Mg), contribute to the normal regulation of bone mineral metabolism [2].
The principal organs involved in calcium homeostasis, by continuous fluxes into and out from bloodstream when necessary, are the following:

- the *small intestine* is the absorption site of dietary calcium, which is linked by means of binding protein expressed on epithelial cells; after being absorbed, calcium is transported into the extracellular fluid (ECF), where it is partially retained and from here it passes both in the bone tissue(from where it is daily reabsorbed) and in the kidneys [4];

- the *bones* which represent a reservoir of calcium. The turnover of calcium in the skeleton is about 250 mg/day and it is performed by bone-forming cells, the osteoblasts and bone-resorbing osteoclasts [2]. The net *resorption* of bone mineral promotes the release of calcium and phosphate into blood and the suppression of this effect causes the deposition of calcium in the bones [4];

- the *kidneys* whose role is crucial for calcium homeostasis. At normal blood calcium concentrations, most of the calcium entering the glomerular filtrate, passes from the tubular system into blood, to preserve blood calcium levels. A reduction of tubular *reabsorption* of calcium determines its lost [4].

The bone mineral metabolism is regulated through mutual actions of three hormones: parathyroid hormone (PTH, Paragraph 1.2.1), calcitonin (CT, Paragraph 1.2.2) and fibroblast growth factor 23 (FGF23, Paragraph 1.2.3) [2].
They exert their action on the bone, kidneys, and GI tract, in order to regulate the distribution of minerals such as calcium, magnesium, and phosphorus.
Moreover, there is a hormone-like molecule, the vitamin D (VD, Paragraph 1.2.4), either synthesised by the skin or introduced into the diet, which, by acting on the intestinal absorption, helps to preserve the calcium levels in the blood [2].

### 1.2.1 Role of PTH

Parathyroid hormone (PTH) is a peptide composed of 84-amino-acids, released by parathyroid glands which are behind the thyroid gland in the neck [2] (Figure 1).

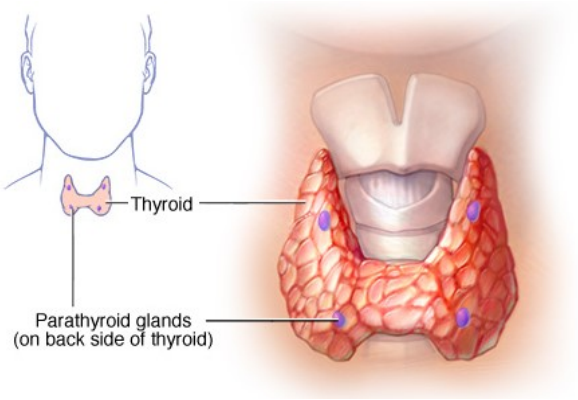


Figure 1: Anatomical position of parathyroid glands [5]

The serum concentration of calcium has an inverse effect on PTH secretion, which occurs to protect against hypocalcaemia [6]. The *cell-surface calcium-sensing receptor (CaSR)* on parathyroid glands detects the extracellular concentration of ionized calcium [2, 7].

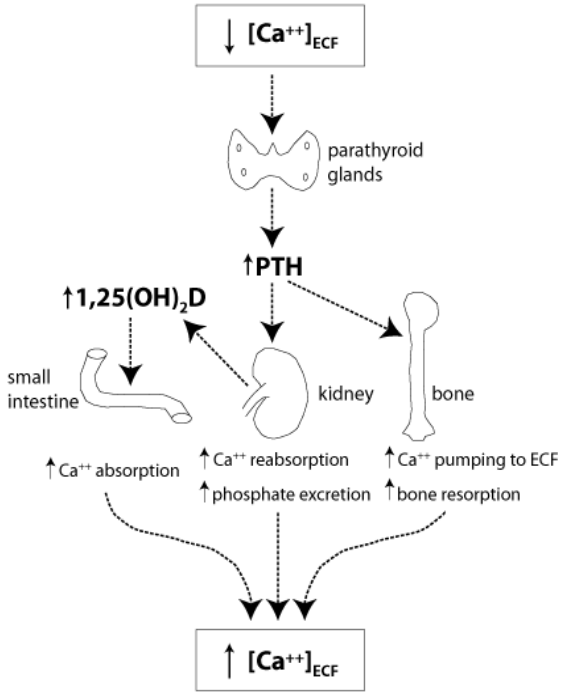


Figure 2: Effects of PTH [8]

As it is shown in Figure 2, the reduction of calcium concentration in the ECF promotes the release of PTH, which contributes to increasing the blood levels of calcium [8].
The parathyroid hormone acts on the bones, by inducing calcium release from bones to ECF (e.g. plasma) through the activation of osteoblasts, which perform bone resorption and consequently determine the differentiation and activation of osteoclasts [9]. At tubular level in the kidney, the PTH stimulates calcium reabsorption (from kidney to the blood) [10]. Moreover, the PTH determines indirectly the absorption increase of calcium from the diet by favouring the renal secretion of *calcitriol* (1, 25-dihydroxyvitamin D), principal active form of vitamin $D_3$ [11]. However, the increase of calcium in the ECF inhibits PTH secretion [12].

### 1.2.2 Role of calcitonin

Calcitonin (CT) is a peptide composed of 32-amino acids, secreted by para-follicular C cells of the thyroid (Figure 3) [2]. It is the hormone which opposes the action of PTH. The secretion of CT increases due to hypercalcemia, while hypocalcaemia causes inhibition [13].

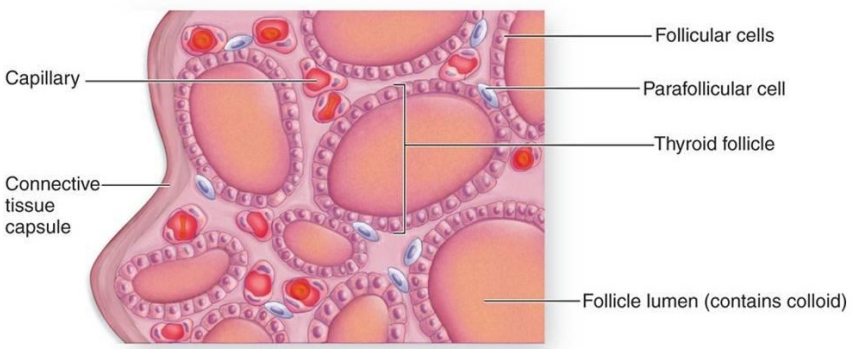


Figure 3: Anatomical location of para-follicular cells,
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As well as for the PTH, CT secretion is regulated by the *CaSR* which in this case responds to high calcium concentration in ECF [2, 14].

| Calcitonin functions | |
|---|---|
| **Target organs** | **Effects** |
| Osteoclasts in the bone tissue | Inhibition of bone resorption [2] |
| Kidney | • Increase of calcium and phosphorus excretion [2]<br>• Calciuresis : hypocalcemic effect (decrease of calcium blood levels) [2] |

Table 2: Summary of calcitonin effects on calcium metabolism

As shown in Table 2, the CT performs its function acting on bones and kidneys. CT can inhibit bone resorption by activation of the CT-receptor expressed on osteoclasts, where it causes contraction thus preventing osteoclasts motility [14].
CT also promotes renal phosphate excretion and induces sodium excretion and calcium excretion, determining its hypocalcaemic effect [2, 14].

### 1.2.3 Role of FGF23

The fibroblast growth factor 23 (FGF23) is a protein of 32-kD (251 amino acids), mostly produced by osteoblasts and osteocytes in bones [15].
The FGF23 participates indirectly to calcium homeostasis. In fact, it decreases levels of 1, 25(OH) $_2$ D, promoter for intestinal calcium absorption, probably by reducing the expression of the 1α-hydroxylase enzyme and increasing the production of the 24-hydroxylase enzyme, responsible for the release of inactive metabolites from the $1,25(OH)_2D$ [2, 16].

### 1.2.4 Role of vitamin D

Vitamin D is the general name given to a group of fat-soluble compounds that are essential to maintain the calcium balance in the body [17].

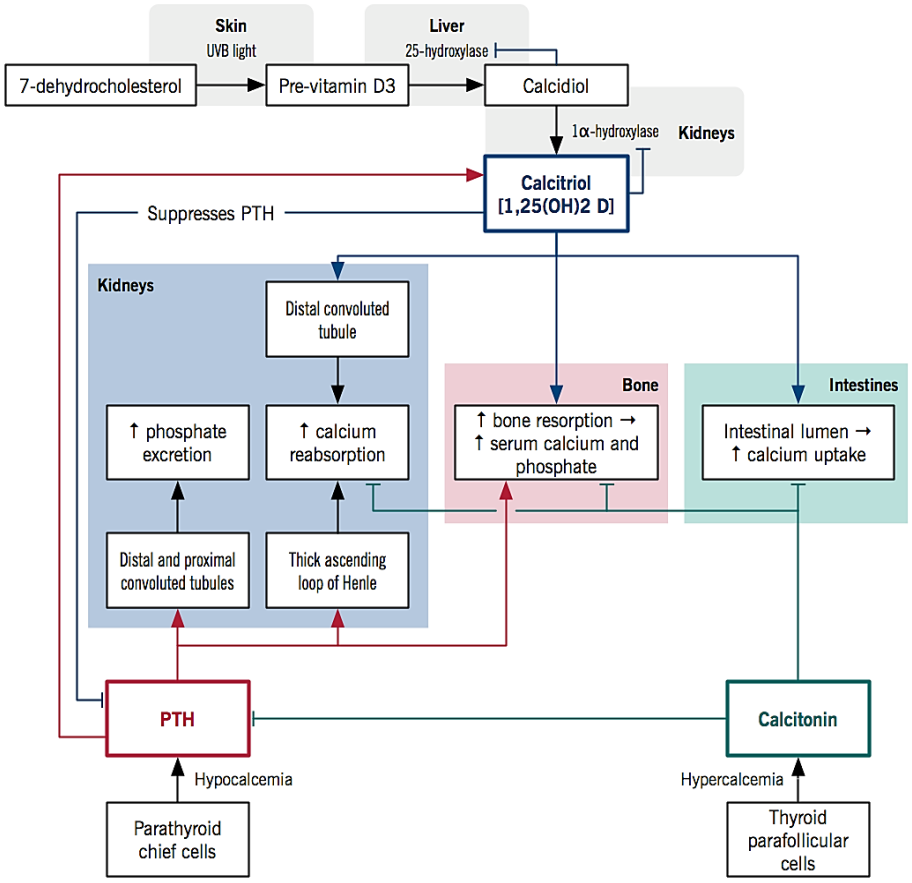



Figure 4: Summary of the involved regulation mechanisms of calcium homeostasis [18]

As shown in Figure 4, vitamin D and in particular its renal metabolite, the *calcitriol* (1,25-$(OH)_2$ D) has an hypercalcaemic effect, which is exerted through the following mechanisms:

1. stimulation of calcium uptake from the intestinal lumen [11];
2. promotion of calcium reabsorption in the distal tubules of kidney [10];
3. induction of osteoblasts in bone cells to express the RANKL (Receptor activator of nuclear factor kappa-B ligand) on their surface, through which they can bind the RANK receptor on the osteoclasts, contributing to their formation, activation and differentiation [19]. They are responsible for bone resorption, which enhances serum calcium levels.

Vitamin D production is suppressed by PTH (red arrow, Figure 4), whose release can occur in response to high levels of the hypercalcaemic calcitonin hormone (green arrow, Figure 4).

## 1.3 Chemistry behind the vitamin D

The main forms of vitamin D are vitamin $D_2$ (*ergocalciferol*: found in plants, yeasts and fungi) and vitamin $D_3$ (*cholecalciferol*: of animal origin) [20].

The vitamin $D_3$ can be either originated by the skin through sunlight action or introduced with the diet, while vitamin $D_2$ is exclusively a dietary form of plant origin [21].

They both have a general chemical structure characterised by the rings A, B, C, and D with different substituents [22].

The ring structure comes from the cyclopentano-perhydrophenanthrene, characteristic for steroids, but the typical classification of vitamin D molecules is that of *secosteroids* [22].

As shown in Figure 5, in secosteroids, one of the rings is broken and in particular in vitamin D structure, the ring B is broken, between 9,10 carbons [22].

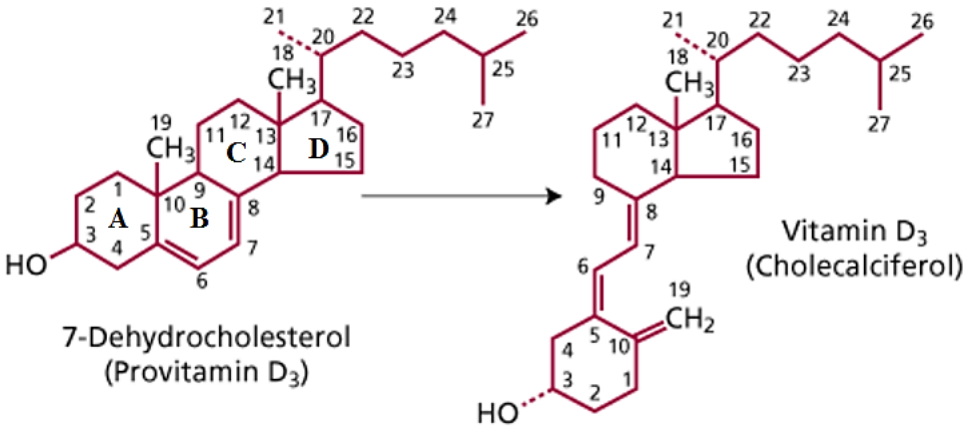



Figure 5: Chemical structures of 7-Dehydrocholesterol (*steroid*) and vitamin $D_3$ (*secosteroid*) [23]

Because of their origin, vitamin $D_3$ (*cholecalciferol*) and $D_2$ (*ergocalciferol*) are synthesised in two different ways.

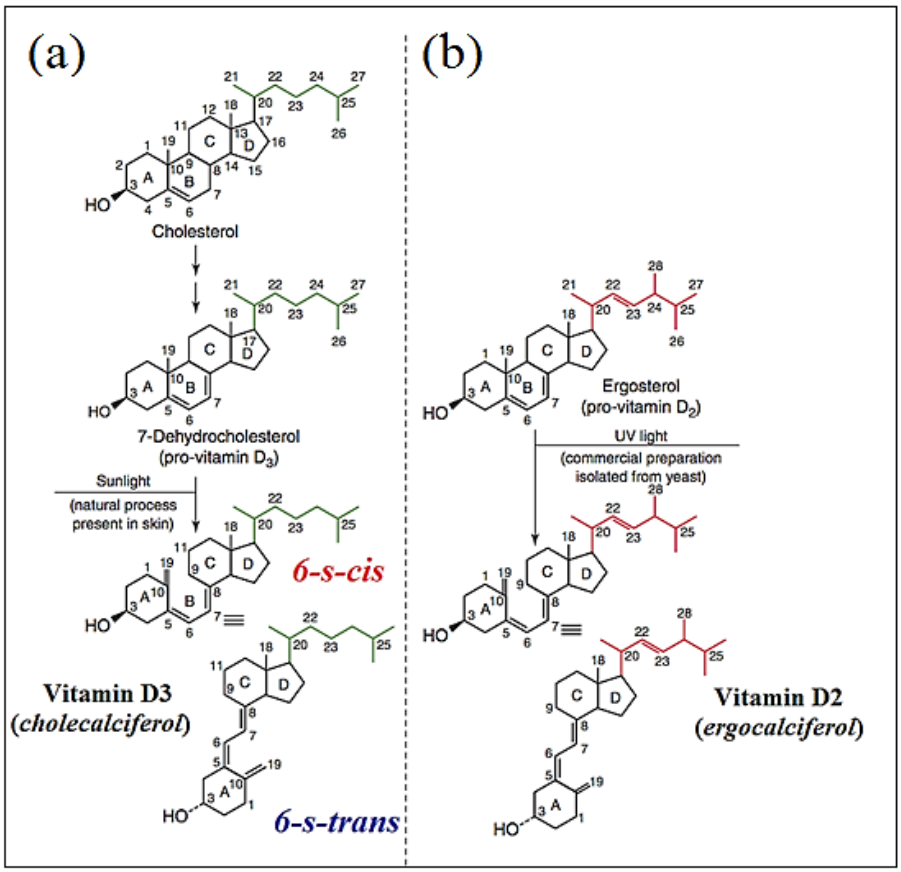



Figure 6: Synthesis of vitamin $D_3$ (a) and $D_2$ (b) [22]

As shown in Figure 6(a), vitamin $D_3$ derives from the photochemical reaction of sterol 7-dehydrocholesterol (pro-vitamin $D_3$, *cholesterol-like molecule*) present in the skin with the sunlight. Otherwise, it can be provided pre-formed by the diet through food (eggs, meat, fish oil) or supplements [24].

Figure 6(b) describes how the commercially produced form of vitamin D, the vitamin $D_2$, can be obtained from the molecule of ergosterol (pro-vitamin $D_2$), contained in yeasts, plants and fungi, under UV light action, as the UVB radiation with wavelength between 290 and 315 nm [20].

The starting point of vitamin $D_3$ synthesis is the irradiation of pro-vitamin $D_3$, whose 5,7-conjugated double bonds (diene, Figure 6a), allows the absorption of sunlight [22].

The principal product is pre-vitamin $D_3$, which undergoes then a 1, 7-sigmatropic hydrogen transfer from C-19 to C-9, yielding the final vitamin $D_3$ (Figure 7).

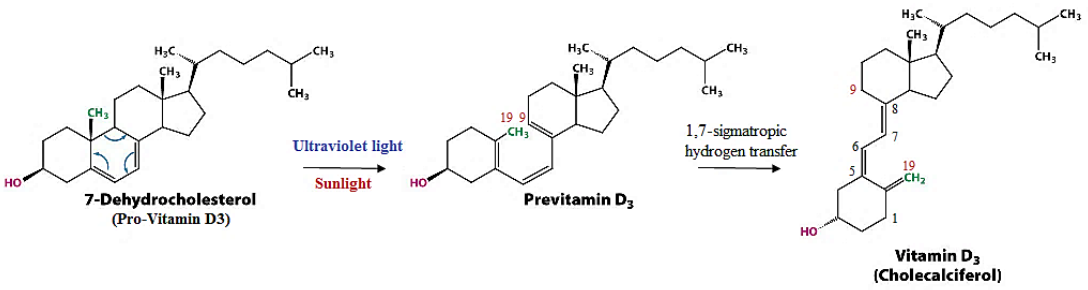



Figure 7: Detail of the vitamin $D_3$ synthesis with the sigmatropic hydrogen transfer [25]

Depending on the rotation around the 6, 7-single bond, vitamin $D_3$ can be drawn in either a 6-s-*trans* or as 6-s-*cis* (Figure 6a) form [22, 26].

In addition to vitamin $D_3$, the activated pre-vitamin $D_3$ can provide other distinct products (Figure 8), whose synthesis prevents to reach toxic levels of vitamin $D_3$ in the skin during sun exposure [22].

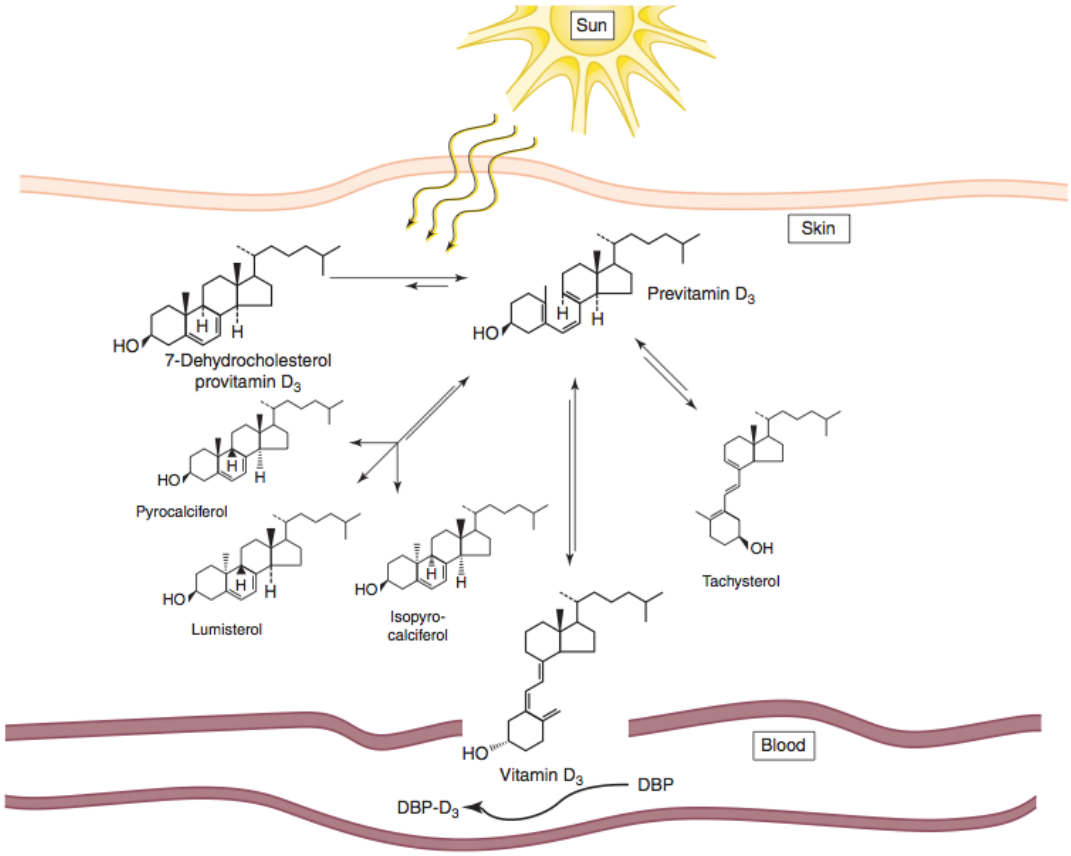



Figure 8: Photochemical pathway of production of vitamin $D_3$ (cholecalciferol) from 7-dehydrocholesterol [22]

As described in Figure 8, the resulting hydrophobic vitamin $D_3$, which is formed in the skin, is removed by binding to the plasma transport protein, the *vitamin D-binding protein* (DBP), present in the capillary bed of the dermis [17, 22]. The DBP- $D_3$ then enters the circulatory system [23].

Both vitamin $D_3$ and vitamin $D_2$ are synthesised commercially and found in dietary supplements or fortified foods [17].

The $D_2$ and $D_3$ forms have just different substituents, but it has no effect on metabolism. In fact, the activated forms of vitamin $D_2$ and $D_3$ undergoes the same physiological mechanism in the body, therefore they can be both used in the treatment of vitamin D deficiency [17].

## 1.4 Metabolism of vitamin D

### 1.4.1 Activation of the vitamin D hormonal form

The pro-hormonal inactive form of vitamin D, both the one originated from the skin and the one coming from dietary intake, must undergo a metabolising process in order to be transformed into the active hormonal form [17].

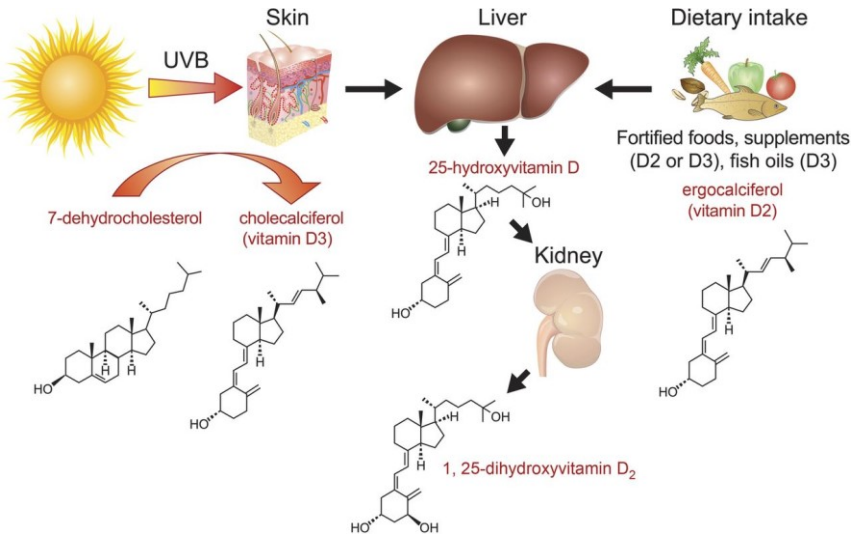



Figure 9: General description of the biochemistry of vitamin D [27]

Therefore, as already described in the previous chapter, once vitamin $D_2$ or $D_3$ enters the systemic circulation, it is transported by the DBP first to the liver and then to the kidney, where active biological substances (e.g. 1, $25(OH)_2D$ ) are released (Figure 9).

Vitamin $D_2$ and $D_3$ are transported as hormones through the plasma by the *vitamin D binding protein* (*VDBP*), a specific α-globulin, which allows them to reach the vitamin D receptor (VDR) on target organs, thus inducing the physiological response associated with the vitamin D [28, 29].

Both vitamin $D_2$ and vitamin $D_3$ vitamin D goes through two successive hydroxylation steps [27]. The following description refers to vitamin $D_3$, but the same occurs for vitamin $D_2$.

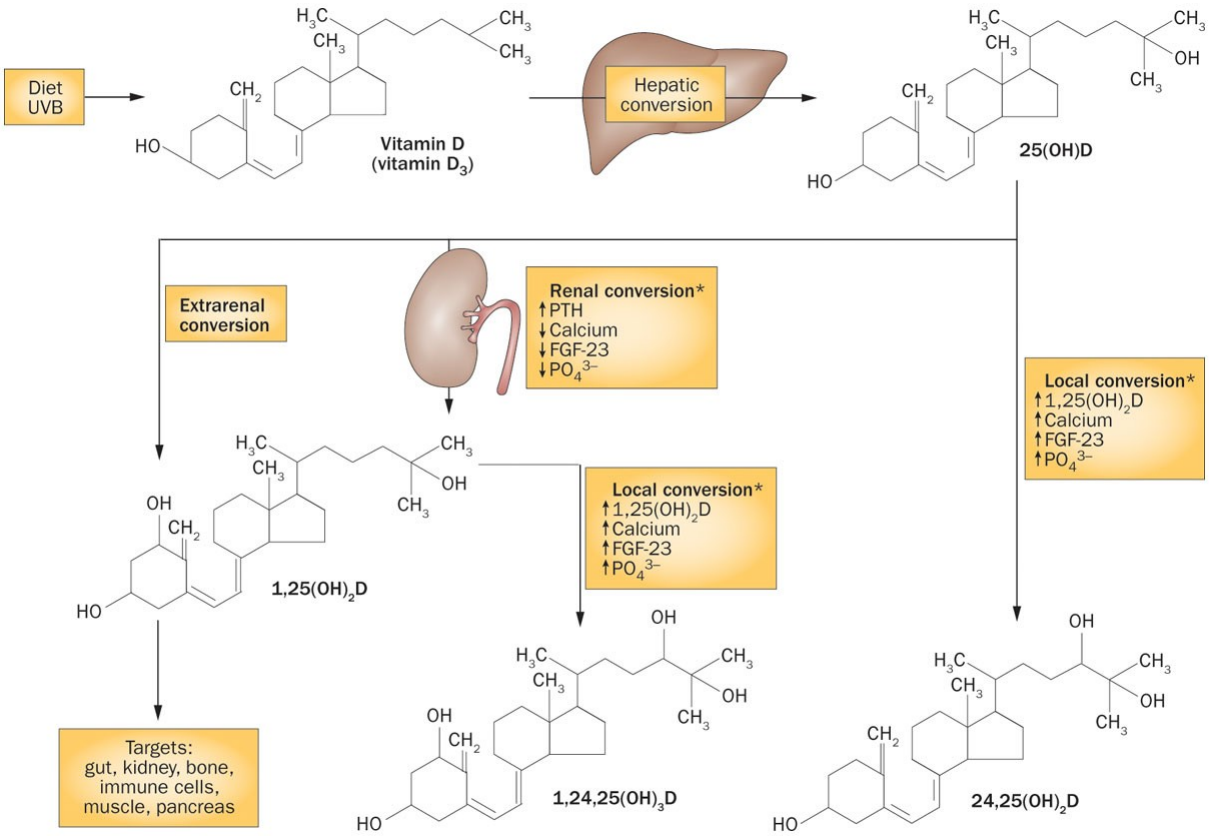



Figure 10: Overview of the hydrolysis steps of vitamin $D_3$ and resulting metabolites [30]

As it can be seen in Figure 10, the first hydroxylation occurs in the liver by a microsomal enzyme, the *vitamin D-25-hydroxylase* (CYP2R1), which can metabolise both vitamin $D_2$ and vitamin $D_3$ [17].

The enzymatic reaction yields the 25-hydroxyvitamin $D_3$ (*calcidiol*), which is the dominant circulating form of vitamin $D_3$ in blood compartment, where it is bound to the VDBP [17].

The second hydroxylation step occurs in the kidney by the enzyme *25-OH-D-1-α-hydroxylase* (CYP27B1), a tightly regulated enzyme found in the mitochondria of the proximal tubules [29]. This enzyme metabolises the 25-OH-$D_3$ into the principal form of vitamin D, the 1α, 25$(OH)_2$ $D_3$ (the hormonal form, *calcitriol*) [29]. As indicated by Figure 10, the renal conversion is stimulated by high levels of PTH and low levels of serum calcium, FGF23 and phosphate ($PO_4^{3-}$).

There is another hydroxylation step performed by *25-(OH) $D_3$-24-hydroxylase* (CYP24A1) which involves the 25-(OH) $D_3$ and 1,25 $(OH)_2D_3$.

Their conversion determines the release of the 1,24,25 $(OH)_3$ $D_3$, which exerts biological activity through the VDR and the 24, 25$(OH)_2$ $D_3$, considered by some studies a degradation product with no relevant biological effects, while according to others, it is important in chondrogenesis and bone formation [2]. An increased release of the 24-hydrolyzed metabolites, it is due usually to high levels of 1,25$(OH)_2D_3$, serum calcium, FGF 23 and $PO_4^{3-}$ [2].

The hormonal regulation of the biologically-active vitamin $D_3$ metabolite, the 1,25-$(OH)_2D_3$, is described in more details in the following paragraph.

### 1.4.2 Vitamin D endocrine system

The principal regulation centre of calcium and phosphate homeostasis of the vitamin D endocrine system is represented by the kidney. In fact, by controlling the activity of the renal 1-hydroxylase, the production of the hormone 1, 25$(OH)_2$ $D_3$ can be changed according to the calcium and other endocrine signals of the organism [31].

Figure 11 describes the principal regulatory factors, which are 1, 25$(OH)_2$ $D_3$ itself, parathyroid hormone (PTH), the serum concentrations of calcium, phosphate ($PO_4^{3-}$) and FGF 23. Referring to the chapter calcium homeostasis, where the main effects of the regulatory hormones have been described, Figure 11 gives an overview of their influence on the vitamin D activity.

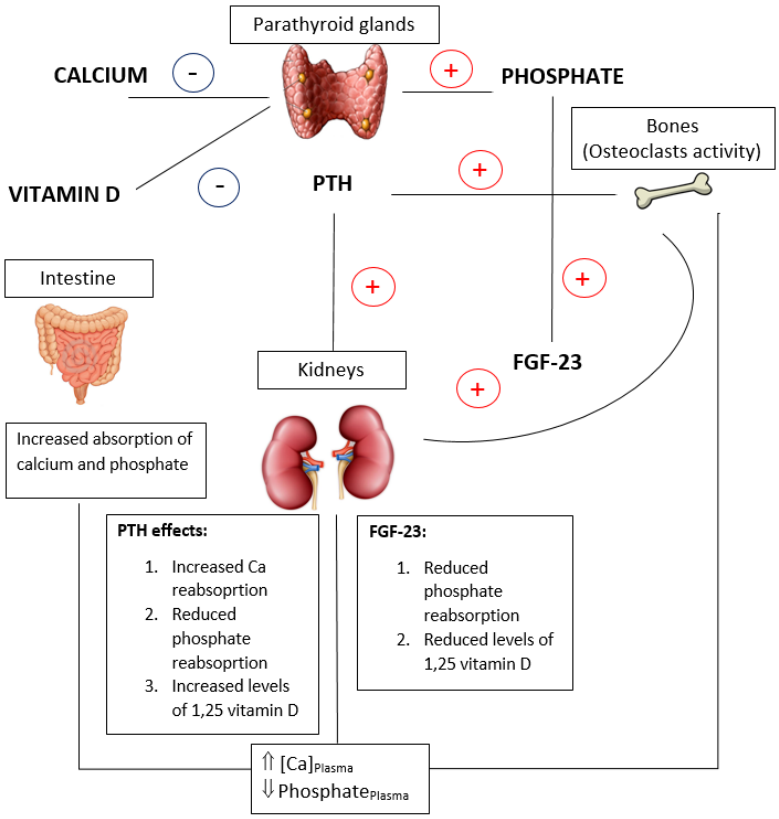



Figure 11: Regulation mechanisms activated by low blood calcium levels [32]

The renal activity of the 25-OH-D-1-α-hydroxylase is stimulated by PTH (Paragraph 1.2.1), whose secretion by the parathyroid glands is promoted by the increased renal phosphate excretion due to the FGF23 (Paragraph 1.2.3).

As a consequence, calcitriol (1, 25$(OH)_2D_3$) production increases and hence high blood calcium levels are achieved (Figure 11) by promoting absorption of dietary calcium from the gastrointestinal tract, increasing renal tubular calcium reabsorption and stimulating the release of calcium from bone [10, 33].

Once the necessary levels of calcium have been reached, serum calcitriol levels decrease both because PTH activity is suppressed through a negative feedback and because calcitriol can destroy itself by different mechanisms comprising [29]:

- inhibition of the activation PTH-mediated of *25-OH-D-1-α-hydroxylase*;
- direct transcriptional repression of the CYP27B1 gene;
- FGF23 and CYP24A1 induction.

Furthermore, by stimulating the enzyme CYP24A1 (*25-(OH)$_2$D$_3$-24-hydroxylase*), calcitriol is degraded to water-soluble and less active metabolites (Figure 10) [34]. Even the phosphate blood levels affect the calcitriol release as follows:

- high phosphate levels activate the FGF23, able to inhibit CYP27B1 (enzyme for calcitriol synthesis) [6, 17];
- a deficiency of serum phosphate stimulates CYP27B1 to produce more calcitriol, which induces the absorption of phosphate in the small intestine[6, 17].

## 1.5 Physiological mechanisms activated by vitamin D

The dihydroxylated and hydrophobic metabolites (Figure 11) such as 1,25(OH)$_2$D$_3$, 1,24,25 (OH)$_3$ D$_3$ and 24,25(OH)$_2$D$_3$ are systematically transported to distal target organs by the plasma *vitamin D binding protein* (VDBP) [17].

The free form of 1,25(OH)$_2$D$_3$ passes through the plasma membrane and links to a specific nuclear receptor known as the *vitamin D receptor* (VDR), a DNA-binding, zinc-finger protein [28, 35]. The VDR occurs in more than 35 different cell types [22], therefore even the effects, resulting from its activation, interest many target organs (Paragraph 1.5.2).

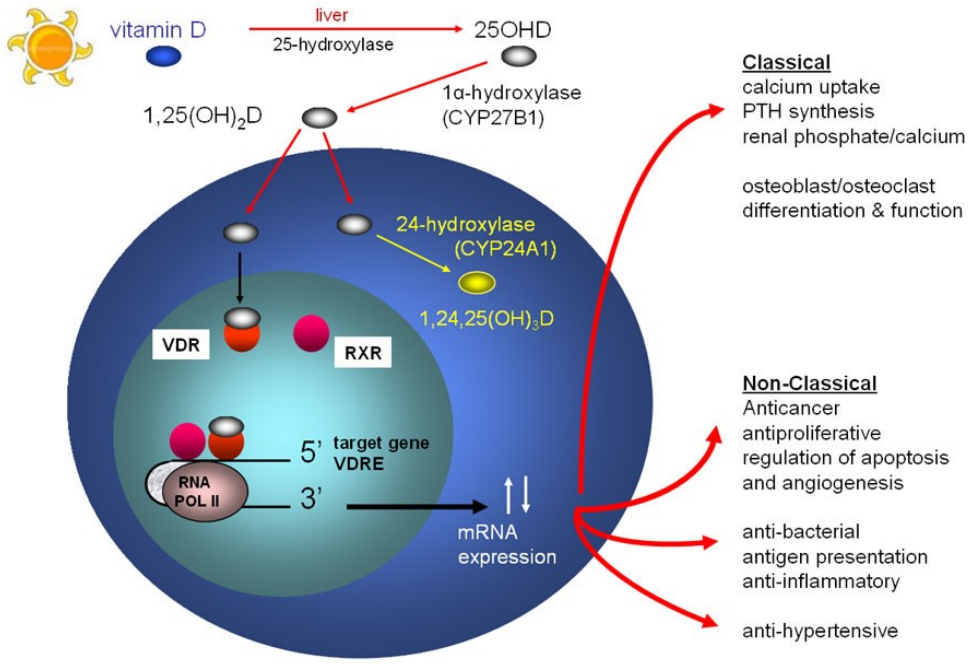



Figure 12: Activation of the VDR and relative physiological responses [36]

The molecular effects of the ligand-VDR complex arise from its dimerization with the retinoid X receptor (RXR) that contributes to transforming the complex into a transcription factor (Figure 12) [35]. This binds to specific promoter regions, the *vitamin D response elements* (VDREs), which act on the expression of ≈ 3% of the 22.000 genes of the human genome, thus enhancing the transcription of mRNAs necessary for the codification of proteins involved in the transport of calcium, in the building of bone matrix, or in the regulation of cell-cycle [22, 29, 37].

As a result of these processes,1,25-$(OH)_2$-$D_3$ stimulates intestinal absorption of dietary calcium (Paragraph 1.4.1) and increases renal tubular reabsorption of calcium, thus reducing the loss of calcium in the urine. It also acts to a lesser extent than the PTH by mobilising phosphate and calcium, promoting their release from the bone tissue [2].

These functions help to re-establish the blood levels of calcium and phosphate, when concentrations are low.

However, there is a negative feedback mechanism which avoids the excessive bone resorption and hence its embrittlement, when the levels of serum calcium are optimal.

In fact, the bioavailability of calcitriol is firmly regulated, as already written in Paragraph 1.4.2, to restrict its biological action in target cells, while keeping calcium and phosphate homeostasis.

### 1.5.1 Intestinal calcium absorption promoted by the vitamin D

The epithelium of kidney, intestine, placenta, mammary glands, and gills are typical sites of calcium absorption [38]. Factors which have influence on the movement of calcium across epithelia are the extracellular calcium concentration, pH and different hormones [39].

Calcium can be absorbed by active transport (*transcellularly*) and by passive diffusion (*paracellularly*) across the intestinal mucosa [39, 40].

As it is shown in Figure 13, active transport of calcium relies on the action of calcitriol, which binds to the intestinal VDR, increasing in the duodenum the expression of calcium channels in the epithelial cells (*calbindins*) and the extrusion systems, represented by calcium transport proteins named *transient receptor potential cation channel, vanilloid family member 6 or TRPV6* [39].

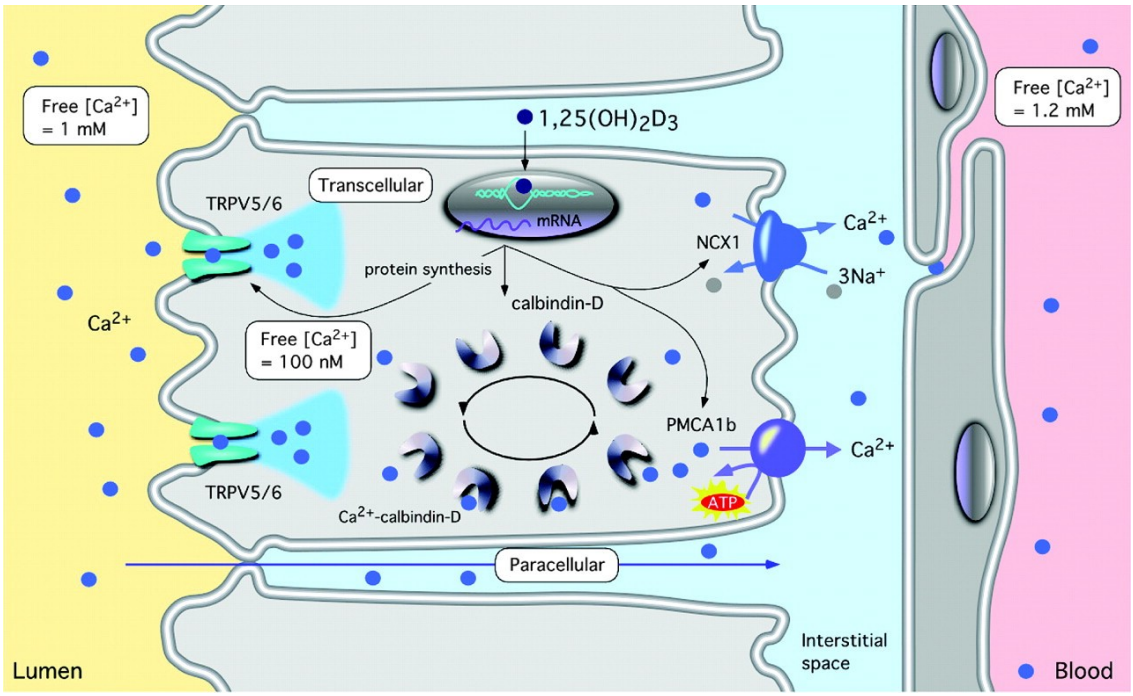



Figure 13: Intestinal calcium absorption mediated by the 1,25 $(OH)_2D_3$ [39]

The calcium transport through an active and transcellular way is a three-step process [41]. Calcium enters through the tetrameric epithelial calcium channels (TRPV5 and TRPV6), binds to *calbindin* (calcium binding protein) and diffuses through the basolateral membrane of epithelial cells, from where it is discharged by means of an ATP-dependent $Ca^{2+}$-ATPase (PMCA1b) and a $Na^{+}/Ca^{2+}$ exchanger (NCX1) [39]. Because of this, calcium moves from the luminal space to the interstitial space and from here to the extracellular compartment (e.g. blood) [39].

Passive and paracellular calcium transport occurs through the tight junctions and the electrochemical gradient for $Ca^{2+}$ (blue arrows, Figure 13) is the driving force for the passage [39]. It takes place in case of high intake of calcium and interests the length of the intestine (Ireland and Fordtran, 1973) [3], where duodenum, jejunum, and ileum (Weaver and Heaney, 2006b) have the highest diffusion rate [3].

Only 10 to 15% of dietary calcium is absorbed by the gastrointestinal tract in subjects suffering from vitamin D deficiency, while it is approximately 30% in healthy adults [2].

In particular conditions, such as during pregnancy, lactation, and growth, high concentrations of 1, 25-D support the intestinal calcium absorption which is around 50-80% [2].

### 1.5.2 Other relevant roles of vitamin D for the health

Lately, the 1, 25-$(OH)_2D_3$ (*calcitriol*) receptors have been discovered in tissues, which are not the classical targets for vitamin $D_3$ (e.g. brain, bone marrow-derived cells, skin, thymus) [42]. These receptors are responsible for non-classical activities associated with vitamin D (Figure 12). A general overview of calcitriol biological effects is shown in the following table:

| Biological functions of calcitriol | |
|---|---|
| **Classical effects** | |
| Bone mineralisation process (Paragraph 1.4.2) | • Increase of calcium and phosphate intestinal absorption<br>• Reduce loss of calcium in the urine<br>• Acting on osteoclasts it promotes the release of calcium from the bones (less than the PTH)<br>• Acting on osteoblasts determines deposition of calcium |
| **Non -classical effects** | |
| Treatment of infections | • Increase of macrophages activity and promotion of their cytotoxic activity [43]<br>• Inhibits T helper cell proliferation and B cell immunoglobulin production [17, 44] |
| Treatment of allergies | Enhancement of anti-inflammatory and anti-allergic cytokine IL-10 secretion [43] |

| | |
|---|---|
| Prevention of diabetes mellitus | • Reduction pancreatic beta-cell destruction and hence the incidence of autoimmune diabetes by suppression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α [43]<br>• Promotion of insulin secretion through VDR receptors on pancreatic ß cells [43] |
| Prevention of cardiovascular diseases | Decrease vascular calcification, reduction of atherosclerosis risk, cardiomyocyte hypertrophy prevention, reduction of systolic blood pressure [43] |
| Multiple sclerosis (MS) | Regulation of gene expression associated with the MS, reducing the risk of disease [43] |
| Anti-cancer effect | Tumor growth retardation as well as tumor regression, due to inhibition of angiogenesis (growth of blood vessels from pre-existing vasculature), in this way the cancer cells cannot have access anymore to nutrients and oxygen for growth and survival [43, 45] |

Table 3: General description of the classical and non-classical effects of vitamin D

# 1.6 Importance of vitamin D intake

## 1.6.1 Dietary intake

Vitamin D can be introduced from the diet by food and dietary supplements, which both contribute to the total vitamin D intake. Vitamin D intake can be measured in two ways: in micrograms (mcg) and International Units (IU) [46]. Table 4 shows the vitamin D content of various foods expressed in IU.

| Food | IUs per serving |
|---|---|
| Cod liver oil, 1 tablespoon | 1360 |
| Swordfish, cooked, 3 ounces | 566 |
| Salmon (sockeye) cooked, 3 ounces | 477 |
| Tuna, canned in water, drained, 3 ounces | 154 |
| Orange juice fortified with vitamin D, 1 cup | 137 |
| Milk, vitamin fortified, 1 cup | 115-124 |
| Yogurt, fortified with 20% of the daily value of vitamin D, 6 ounces | 80 |
| Margarine, fortified, 1 tablespoon | 60 |
| Sardines, canned in oil, drained, 2 sardines | 46 |
| Liver, beef, cooked, 3 ounces | 42 |
| Egg yolk, 1 large | 41 |
| Cereal, fortified with 10% of the daily value of vitamin D, 1 cup | 40 |
| Cheese, Swiss, 1 ounce | 6 |

Table 4: Content of vitamin D in some foods and possible percentage of fortification (National Institutes of Health; Office of Dietary Supplements, June 24, 2011) [24]

The production of dietary supplements fortified with vitamin D has been increased in the last years [17]. Usually, many marketed dietary supplements are designed to provide 400 IU per daily dose, but levels in supplements tend to be increased [17].

### 1.6.2 Recommended intake of vitamin D

The recommended intakes of vitamin D throughout life in Europe are shown in the Table 5.

| Age | Male | Female | Pregnancy | Lactation |
|---|---|---|---|---|
| **0-12 months** | 400 IU (10μg) | 400 IU (10 μg) | | |
| **1-13years** | 600 IU (15 μg) | 600 IU (15 μg) | | |
| **14-18years** | 600 IU (15 μg) | 600 IU (15 μg) | 600 IU(15 μg) | 600 IU (15 μg) |
| **19-50 years** | 600 IU(15 μg) | 600 IU (15 μg) | 600 IU (15 μg) | 600 IU(15 μg) |
| **51-70 years** | 600IU(15 μg) | 600 IU (15 μg) | | |
| **>70 years** | 800 IU(20 μg) | 800 IU (20 μg) | | |

Table 5: Recommended dietary allowances (RDAs) for vitamin D [24]

Even with a balanced diet, high levels of intake (e.g. 800 IU) are difficult to achieve. For this reason, supplementation, fortified foods and pharmaceutical products are necessary.
Certainly, in the case of people suffering from vitamin D deficiencies (e.g. age, particular disease, osteoporosis) a blood test for vitamin D levels is determinant to decide the most appropriate daily intake.

### 1.6.3 Determination of the status of vitamin D deficiency

A blood test can be useful to detect the vitamin D status of a person by controlling the plasma levels of 25-(OH)-D [47]. In this way, it is possible to evaluate whether the daily vitamin D intake both from sunlight and dietary sources is enough.
The vitamin D content in the blood for medical investigations is measured in either nmol/L or ng/ml.

| Characterization | Serum or plasma 25-(OH)-D |
|---|---|
| **Optimal** | > 20 ng/ml |
| **Insufficiency** | 11-20 ng/ml |
| **Deficiency** | ≤ 10 ng/ml |

Table 6: Reference values of 25-(OH)-D for evaluation of vitamin D-status [48]

Table 6 shows what are the corresponding clinical conditions of the patient at a certain plasma level of 25-(OH)-D. However, the classification listed in the table above is indicative, because changes can occur due to the chosen vitamin D assay, the season or months in which blood samples are taken and then the geographic latitude, the age range, ethnicity and sex of the study populations (Wahl *et al*.2012; Hilger *et al*.2014) [47].

### 1.6.4 Causes of vitamin D deficiency

Foods naturally containing or fortified with vitamin D are not very widespread on the market [47, 49]. Therefore, the major source of vitamin D for kids and adults is represented by the exposure to natural sunlight.
In order to produce a sufficient amount of vitamin D, the human body needs that the correct sunlight (UVB) hits the skin for at least 3-15 minutes a day in case of white skin and 15-30 minutes in case of black skin [50]. Hence, inadequate exposure to sunlight can be one of the causes of vitamin D deficiency [50]. It should be known that a sunscreen with a sun protection factor of 30 is responsible for the 95% reduction of vitamin D synthesis in the skin [21].

There are many factors which can affect vitamin D synthesis through the skin [17]. For example, the sun protection is higher for people with dark skin and they require a longer exposure to produce the same amount of vitamin D as a person with a white skin tone [21]. Moreover, in case of body mass index (BMI) greater than 30 kg/m$^2$, low levels of serum 25-(OH)-D can occur. Therefore, obesity is associated with vitamin D deficiency [51].
Other causes for vitamin D deficiency are listed in Table 7.

| Specific medical conditions | Effects |
|---|---|
| **Kidney (e.g. Chronic kidney disease) and liver diseases** | Patients with CKD suffer from vitamin D deficiency which is further intensified by the reduced ability to convert 25-(OH)vitamin D into the active form, 1,25 dihydroxy-vitamin D [52]. |
| **Cystic fibrosis, Crohn's disease, celiac disease** | These diseases do not allow the intestines to absorb enough vitamin D [53] |
| **Gastric bypass surgery** | The removal of a part of the stomach and/or the intestines causes a size reduction, decreasing the absorbable amount of vitamin D-containing nutrients [54] |
| **Obesity** | A body mass index greater than 30 kg/m$^2$ is associated with lower vitamin D levels, because the fat does not allow the vitamin to be released into the bloodstream [51] |

Table 7: Medical conditions which can lead to vitamin D deficiency

Besides the insufficient sunlight exposure, skin pigmentation, pathological conditions, other factors can determine vitamin D deficiency, such as the age. In fact, the synthesis from the skin decreases over time due to a reduced mobility (e.g. elderly age) and possibility of sun exposure.

Moreover, in the lactation period, the woman's breast milk contains a small amount of vitamin D, therefore infants are more at risk for vitamin deficiency [55].

### 1.6.5 Effects associated with vitamin D deficiency

Vitamin D contributes to preserving bone health, by acting in the regulation of calcium and phosphate levels in the blood. As already described, vitamin D is needed to promote the calcium absorption in the intestine and hence to restore calcium, that would otherwise be excreted through the kidneys. Vitamin D deficiency causes insufficient mineralisation of the skeleton, bringing the kids to *rickets* and the adults to *osteomalacia* [17].
The *rickets* is a disorder of the bone growth, where the cartilage does not complete the process of mineralisation, therefore the bones result soft and ductile [3]. In adults, the deficiency of vitamin D causes *osteomalacia*, where regions, similar to seams, in the bone matrix are formed [17], due to inappropriate mineralisation.
The intake of small amount of calcium is associated with other critical conditions as the secondary increase of PTH release (*secondary hyperparathyroidism*) in response to the low serum calcium levels [3].
As a consequence, the episodes of bone resorption and suppression of bone formation become more frequent [3].
Another pathology associated with vitamin D deficiency is *osteoporosis* , characterised by bone fragility as well as by the risk of fracture, due to a reduced bone mass, indicated by low bone mineral density (BMD) [3].

## 1.7 Potential health risks of vitamin D excess

The intake of high levels of vitamin D causes the increase of the circulating 25-(OH)-D, determining intestinal absorption and bone resorption of calcium, which may cause hypercalcaemia with possible renal failure and cardiac arrest [47].
Tolerable upper intake levels (UL) have recently been set both by the EU Scientific Committee on Food/EFSA (SCF - Scientific Committee of Food, 2006) and by IOM (US Institue of Medicine) to ensure no risks for public health (Table 8).

| EFSA | | IOM | |
|---|---|---|---|
| **Tolerable upper intake level µg/day (IU/day)** | | | |
| 0–12 months | 25 (1000) | 0–6 months | 25 (1000) |
| 1–10 years | 50 (2000) | 7–12 months | 37.5 (1500) |
| 11 years to adult | 100 (4000) | 1–3 years | 62.5 (2500) |
| | | 4–8 years | 75 (3000) |
| | | 9 years to adult | 100 (4000) |

Table 8: Upper tolerable limits of vitamin D in Europe (SCF) and upper intake levels in the US [47]

Referring to the Table 8, it can be seen as EU established a UL of 25 µg/day for infants until 12 months and 50 µg/day for kids from one year up to 10 years. The level increases to 100 µg/day starting from11 years to adults.

The UL of 100 µg/day for adults is the limit above which the risk of hypercalcaemia increases [47]. Signs of vitamin D intoxication according to National Institutes of Health (NIH) are listed in the diagram below.

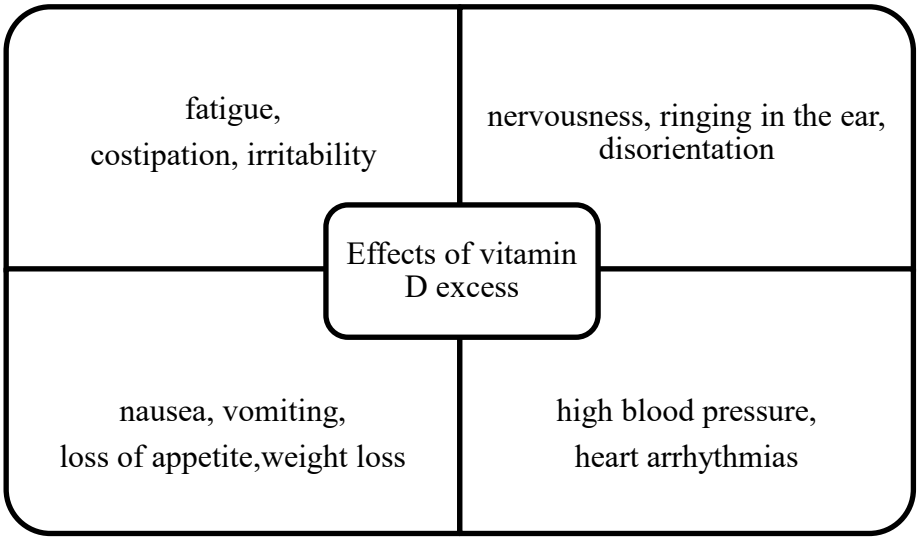



Figure 14: Schematic overview of principal effects associated with vitamin D excess [56]

## 1.8 Advantages of the vitamin $D_3$ spray

The vitamin D deficiency can be particularly harmful to infants, kids and elderly people, who have difficulties to keep adherence to the therapy due to unsuitable dosage forms. Swallowing a tablet or a capsule is not always the best administration form even for young adults. Moreover, for infants and kids a relevant issue to consider is the taste.

The manufacturing process also represents a drawback of the currently existing solid dosage forms. On one hand the physical mixing may give heterogeneous dispersions with uneven distribution of the active component, providing a high local drug concentration able to produce toxic side effects. On other hand the drying step required before tabletting or encapsulating, affects the thermal stability of the vitamin D due to the high temperatures used (e.g. 60-80°C). Furthermore, vitamin D and analogues can produce strong physiological effects already at small amounts therefore a strictly control of product quality is necessary during the production process.

For these reasons, the improvement of dosage forms containing vitamin D has become necessary, in order to make them more tasty and practical in the administration phase (e.g. spray) thus increasing the compliance by special categories of patients.

The development and implementation of a new simplified manufacturing process have also been experimented, in order to enhance bioavailability and chemical stability.

Up to now, the spray formulation is a unique dosage form, which better meets these requirements.

### 1.8.1 Faster systemic effect of oral absorption

In the past few years, drug delivery systems acting on the oral mucosa, such as sprays, have been taken into consideration as route of administration for several drugs, due to their numerous advantages over the other frequently used routes (e.g. peroral, oral, parenteral). The principal reasons for this choice are listed in Table 9.

| |
|---|
| ✓ No passage through the gastrointestinal tract and the hepatic portal system. Therefore, neither the degradation due to pH and digestive enzymes nor the hepatic first-pass metabolism occurs [57] |
| ✓ Improvement of patient compliance: elimination of pain due to injections; possibility of administration to unconscious patients or with motility problems [57] |
| ✓ Sustained drug delivery [57] |
| ✓ Rapid onset of action [57] |
| ✓ Easiness of drug administration [57] |
| ✓ Immediate systemic action thanks to the rapid absorption by the venous system under the oral mucosa [57] |
| ✓ Fast drug absorption due to the extended contact surface of oral mucosa (200 $cm^2$) [57] |

Table 9: Principal advantages of oral mucosal drug delivery system

The oral cavity has many different physiological functions and this is reflected in the complex anatomical structure. It comprises the lips, cheek (buccal), tongue, hard palate, soft palate and floor of the mouth and each region varies in thickness and permeability [57]. Three principal absorption areas can be distinguished in the mouth:

- *sublingual area*: located under the tongue; the membrane of the underneath (ventral surface) of the tongue and the floor of the mouth; it provides a high vascularized absorption region [57];
- *buccal area* : absorption occurs through the lining of the cheeks (buccal mucosa) [57];
- *gingival area:* the mucosa of the gingivae is responsible for the absorption in this area [57].

Since sublingual and buccal mucosa are non-keratinized, they are the principal site of absorption in the oral cavity [57]. In fact, the gingival mucosa is keratinized and hence is less permeable [57].
In the specific case of spray formulations, the interested area is the buccal mucosa, which shows good permeability and blood supply [58].

In fact, as displayed in Figure 15, micro-sized droplets are sprayed in the mouth, where they distribute mostly on the internal side of the cheeks.

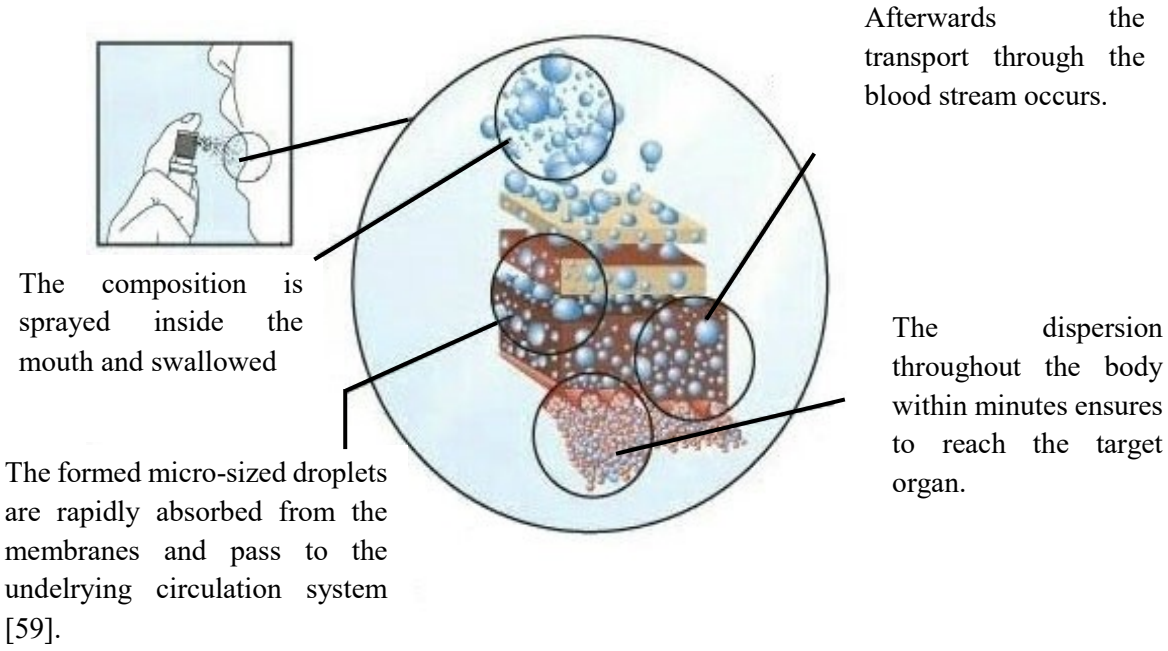



Figure 15: Schematic overview of buccal absorption [59]

The droplets are absorbed through the different superficial layers (epithelium; lamina propria). The permeation phase occurs through the epithelium layer both through the cells (*transcellular*) and around the cells (*paracellular*), but the main mechanism of the absorption is the passive diffusion described by the following Fick's first law [57, 60]:

$$P = \frac{DK_P}{h} \qquad \text{Equation 1}$$

Where P is the permeability coefficient, D is the diffusion coefficient of the drug in the oral mucosa, Kp is the partition coefficient of the drug between the delivery medium and the oral mucosa, h is the thickness of the oral mucosa [57, 60]. The amount of drug absorbed is given by:

$$A = PCSt = \frac{DK_P}{h} CSt \qquad \text{Equation 2}$$

With A the drug absorbed, C is the free drug concentration dissolved in the formulation medium (e.g. lipophilic excipients), S is the surface area of the delivery system (e.g. drops particle size) or the absorption site on the mucosa, and t is the contact time of the drug stays with the mucosa [57, 60].
The tissue thickness, partition coefficient, and the diffusion coefficient characterize the mucosa and the drug, but the surface area for delivery of the drug, time of contact, and the free drug concentration can be modified using the appropriate formulation [57].
Lipophilic compounds, mostly unionised and with high partition coefficient, such as vitamin $D_3$, permeate easily trough transcellular route, because their lipid solubility allows them to pass easily through the lipophilic cell membranes [57, 60].
Therefore, spray systems containing oils and surfactants, contribute to enhancing the absorption of dissolved APIs, characterised by high molecular weight, poor aqueous solubility and high membrane permeability [61].

### 1.8.2 Enhancement of bioavailability through lipid-based formulations

The US FDA has conducted studies which demonstrate that the lipid component of the food promotes the absorption of lipophilic drugs and contributes to increasing the oral bioavailability [62]. In fact, it has been proved that molecules, like triglycerides and long chain fatty acids, enhance membrane fluidity and hence wall permeability, favouring the transcellular absorption of drugs [57, 63].
Therefore, many formulations have been developed as lipid-based drug delivery (LBDD) systems, composed of specific additives, such as penetration enhancers (e.g. fatty acids and their salts and esters, surfactants), which promote the dissolution of drugs and facilitate the absorption [61, 64].

#### 1.8.2.1 Triglycerides as lipid excipients in lipid based drug delivery

The principal constituents of lipid-based drug delivery systems are triglycerides, especially in the form of vegetable oils [64]. This kind of lipids is not hazardous to health, since they can be digested and absorbed [64].

Triglycerides can be classified as long chain triglycerides (LCT, with 13-21 carbons), medium chain triglycerides (MCT, between 6 and 12 carbons) and short chain triglycerides (SCT, with less than 6) [61, 65].The triglycerides are composed of a glycerol backbone, whose hydroxyl groups undergo an esterification reaction with three fatty acids (Figure 16).

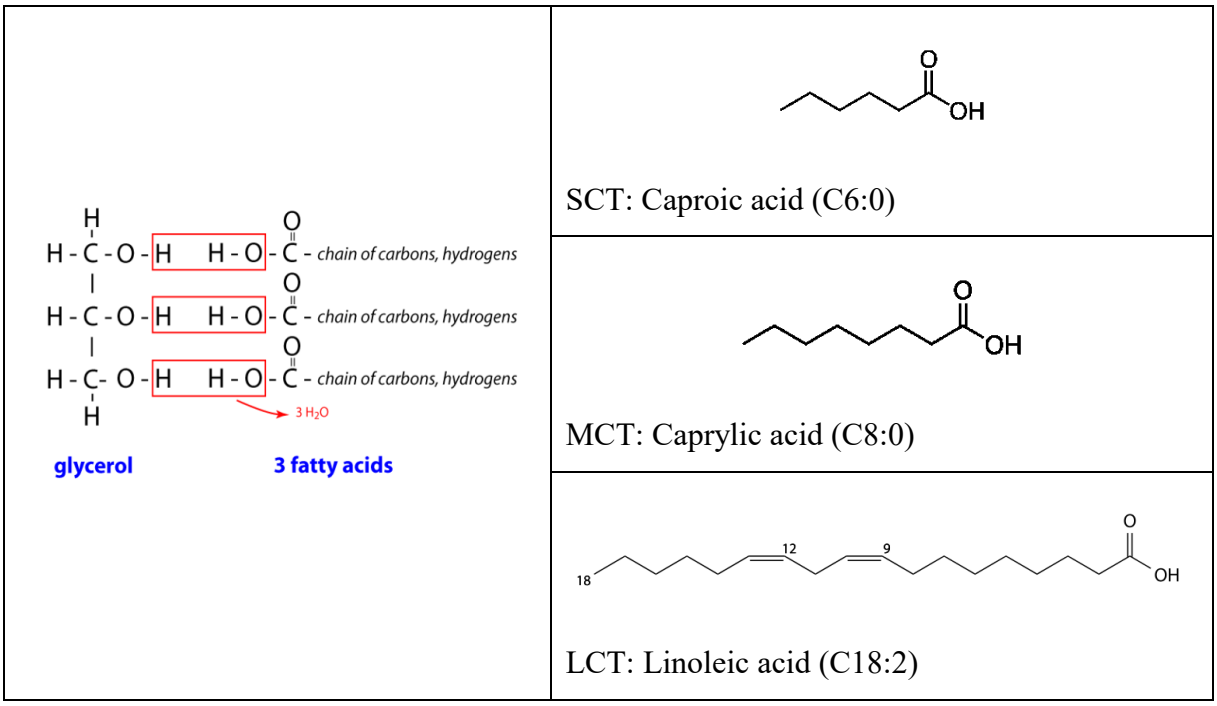



Figure 16: General chemical structure of SCT, MCT and LCT [66–69]

Recently many oil based delivery systems, such as drugs and dietary supplements containing vitamin D (Paragraph 1.9), are directed towards the use of MCT instead of LCT, while SCT are not considered for this use. Table 10 describes some reasons for this choice.

<table>
<tr><th colspan="2">Comparison between MCT and LCT</th></tr>
<tr><th>MCT</th><th>LCT</th></tr>
<tr><td colspan="2">Fatty acids composition</td></tr>
<tr><td>Class of lipids in which three saturated fats are bound to a glycerol backbone (Figure 16).</td><td rowspan="2">Most of the fatty acids contain 16 to 18 carbons in their fatty acid chains. In addition to energy, these oils also provide essential fatty acids such as linoleic acid, a precursor to arachidonic acid, an initial substrate for synthesis of prostaglandins and leukotrienes [65]</td></tr>
<tr><td>Fatty acids between 6 and 12 carbons in length; typical composition: caprylic (C8; 50-80%), capric fatty acids (C10; 20-50%), caproic (C6; 1-2%) and lauric (C12; 1-2%) fatty acids [65]</td></tr>
<tr><td colspan="2">Solubility</td></tr>
<tr><td>Higher water solubility due to shorter length [65]</td><td>Low water solubility [65]</td></tr>
<tr><td colspan="2">Metabolism</td></tr>
<tr><td>Unique metabolism: absorption without the need for micelle formation [65]: MCT are broken down into medium-chain fatty acids (MCFA) after digestion and are carried to the liver by venous circulation, which is faster compared to the lymphatic system [65]</td><td>For the absorption, lipase enzyme operates separating fatty acid chains from the glycerol backbone [65]. Fatty acids form micelles which are absorbed and go to reconstitute triglycerides, travelling through the lymphatic system to the bloodstream [65].</td></tr>
<tr><td>Higher water solubility facilitates the distribution through the bloodstream [65].</td><td>LCT require many steps to be digested before they can reach the bloodstream [65].</td></tr>
<tr><td>MCT are faster than LCT in leaving the circulation [65]</td><td>Once in the bloodstream, LCT are stored as body fat in the adipose tissue [65].</td></tr>
</table>

| Origin | |
|---|---|
| Derivation from edible oils, such as coconut oil or palm seed oil. Trade name of a capric and/or caprylic triglyceride oil is Miglyol (810,812) [65]. | They mostly derive from the following oils: corn, peanut, soyabean, sunflower, olive. |

Table 10: Comparison between medium chain triglycerides (MCT) and long chain triglycerides (LCT)

### 1.8.2.2 Water-insoluble and water-soluble surfactants

The lipid-based delivery systems improve absorption of lipophilic drugs, but they need special additives to promote *in vivo* dispersion. In fact, the simple dissolution of the API in oil alone is not enough to ensure an optimal bioavailability, due to the poor miscibility of the oil with the aqueous extracellular fluids of the body (e.g. saliva in the mouth, blood), which contribute to the transport of the drug and the absorption.

In order to guarantee a better dispersion of the oil in aqueous fluids, surfactants play an important role in the oil-based pharmaceutical formulations.

There are two categories of surfactants [70] (Table 11): *lipophilic surfactants* (oil soluble, HLB<10), which are w/o emulsifying agents and *hydrophilic surfactants*, (water soluble, HLB>10), o/w emulsifying agents.

| Lipophilic surfactants | Hydrophilic surfactants |
|---|---|
| **Fatty acids:**<br>e.g. oleic acid (preferred), linoleic acid, stearic acid, lauric acid, palmitic acid, capric acid and caprylic acid | **Phospholipids** in particular lecithins<br>e.g. soyabean lecithins |

| | |
|---|---|
| **Mono and/or diglycerides of fatty acids:** e.g. Imwitor (glycerol mono or di-caprylate, glycerol mono-stearate); Capmul GMO (glycerol mono-oleate); Peceol (glycerol mono-oleate) | **Polyoxyethylene sorbitan fatty acid derivates:** e.g. Tween 20 (polyoxyethylene (20) monolaurate), Tween 80 (polyoxyethylene (20) monooleate) |
| **Propylene glycol mono and/or diesters of fatty acids:** e.g. Miglyol 840 (propylene glycol dicaprylate/ dicaprate); Neobee M20 (propylene glycol dicaprylate/ dicaprate) | **Castor oil or hydrogenated caster oil ethoxylates (HLB>10):** e.g. Cremophor EL (polyoxyethylene (35) castor oil), Cremophor RH40 (polyoxyethylene (40) hydrogenated castor oil) |

Table 11: List of some surfactants described in the patent EP 0750495 B1 for oil-based pharmaceutical formulations

Both kinds of surfactants are *amphiphilic* compounds, having the same general structure, represented by the hydrophilic *head*, which interacts with the aqueous phase and the hydrophobic *tail*, which interacts with the oil phase, allowing the passage through the lipophilic membrane cells.

| **Component** | **Generally preferred** | **More preferred** | **Most preferred** |
|---|---|---|---|
| | w/w% | | |
| Digestible oil | 10-90 | 20-60 | 25-45 |
| Hydrophilic surfactant | 10-60 | 25-50 | 30-45 |
| Lipophilic surfactant | 5-60 | 10-45 | 20-40 |

Table 12: Relative concentrations by weight of the digestible oil, hydrophilic surfactant and lipophilic surfactant [70]

In many oil-based drug formulations, a mixture of lipophilic and hydrophilic surfactants (Table 12) is usually preferred, because this allows a mutual support.
In fact, on one side the hydrophilic surfactants are important to get better emulsions, but on the other side their low miscibility with the oil component can compromise the homogeneity [70]. Moreover, there is a drawback related to the use of hydrophilic surfactants alone, which is the reduction of lipolysis process (lipolysis-inhibiting effect) and absorption of a drug in vivo [70]. In order to solve these issues, the use of lipophilic surfactants is required.
They promote the lipolysis of fatty components (e.g. MCT oil) by the lipase enzymes, which hydrolyse the triglycerides to fatty acids and glycerol, thus enhancing the dissolution rate of the hydrophobic drug dispersed in the oil phase (e.g. vitamin $D_3$) [64, 70].

## 1.9 Oily formulations with vitamin $D_3$ currently on the market

The lipid-based drug delivery systems have been recently adopted by pharmaceutical industries as a new approach for the development of medicaments containing vitamin $D_3$. The main forms currently on the market are represented by soft gelatine capsules with vitamin $D_3$ in oil (Paragraph 1.9.1) and simple oily solutions administered either as drops (Paragraph 1.9.2) or as spray (Paragraph 1.9.3). In the next paragraphs, a general description of some patented formulations is proposed.

### 1.9.1 Capsules containing oil-based drug formulation

The patent *WO 2012117236 A1* refers to a pharmaceutical composition for oral delivery which is characterised by around 20,000 IU of vitamin D, a lipophilic excipient as carrier that stabilizes vitamin D and a non-animal derived polymer as outer layer.
Vitamin D is very sensitive to light and oxidative degradation. In the considered formulation, the high concentration of vitamin $D_3$ has required the adoption of specific antioxidants and protective barriers against oxygen, such as the gelatinised capsules, composed of HPMC (hydroxypropylmethylcellulose).

The lipophilic excipient used in the patented formulation is represented by Miglyol® 812N, a mixture of caprylic and capric oils, which solubilizes vitamin D and has antioxidant effect, together with butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT).
The following table proposes the possible compositions as reported in the patent.

| **Compositions** | **Vitamin $D_3$** | **Miglyol® 812N** | **BHA or BHT** |
|---|---|---|---|
| | w/w% | | |
| Suitable | 0.25-1 | 98.95-99.7 | 0.02-0.08 |
| Preferable | 0.25 | 98.5 | 0.05 |
| Alternative | 1 | 98.5 | 0.05 |

Table 13: Possible combinations of formulation ingredients as described by the patent WO 2012117236 A1

The preparation method of formulations stated in the patent is explained in the table below.

| |
|---|
| 1. Miglyol® 812N is placed into a suitable container |
| 2. Vitamin $D_3$ is poured in the same container where is mixed to form a clear solution |
| 3. BHA is blended with the vitamin $D_3$ and Miglyol® 812N solution |
| 4. Application of low shear until obtaining homogeneity |
| 5. Discharge of the gas from the solution if necessary |
| 6. Filling under nitrogen atmosphere of solution into HPMC capsules |

Table 14: Method for preparing formulations containing vitamin $D_3$, Miglyol® 812N and BHA as reported in the patent WO 2012117236 A1

### 1.9.2 Vitamin D in oily drops

Drops are widely preferred to capsules or tablets not only for the easier administration but also for the faster absorption. In the patent *CA 2558202 A1*, it has been described a pharmaceutical preparation for the delivery of vitamin D to infants. It consists of vitamin D dissolved in a solution containing medium chain triglycerides (MCT) and antioxidants.
According to the patent, one drop (33µL) volume of solution can be applied to a woman's nipple or pacifier, which enters the mouth of the infant.
With this method, vitamin D is made available to the infant in a safer and efficient way, which allows avoiding the excessive exposition to sunlight.
The MCT used are natural constituents of breast milk and are hydrolysed by lipases present in the saliva of the infant, which determine the release of free fatty acids and monoglyceride with a further antimicrobial action [71].

| |
|---|
| 1. Carbon chain lengths of 6 -12 and, preferably, the medium contains at least 95% triglycerides with a carbon chain length from 8 to10. |
| 2. Medium chain triglycerides, extracted from the endosperm of Cocos nucifera L. or Elaeis guineehsis Jacq, represented by a mixture of triglycerides of saturated fatty acids, principally caprylic acid and caproic acid |
| 3. Not less than 95 % of saturated fatty acids having 8 to 10 carbon atoms. |
| 4. MCT is a clear solution |

Table 15: General characteristics of MCTs as described by the patent CA 2558202 A1

The medium chain triglycerides used in this invention (Table 15) are liquids rather viscous, which remain on the nipple or pacifier, but not adhere to them, allowing an easy removal through sucking.
The formulation results infant safe due to the natural occurring MCT oil, whose resistance to rancidity and oxidation over the shelf life, makes the formulation very stable.

The use of MCT oils for the development of vitamin D dosage forms has also been described by the patent *US20030191093A1*, which refers to a *pre-concentrate* emulsion comprising vitamin D.

It is a non-aqueous formulation which becomes emulsion after contact with water or other aqueous solutions. In the table below have been reported the principal components of *pre-concentrate* emulsions.

| **Vitamin D or its active compounds** |
|---|
| Calcitriol, calcidiol, cholecalciferol |
| **Lipophilic phase component** |
| Preferably: MCT type; e.g. Labrafac CC, Miglyol 810/812; CAPMUL MCT |
| **One or more surfactants** |
| **Hydrophilic surfactants:** e.g. TWEEN 40/80; Lecithins (soya bean lecithins)<br>**Lipophilic surfactants:** e.g. Monoglycerides (glycerol monooleate, glycerol monopalmitate, glycerol monostearate); tocopherol PEG-1000 succinate (Vitamin E TPGS) |
| **Additives** |
| **Antioxidants:** e.g. BHA; BHT; vitamin E<br>**Flavorants**<br>**Viscomodulators:** cellulose and cellulose derivatives (e.g methyl-,ethyl-,propyl-celluloses); hydroxyalkyl-celluloses (e.g. hydroxypropyl-celluloses); polyacrylate and polyacrylate co-polymer resins (e.g. poly-acrylic acid/methacrylic acid resins ) |

Table 16: Components of the pre-concentrate emulsions described by the patent US20030191093A1

The relative proportion of ingredients (Table 17) in the composition is variable according to the dosage form and the route of administration.

| Components | Based upon the total weight of the composition (w/w %) |
|---|---|
| Active vitamin D compound | General: 0.005-20%<br>Preferably:0.01-15% |
| Lipophilic phase component | Suitably: 30-90%<br>Preferably: 50-85% |
| Surfactants | Suitably: 1-50%<br>Preferably: 5-40% |

Table 17: Relative proportions of the ingredients in the patented formulation

In the case of oral liquid formulations, the following percentages, always based on the total weight of the composition, have been tested:

- 50-60% of lipophilic phase
- 4-25% of surfactants (e.g. hydrophilic surfactant: 25% Cremophor RH 40)
- 0.01-15% of active vitamin D compound

The pre-concentrate emulsions could be administered orally as solutions or encapsulated in soft gelatine capsules.
Another interesting patent, the *DE202014006889 U1*, refers to a vitamin D composition containing tocopherols and sunflower oil (Table 18).

| Components | Overall range | Preferably | More preferably: |
|---|---|---|---|
| Vitamin D | 1µg to 100 µg | 10 µg to 40 µg | 15 µg to 25 µg |
| Tocopherols | 0.03 mg to 10 mg | 0.1 mg to 3 mg | 0.5 mg to 1 mg |
| Sunflower oil | 20mg to 40 mg | 25 mg to 35 mg | 28mg to 32 mg |

Table 18: Components and respective proportional amounts as described in the patent DE202014006889 U1

As stated in the patent, the use of sunflower oil with strong tocopherol extracts, in particular, gamma-alpha-delta –beta-tocopherol, provides adequate protection of vitamin $D_3$ against oxidation. In fact, as already specified before, vitamin D is highly sensitive to oxidation; therefore, a vitamin $D_3$ solution could not be long exposed to air. However, this can accidentally happen when administered in the form of drops, through the daily opening of the bottle.
According to the considered patent, the stability of vitamin D oil is preserved for two years without being opened and over six months, when it is regularly used.
A more detailed overview of the patented formulation is reported in the following table.

| Ingredients | mg/per drop | g per 100 ml |
|---|---|---|
| **Tocopherols extract** | 0.74 | 2.23 |
| **Vitamin $D_3$ oil** | 0.83 | 2.5 |
| **Sunflower oil** | 29.5 | 88.35 |

Table 19: Example of vitamin $D_3$ in sunflower oil as stated in the patent DE202014006889 U1

### 1.9.3 Oil spray vitamin D

The spray formulation, as already discussed, is considered one of the most suitable dosage forms for its easiness in the administration and the faster absorption through the buccal mucosa. In addition to this, by a suitable drops atomization and taste-masking effect through flavouring agents, the spray allows reducing the oil feeling characteristic of simple drops, thus improving the palatability of the dosage form.
One of the first flavoured sprays has been described in the Chinese patent *CN 1732958 A*, which refers to a composition containing vitamin D (vitamin $D_2$, $D_3$) or similar (including, calcidiol, calcitriol) and an oily liquid carrier.
In the previous spray formulations developed in US by the company Mayor (1985), the carrier liquid was alcohol, aqueous alcohol or water. The use of ethanol as solvent allows getting a low viscous solution ideal to be sprayed and a higher microbiological stability as well as a better absorption of the drug, due to a dilatant effect of ethanol on the oral mucosa blood vessels.

However, this made this kind of spray not accessible to certain populations sensitive to ethanol and furthermore the resulting oil in water emulsion, even with the introduction of an emulsifier (e.g. Tween 80) did not achieve the desired stability.
The invention of the considered patent deals with the development of an ethanol-free vitamin D spray, where medium chain fatty acid triglycerides are used as solvent (Table 20).

| **Preferably** |
|---|
| Medium chain length C6~C14 |
| **More preferably** |
| Medium chain length –MCT C8~C12 |
| From CONDEA Chemie Gmbh:<br>• MIGLYOL 812: 50 - 65% caprylic acid (C8), 30-45% capric acid (C10);<br>• MIGLYOL 810: 65 -80% C8; 20-35% C10<br><br>From GATTEFOSSE, France:<br>LABRAFAC CC 50 to 80% C8; 20-50% C10 |

Table 20: Components of the oily phase described in the patent CN 1732958 A

Natural fatty oils, which are mostly unsaturated fatty acids (e.g. canola oil) may also be used. The use of pharmaceutically acceptable additives such as flavouring agents, antioxidants, surfactants agents and thickeners is advantageous in the spray formulation (Table 21).

| | |
|---|---|
| **Flavouring agents** | Menthol, peppermint oil, milk chocolate flavour (e.g. BFL1227 type, International Flavors & Fragrances Inc.), sorbitol |
| **Antioxidants** | p-tert-butyl hydroxyl anisole (BHA), di-t-butyl-p-cresol (BHT) |

| | |
|---|---|
| **Surface active agents** | Tween 80, etc.; Span |
| **Thickeners** | Glycerol, propylene glycol, polyvinylpyrrolidone (PVP) |

Table 21: Pharmaceutically acceptable additives described in the patent CN 1732958 A

According to the patent, the use of oleaginous carriers requires a suitable material for the spray system, such as high-density polyethylene, polypropylene, metal (e.g. aluminium), amber glass, in order to preserve the stability of the API. Information regarding spray amount and hump size are described in the following table.

| | |
|---|---|
| Spray amount | Generally 20~500 mg<br>Preferably 40~250 mg |
| Hand pump size | 0.1ml, thread 18/415, Pfeiffer, Germany |

Table 22: Characteristics of spray apparatus as described in the patent CN 1732958 A

Table 23 shows the amounts of different excipients, as reported in the considered patent.

| 0.1 mg~10g vitamin D or analogues added to 100 ml of oily carrier | |
|---|---|
| **Generally** | **Preferably** |
| 0.1 mg ~10 g vitamin D | 0.25 mg~5g vitamin D |
| [0~40 g ethanol if required] | [0~30 g ethanol if required] |
| 0~10 g pharmaceutical acceptable additives | 0~5g pharmaceutical acceptable additives |
| 100 ml oily carrier | 100 ml oily carrier |

Table 23: General description of spray formulation as provided in the patent CN 1732958 A

The preparation procedure proposed in the patent consists of a dissolution step of vitamin D in the oleaginous liquid carrier, with eventual addition of other pharmaceutical agents. Afterwards glass filters or porous membrane can be used in order to remove contaminants before filling the high-density polyethylene spray bottles, tightened manually by means of spray pumps. It follows a detailed description of the formulation and related procedure (Table 24).

| **Formulation example** | | |
|---|---|---|
| **Components** | **Amounts** | **Procedure** |
| **Vitamin $D_3$** | 10 g | 1. Add propylene glycol, peppermint oil to vitamin $D_3$<br>2. Then add 40 g of MCT, while stirring for the dissolution<br>3. Addition of MCT up to 100 ml<br>4. Stir with a dry G6 sintered glass filter<br>5. Distribution into a spray-bottle sealed by a spray pump |
| **Peppermint** | 0.5g | |
| **1,2-glycol** | 9.5g | |
| **MCT**<br>(e.g. LABRAFAC CC) | To 100 ml | |

Table 24: Example of formulation and relative procedure
as described in the patent CN 1732958 A

The chemical stability of patented formulations has been tested by means of HPLC using as references for controlling, commercially available solid dosage forms (e.g. vitamin $D_3$ tablets 1.25mg / piece). The sealed spray bottles have been placed in a 40° C thermostat for three months (e.g. from March to May) and the liquid content (μg/ml) of vitamin D has been monitored in order to calculate the residual ratio (%). As well as the sprays, also the controlling dosage forms have been placed for three months in a thermostat 40°C and the amount of vitamin D (μg/tablet) has been calculated.
According to the patent, the spray uniformity and the spray volume have to be guaranteed for consecutive administrations. Therefore, they have been examined by conducting the following investigations (Table 25).

| Definition of sprayed volume |
| --- |
| 1. Spray weighing<br>2. Spray 10 seconds for 10 times<br>3. Total weight after each spraying<br>4. Calculate the average weight per spray<br>5. Spray placed at room temperature for 7 days<br>6. Repeat steps from 1 to 4 |
| 10 bottles of repeated measurements and calculation of the average |

Table 25: Procedure to determine the spray volume and the difference among consecutive sprayings

Another interesting invention is described in the patent *EP 2942050 A1*, which relates to spray compositions containing lipophilic vitamins and suggests expedients to improve the preparation procedure.

As already written before, several oily solutions of vitamin D in drops can be already found on the market, but they have some limits, such as the difficulty of administration in paediatric and adolescent age. Furthermore, the daily intake becomes unpleasant, due to absence of tasty flavour and the bad palatability of the oil.

In order to reduce these constraints, the development has been directed toward solid or aqueous dispersions of vitamin D, but the bioavailability of the vitamin has shown a reduction, respect to the oil solutions.

For these reasons, the research of the patent has been focused on new formulations having preparation methods easy to implement, reproducible and economic and therefore a spray system has been chosen. In this way, the oil-feeling can be reduced since the oil is highly divided into a homogeneous fine dispersion of particles.

The mixture of the patented formulation comprises a medium chain triglyceride oil (MCT oil), in association with other medium chain acids triglycerides, used to improve oil compressibility. Moreover, excipients, sweeteners and flavourings have been adopted in the development.

| | |
|---|---|
| Miglyol 810/812 | 1-3% fatty acids C6 |
| Crodamol GTC/C | 1-5% fatty acids C12 |

Table 26: Types and composition of the MCT oils described in the patent EP 2942050 A1

The MCT oils shown in Table 26 derive from the hydrolysis of coconut oil with successive fractionation of the fatty acids.
The used MCT oil has a low purity, due to the higher content of lipases, which participate to its synthesis and thus can be found in the finished oil, despite the filtration process.
In fact, lipases cause oil hydrolysis of triglycerides contained in the MCT oil and this has some disadvantages (Table 27).

| **Drawbacks of oil hydrolysis** |
|---|
| Worse palatability due to the increase of oil-feeling |
| Reduction of the amount of triglycerides which reduces the quality of atomization |

Table 27: Drawbacks related to oil hydrolysis as reported in the patent EP 2942050 A1

In order to prevent these shortcomings, the patent describes as convenient the use of 1:1 weight ratio of triglycerides of medium chain acids, for instance *Labrafac Lipophile WL 1349*, to the medium chain triglyceride oil (MCT oil), like *Miglyol 810* or *812* or *Crodamol GTC/C*. Moreover, the heating step for the medium chain triglyceride oil is necessary, before mixing with vitamin D (e.g. vitamin $D_3$) and triglyceride of medium chain acids (e.g. Labrafac Lipophile WL 1349) (Table 28).

| 1:1 Weight ratio between triglycerides of medium chain acids and MCT oil |
|---|
| 1. Heating of MCT oil ( *Miglyol 810* or *812 or Crodamol GTC/C*)<br>2. Mixing with vitamin D and triglycerides of medium chain acids ( *Labrafac Lipophile WL 1349* ) |

Table 28: Amount and mixing steps of the lipophilic phase with vitamin D as reported in the patent EP 2942050 A1

This kind of triglycerides composition is also useful to improve the oil compressibility, but for this purpose specific amounts have to be taken into account.
In fact, the triglycerides mentioned above should have a higher amount of caprylic acid (C8) than capric acid (C10) (Table 29).

| | | | |
|---|---|---|---|
| **CAS 73398-61-5**<br>**CAS 85409-09-2**<br>**CAS 438544-49-1**<br>**EINECS 277-452-2** | Caprylic acid (C8) | 50-80% | **Sum of saturated fatty acids (C8+C10)** |
| | | Preferably at least 59% | |
| | Capric acid (C10) | 20-50% | 96% or above; preferably of 98 or above 99.5% |
| | | Preferably at least 40% | |

Table 29: Triglyceride of medium chain acids described in the patent EP 2942050 A1

Moreover, they should present the following chemical-physical properties (Table 30).

| | |
|---|---|
| **Viscosity (20°C)** | 25 to 33 mPa*s |
| **Acid number** [1] | 0.20 mg KOH/g or below |
| **Saponification value** | 310 to 360 mg KOH/g |
| **Water content** | 0.20% or below |

Table 30: Chemical-physical properties of the triglyceride of medium chain acids according to the patent EP 2942050 A1

In particular the triglyceride of medium chain acids used in the considered patent is a triglyceride of capric acid (C10) and/or caprylic acid (C8) having the commercial name of *Labrafac Lipophile WL 1349 Code 3139* (European Pharmacopeia no.0868) and produced by the company Gattefossè SAS. The composition of the mentioned triglyceride is shown in the Table 31.

| | |
|---|---|
| Caproic acid C6 | 2% or below (e.g. below 10%) |
| Caprylic acid C8 | 50-80% (e.g 59.2%) |
| Capric acid C10 | 20-50% (e.g. 40.5%) |
| Lauric acid C10 | 3% or below (e.g. 0.2%) |
| Myristic acid C14 | 1% or below (e.g. 0.1%) |

Table 31: Composition of the mentioned triglycerides as described in the patent EP 2942050 A1

In Table 32, one formulation procedure used for the patented spray is described.

[1] The acid number refers to the amount (mg) of sodium hydroxide necessary to neutralize the free fatty acids in one gram of substance. The increase of the acid number indicates the increase of free fatty acids.

| | |
|---|---|
| 1 | Dissolution of vitamin D in the MCT oil heated up to 40-60°C (preferably 45-55°C; e.g. 50°C) under stirring until the dissolution of bottom body in order to obtain an oily solution |
| 2 | Addition of the triglyceride of medium chain acids *Labrafac Lipophile WL 1349* in a ratio 1:1 to the MCT oil |
| 3 | The whole mixture is cooled to a room temperature of about 20°C under stirring |

Table 32: Example of a possible formulation procedure as stated in the patent EP 2942050 A1

Another way to proceed consists in adding the triglyceride of medium chain acids directly to the vitamin $D_3$ and afterwards the MCT oil heated up to 40-60°C (preferably 45-55°C; e.g. 50°C) is added under stirring. The whole mixture is cooled to a room temperature of about 20°C under stirring.

In both formulation procedures previously described, the desired flavourings and sweeteners have been added, before the distribution of formulation in the bottles (preferably 10 ml).

As written in the patent, the heating step in the preparation procedure guarantees a better atomization of the product, which is then related to a better palatability as well, thanks to the removal of oil feeling. In fact while heating, the lipases in the MCT can be disabled, avoiding the change of triglycerides present in the oil.

According to the patent, the acid number of formulations obtained without heating is higher than that of the formulations, in which the lipases have been deactivated. The reason for that is the increased percentage of triglycerides, deriving from *Labrafac* and *Miglyol*, which are hydrolysed by lipases, releasing fatty acids in the formulation.

This change in the composition of formulations leads to a worse atomization, which means that there is a higher percentage of particles smaller than 10 µm, representing the fraction which can be breathed (Table 33), and, therefore has to remain very low.

| | |
|---|---|
| **Process with heating step** | 0.36 % of particles < 10 µm |
| **Process without heating step** | 0.45% of particles < 10 µm |

Table 33: Effect of heating step on atomization performance

Drop size distribution analysis has been performed to this purpose (Table 34).

| **Pump** | **Protocol** |
|---|---|
| PUMP 50 VP6 THREAD PFP 18-1 R-26,0 | 1. The pump is charged with at least 5 sprays.<br>2. The drop size distribution is measured with a laser diffraction instrument as the MASTERSIZER S of MALVERN SQ203 |
| **Actuator** | **Set up:** |
| ACT_ASM 161C-15,10 –M-3,90-5,34-WHIT | • software: 2.19;<br>• sweep no.: 75;<br>• radiation wavelength: 2.40 nm;<br>• lens/actuator distance: 70 mm;<br>• laser beam/actuator distance:40 mm; pump position: 90 degrees |

Table 34: Analysis made at room temperature (22.5±1 °C) on 5 pumps, carrying out 2 tests for each pump

From the conducted investigations, it results that the atomization quality of the particles depends on the amount of triglycerides in the composition and on their stability to lipases.
A general description of component amounts, density of solution and sprayed volume follows in the Table 35.

| **Component** | **Amount** | **Density** |
|---|---|---|
| Vitamin $D_3$ | 0.0060 mg | 899.618 mg/ml |
| Triglyceride of medium chain acids | 23.647 mg | **Sprayed volume** |
| MCT oil | 23.647 mg | 1 puff =0.050 ml;<br>mg/puff<br>(50 µl puff = 47.30 mg) |
| Total weight | 47.30 mg | |

Table 35: Composition of the patented oil spray formulation

In order to evaluate the plasma levels of 25-(OH)-$D_3$ obtained by administering the sprayable oil, a comparison has been conducted by using the same dose of oil drops already on the market and the patented formulation (Table 36).

| | |
|---|---|
| Orally administered drops | 1 dose of 400 IUs of vitamin $D_3$ (10μg/die) in 5 drops diluted in a half glass of water |
| Sublingual spray of oily solution | 1 dose of 400 IU of vitamin $D_3$ (10μg/die) in two sublingual puffs |

Table 36: Doses administered in the morning after breakfast

According to the study, the sprayable oily solution described in the patent *EP 2942050 A1* is able to produce the same plasma levels of 25-(OH) of vitamin- $D_3$ as the oily drops. Therefore, the bioavailability of vitamin $D_3$ in the oily spray and vitamin $D_3$ in drops is comparable.
Another vitamin $D_3$ oral spray recently patented can be found in the Australian market.
The patent *AU 2014101500 B4* refers to the invention of a low dose, liquid vitamin $D_3$ composition for oral delivery. The patented formulation is addressed in particular to people who have adopted a vegetarian/vegan diet and hence can show a decrease in serum levels of *calcidiol* (25-OH-D) as well as of calcitriol and 1,α,25-hydroxy vitamin $D_3$, due to the low assumption of the animal protein. As reported in Table 37, a non-animal derived vitamin $D_3$ has been used and the preferred sources for it are plants or Lichen[2] species, from which the vitamin is extracted.

| |
|---|
| Non animal derived vitamin $D_3$ (e.g. cholecalciferol or a salt) |
| Oil-based excipient (e.g. soybean, corn, canola, cottonseed, safflower, rice bran, coconut oil) |
| Free of artificial preservatives |
| 50 IU to about 1000 IU per metered dose of approximately 180 to 200 μL |

Table 37: Vitamin $D_3$ oral spray described in the patent AU 2014101500 B4

[2] Lichen are organisms formed by the symbiotic association of a fungus and an algae or cyanobacterium.

Before being used for the manufacturing procedure, the raw solution containing vitamin $D_3$ is diluted, yielding 0.00617mg/mL of α- Tocopherol (Vitamin E) which can act as antioxidant and 6.00mg/mL of vegetable oil.

Regarding the lipophilic excipient, no MCT oil has been employed, but a specific type of oil chosen on the base of gamma oryzanol and ferulic acid content, because of its antioxidant and anti-microbiological function against decomposition of cholecalciferol over 24 month shelf-life. In particular, the preferred excipient is the rice bran oil for its shelf-life and antioxidant stability, as well as antimicrobial action. In this way, the use of preservatives like benzoates, bisulphites and analogous, usually responsible for allergic reactions (e.g. asthma), can be avoided, guaranteeing, however, the absence of microbiological contamination, during the considered shelf-life (minimum of 24 months). Table 38 shows the general preparation procedure of the patented vitamin $D_3$ composition.

| |
|---|
| 1. Selection and microbiological test for vitamin $D_3$ (Vitashine, Nottingham, United Kingdom: containing cholecaliferol, D-α- tocopherol and vegetable oil) and rice bran oil |
| 2. Dispensation of raw materials into containers by means of a computerised production system (Wayahead Systems, Jersey City, NJ, United States of America) under GMP conditions. |
| 3. The rice bran oil is mixed in a principal tank for 10 minutes in order to assure homogeneity |
| 4. Addition of vitamin $D_3$ and mixing until reaching uniformity |
| 5. The sides of the main tank are washed with rice bran oil and the resulting solution is mixed until uniformity |
| 6. The mixed solution is then filtered and poured into a holding tank .The solution is distributed into 50mL white round HDPE bottles with a spray cap. |

Table 38: Preparation method of vitamin $D_3$ composition as described in the patent AU 2014101500 B4

In the Australian patent as well as in the patent *EP 2942050 A1*, the delivery target of the spray formulation is the sublingual area of the mouth, which allows a rapid absorption of the drug, due to the high concentration of veins and arteries in the oral mucosa. As shown in Table 39, 180.5µL dose contains:

- 25µg (1000 IU) vitamin $D_3$ (cholecalciferol)
- 164.72 mg rice bran oil

| Component | Amount per 1 mL | Amount per 50mL |
|---|---|---|
| Vitamin $D_3$ (as cholecalciferol) | 5540 IU | 55,400 |
| | (Calculated as 1000IU per dose = 25µg/ 180.5µL = 0.138mg/mL) | 6.9 mg |
| Rice bran oil | 912.6 mg/mL | 45.63g |

Table 39: Vitamin $D_3$ composition containing 1000 IU per dose
calculated for 1ml and for 50 ml dispensing batch (containing 277 doses per bottle)

The product described in table is a pale yellow liquid and has shown a shelf life of two years during storage at 25°C. The stability testing (storage at 25°C; 60% humidity) of the described formulation has been performed after six months storage and Table 40 shows the results of stability analysis.

| Test Conducted | Method | Initial | 6 months |
|---|---|---|---|
| **Appearance** | Visual observation | Clear pale yellow free flowing liquid | Clear pale yellow free flowing liquid |
| **Relative Density (0.88 - 0.97)** | BP | 0.92 | 0.92 |
| **Cholecaliferol (Vit $D_3$) (154-183 µg/ml)** | HPLC analysis | 0.178mg/ml | 0.182mg/ml |

Table 40: Stability testing at 6 months as reported in the patent AU 2014101500 B4

# 2 Design of vitamin $D_3$ spray formulation

The work of this master thesis focused on the development of an oil-spray containing vitamin $D_3$ as active ingredient, MCT oil as lipophilic excipient, a flavouring agent as palatability enhancer and an emulsifier as promoter of the oral film formation as well as of the buccal absorption (Table 41).

| Components | Function |
|---|---|
| Vitamin $D_3$ | API |
| PECEOL™ 1% w/w | Emulsifier |
| Tetrarome(R) Orange 0.5% w/w | Flavouring |
| Medium chain triglycerides (MCT) | Lipophilic phase |

Table 41: Composition of the oil-spray developed

The dosage of vitamin $D_3$ was established taking into account the recommended daily intakes (Paragraph 1.6.2) and the tolerable upper intake levels, suggested by the National Institutes of Health, as described in Paragraph 1.7. The lowest, suggested dosage is 400 IU, usually recommended in the treatment of rickets due to vitamin D deficiency. In the case of the developed formulation, the administration of this dose is provided by one puff, corresponding to 120 µl.

## 2.1 Selection of lipophilic phase

Vitamin $D_3$ is a lipophilic molecule. Therefore, the starting point of the development was the selection of the most appropriate lipophilic phase to dissolve it. As already explained in Paragraph 1.8.2, the lipid-based drug delivery systems give the advantage of an enhanced bioavailability and a faster absorption of the API.

The lipophilic excipient used in the developed formulation was the medium chain triglycerides (MCT) oil, usually classified as vegetable oil. The term of medium-chain triglyceride, as described in Paragraph 1.8.2.1, relates to a mix of triglycerides with saturated fatty acids, characterized by a chain length of 6–10 carbons, i.e., hexanoic acid (C6:0, common name caproic acid), octanoic acid (C8:0, common name caprylic acid), and decanoic acid (C10:0, common name capric acid) [72]. Dodecanoic acid (C12:0, common name lauric acid) can also be found [72]. The natural sources of medium-chain fatty acids (MCFAs), described in the table below (Table 42), are really important.

| | | |
|---|---|---|
| **Coconut and palm kernel oil** | >50 w% of fatty acids [72] | |
| **Bovine milk** | C6:0–C10:0 [72] | 4–12% of all fatty acids |
| | C12:0 [72] | 2–5% of all fatty acids |

Table 42: Natural sources of MCFAs and relative amount

The most diffused process of synthesis for MCT oils consists in the hydrolysis of coconut or palm kernel oil, with successive filtration of the medium chain fatty acids (MCFAs) released, and their re-esterification. The resulting MCT oils are a mixture of octanoic and decanoic acid, at a ratio from 50:50 to 80:20 [72].

| **Fatty acids** | **Content [%]** |
|---|---|
| Myristic acid | <=1.0 |
| Caproic acid | <=2.0 |
| Lauric acid | <=3.0 |
| Capric acid | 20-50 |
| Caprylic acid | 50-80 |

Table 43: Estimated content of fatty acids in the used MCT oil

Table 43 describes the composition of MCT oil of the developed formulation, which derives from the performed quality tests and refers to the values of general health (GH) provided by Krankenfürsorgeanstalt (FKA) in Graz.
Compared to triglycerides containing mainly saturated long-chain fatty acids (LCT), MCT have different characteristics as listed in Paragraph 1.8.2.1, which make them preferable to the LCT. Furthermore, since 1994, the use of MCT in food products is *generally recognised as safe* (GRAS status) by the US Food and Drug Administration for the reasons explained in the table below (Table 44) [72].

- ✓ MCT originate from natural sources [65]
- ✓ MCT show low toxicity in animals [65]
- ✓ Conducted studies (in animals and humans) indicate that dietary MCT has no troublesome health effects [65]
- ✓ MCT do not result carcinogenic [65]

Table 44: Basis for GRAS determination of MCTs

The MCT oil used for the development of this formulation was provided by the company Fresenius Kabi Austria GmbH in Linz with the following identification codes:

| | |
|---|---|
| **Item number** | M011405 |
| **QSD number** | 00002808 |
| **Product identification (PID)** | 09000061 |

From the conducted measurements, literature data and optical investigations, the following relevant physical properties were determined (Table 45).

| | |
|---|---|
| **Physical State** | oily liquid |
| **Colour** | colorless or slightly yellowish |
| **Odour** | odorless |
| **Melting point [°C]** | < -5°C |
| **Density [kg/m3]** | 940 (20ºC) |
| **Viscosity** | 24 mPa*s (20ºC) |
| **Flash point [°C]** | 235 – 260 [73] |

Table 45: Physical and chemical properties of MCTs contained in the spray formulation

As described in the next paragraphs, the values regarding the melting point, the density and the viscosity were measured directly in the lab.
Regarding the chemical properties, in addition to the water insolubility, other more important indicators (Table 46), analysed by the manufacturer, were considered to evaluate oxidation stability and thus the tendency to rancidity of MCT oil.

| | |
|---|---|
| **Peroxide value (PV)** | <=1 mmol/Kg |
| **Acid value** | <=0.2 mgKOH/g |
| **Iodine value (IV)** | <=1 $gI_2$/110g |

Table 46: Some relevant chemical properties of MCTs contained in the spray formulation

Table 46 shows very low levels of peroxide value (PV), acid value, iodine value (IV), confirming a significant stability of MCT oil, as expected. In fact, the absence in the chains of fatty acids with unsaturated bonds reduces the probability of oxygen linkage.

## 2.2 Selection of the aroma

The main purpose of the new oil-spray formulation was to improve the compliance in the course of treatment, not only of kids or infants, but also of adults and elder people. For this reason, the taste was the first element to be considered, because it can compromise the compliance.

| **Tested Aroma** | **Concentration [%w/w]** |
|---|---|
| Lemon flavor 504196 ANS | 0.1-0.5-2 |
| Tetrarome (R) Citron 987317 | 0.1-0.5-2 |
| Citronova 533 Orange FAB 926477 | 0.1-0.5-2 |
| Tetrarome (R) Orange 987431 | 0.1-0.25-0.5-2 |
| **Combination of Aroma** | |
| Tetrarome (R) Orange/ Tetrarome (R) Citron | |
| 1:1 | 0.5/0.1 |
| 2:1 | 1/0.1 |
| 1:2 | 0.5/0.2 |
| 3:1 | 1.5/0.1 |
| 1:3 | 0.5/0.3 |
| Tetrarome (R) Orange/ Tetrarome (R) Citron [%w/w] | |
| 0.25/0.05 | |
| 1/0.2 | |
| 0.5/0.1 | |

Table 47: Tested flavouring agents

As shown in Table 47, four different types of flavouring extracts, produced by the company Firmenich SA, in Switzerland, were used for the test. Solutions, differently concentrated, were prepared for each of them, in order to find the most suitable one.
The aroma which gave the best feeling during tasting was the Tetrarome (R) Orange (987431) at a concentration of 0.5 % w/w. The choice of this flavour was also promoted by the presence of vitamin E (E 307 Alpha-Tocopherol), whose content is equal to 0.05 % w/w, as attested in the technical datasheet released by the manufacturer. In fact, vitamin E is helpful to counteract the oxidation processes, which both lipophilic components and vitamin $D_3$ can undergo. In the following table (Table 48), the principal technical characteristics of the chosen flavour are described.

| Specifications | Values |
|---|---|
| **Appearance** | Liquid |
| **Color** | Yellow until dark yellow |
| **Smell** | Firmenich Standard |
| **Taste** | Firmenich Standard |
| **Flash Point** | 44°C |
| **Relative Density (D20/20)** | 0.841 ... 0.851 |
| **Relative Density (d25/25)** | 0.838 ... 0.848 |
| **Refraction index (ND/20)** | 1.466 ... 1.474 |
| **Refraction index (nD/25)** | 1.464 ... 1.472 |

Table 48: Technical information about TETRAROME(R) ORANGE 987431-Firmenich

## 2.3 Selection of the emulsifier

The distribution of the sprayed oil in the mouth relies on the interfacial tension between the hydrophobic oily formulation and the hydrophilic body fluids, principally composed of water (e.g. saliva). Therefore, the role of the emulsifier is to reduce the interfacial tension and promote in vivo dispersion of the oil.

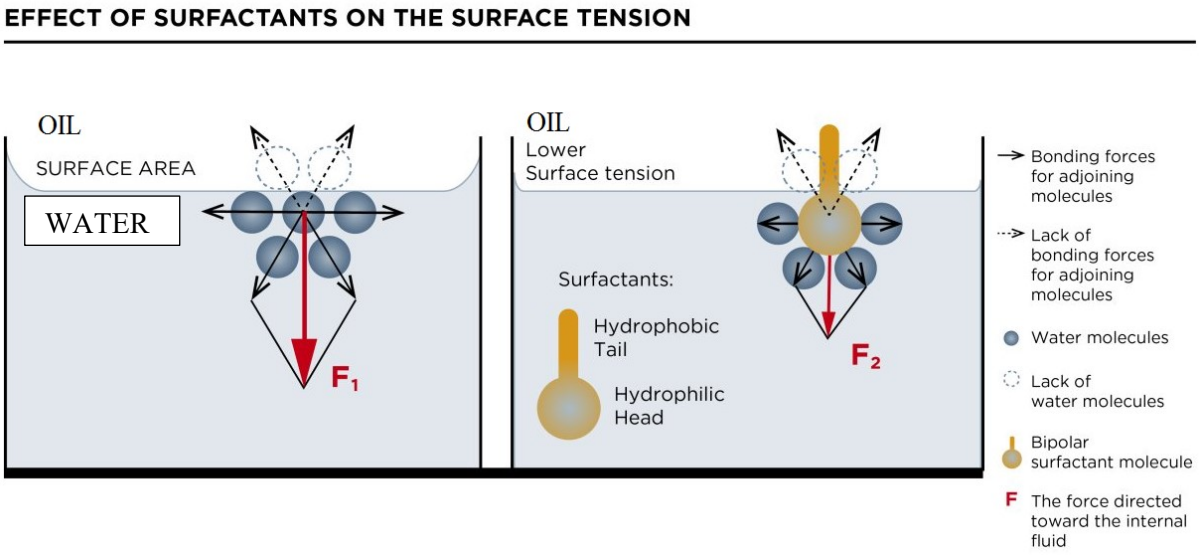



Figure 17: Effects of surfactants on the surface tension [74]

As shown in Figure 17, the surfactant, located at the contact interface between oil and water, decreases the surface tension, represented by the vector force F. The *hydrophilic head* of the surfactant causes interference in the hydrogen bonds which held the water molecules together in a surface film, determining a rearrangement, which allows the oil molecules' distribution.
The presence in the formulation of a lipophilic API, the vitamin $D_3$ and a lipophilic dispersion medium, represented by the MCT, makes necessary the choice of a lipophilic emulsifier (Paragraph 1.8.2.2) with HLB < 10.
The list in Table 49 shows some emulsifiers which have HLB in the appropriate range.

| Emulsifier | HLB Value |
|---|---|
| Mono and diglycerides | |
| • palmitate-stearate | 3 |
| • oleate | 2.5 |
| • oleate-palmitate | 2.5 |
| • stearate | 2.5 |
| • stearate-oleate | 3.8 |
| Sorbitan fatty acid esters | |
| • SPAN 20, laurate | 9 |
| • SPAN 40, palmitate | 7 |
| • SPAN 60, stearate | 5 |
| • SPAN 80, oleate | 4 |
| Polyhoxyethylene sorbitol esters | |
| • beeswax | 5 |
| Polyhoxyethylene alcohols | |
| • cetyl; stearyl; oleyl | 5 |

Table 49: List of some emulsifiers having HLB<10 [75]

The investigation was concentrated on *glycerol monostearate* and *monooleate*, because they usually show good compatibility with drugs and excipients and behave as permeation enhancers [76].

Effectively, they act in the buccal cavity by extracting the intercellular lipids of the mucosa membrane, causing the increase of the lipid layers fluidity and thus the *paracellular* permeability [63].

For each of them, two commercial forms were compared (Table 50), by testing different concentrations with the aim to investigate the saturation level of the MCT.

| **Emulsifier** | **Commercial form** |
|---|---|
| Glycerol monostearate | IMWITOR 491 CHG.607 395 E (MP 76.2°C) Sasol Germany GmbH |
| | IMWITOR 900 K CHG.602008 (MP 54-64°C) Sasol Germany GmbH |
| Glycerol monooleate | Monomuls 90-O 18 BASF |
| | Peceol™ Gattefossè |

Table 50: List of emulsifiers involved in the comparison

The samples of glycerol monostearate were prepared, by solubilizing IMWITOR 491 in MCT in hot water bath at 70°C and IMWITOR 900 K at 60°C, while stirring slowly for about 15 minutes using a glass stick.


Figure 18: Solution of MCT with IMWITOR 491 CHG.607 395 E (MP 76.2°C) Sasol Germany GmbH

As it can be seen in Figure 18, the saturation level of MCT respect to IMWITOR 491 was already reached at concentration 1% w/w after ten minutes from the mixing and the solution became more and more milky, until obtaining a complete white cloudy mixture at 5% w/w.

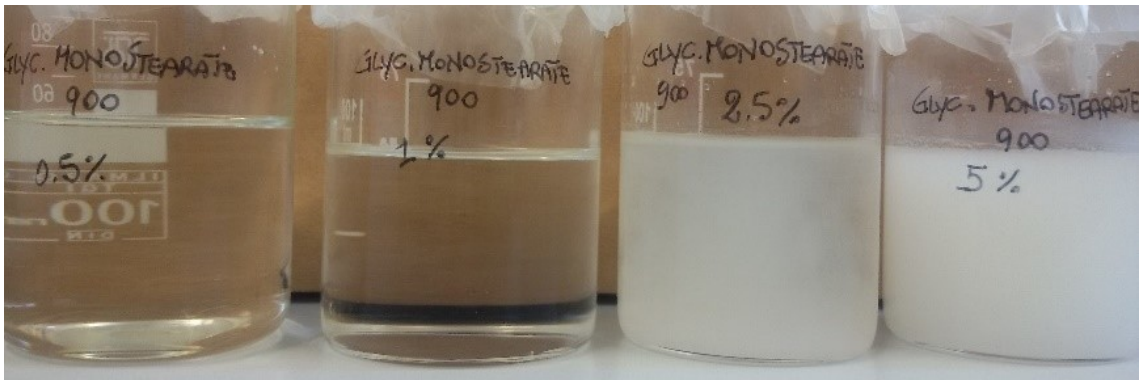



Figure 19: Solution of MCT with IMWITOR 900 K CHG.602008 (MP 54-64°C) Sasol Germany GmbH

Similar behaviour was shown in IMWITOR 900 K (Figure 19), with the only difference that the solution was still stable at 1% w/w, but starting from concentration 2.5% w/w moving to 5% w/w, it acquired the same milky appearance of IMWITOR 491.

Conversely, the solutions resulting from the dissolution of glycerol monooleate in MCT appeared clear, without any residue of precipitation.

The comparison was conducted between the Monomuls 90-O 18 BASF and Peceol™ Gattefossè. At ambient temperature (20°C) they show different physical states, because the former is solid with paste consistency, while the latter is liquid with oily consistency.

Therefore, the solubilisation of Monomuls 90-O 18 BASF was performed in hot water bath at 70°C, while stirring for 15 minutes, while Peceol™ Gattefossè was mixed with MCT, first out of the water bath and then introduced and kept in it at 50°C for only 3 minutes, always stirring until there was no more visible division between the two phases.

Figure 20 shows the behaviour of Monomuls 90-O 18 BASF in MCT. The only solution with a slight turbidity was the one at 5% w/w.


Figure 20: Solution of MCT with Monomuls 90-O 18 BASF

With respect to Monomuls 90-O 18 BASF, the Peceol™ Gattefossè solutions, presented in Figure 21, gave optimal stability until the 5% w/w.

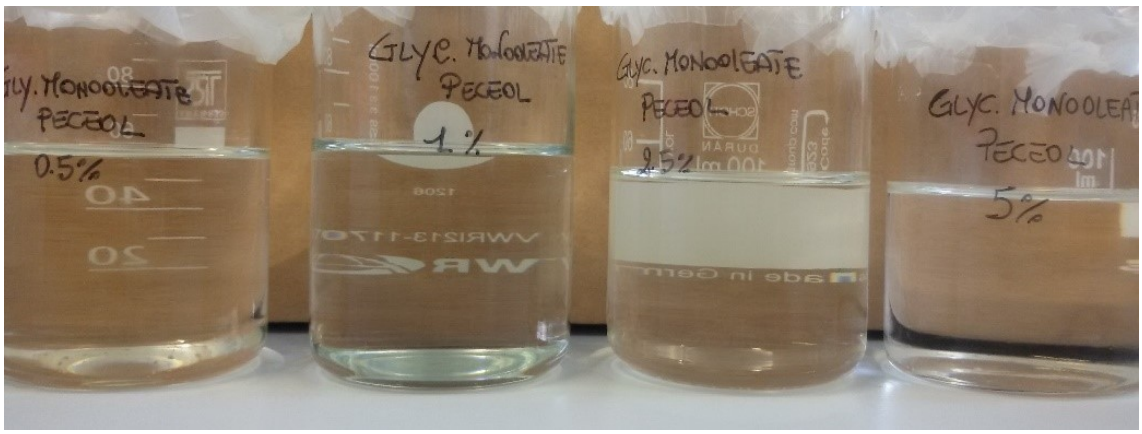



Figure 21: Solution of MCT with Peceol™ Gattefossè

By performing a control test after three days of storage at room temperature, it was seen that Peceol™ solutions, preserved their original appearance, unlike the Monomuls 90-O 18 BASF solutions, which had crystallized at 5%w/w, as it is shown in Figure 22.

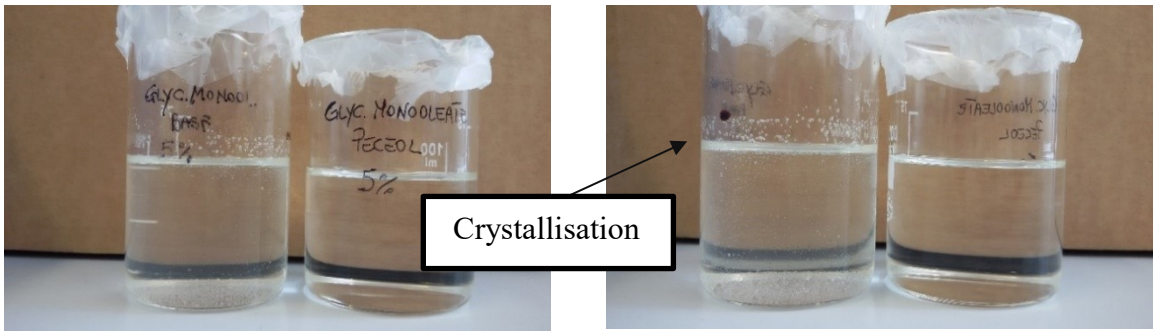



Figure 22: Comparison of 5% w/w solutions of Monomuls 90-O 18 BASF and Peceol™ Gattefossè after three days

The ease of dissolution, the clarity of solutions and the stability at high concentrations are characteristics which contributed to preferring Peceol™ to IMWITOR 491, IMWITOR 900 K, and Monomuls 90-O 18. Therefore, Peceol™ was introduced as emulsifier in the formulation of vitamin $D_3$ spray to be developed. In the table below (Table 51), the principal features of Peceol™ are listed.

| | |
|---|---|
| **Definition** | Glycerol mono-oleate (Type 40) EP / Glyceryl monooleate (Type 40) NF |
| **Solubility at 20°C (Eur. Ph.)** | Water: Insoluble<br>Mineral oils: Very soluble |
| **Appearance (at 20°C)** | Partially crystallized liquid |
| **Crystallisation temperature (first clusters)** | ~ 30° C |
| **Recommended heating temperature** | 50-60° C |
| **HLB Value** | 3 |

Table 51: Principal chemical and physical characteristics of Peceol™

As described in the Table 52, Peceol™ is mainly composed of mono and diglycerides of oleic acid (>= 60.0 %) and the similarity to the end-products of lipid digestion, makes it more tolerable by the organism.

| | |
|---|---|
| **Free glycerol content** | <= 6.0 % |
| **Total monoglycerides content** | 32.0 to 52.0 % |
| **Total diesters content** | 30.0 to 50.0 % |
| **Total triesters content** | 5.0 to 20.0 % |
| **Total glycerol content (E471)** | 16 to 33 % |
| **Palmitic acid (C16)** | <= 12.0 % |
| **Stearic acid (C18)** | <= 6.0 % |
| **Oleic acid (C18:1)** | >= 60.0 % |
| **Linoleic acid (C18:2)** | <= 35.0 % |
| **Linolenic acid (C18:3)** | <= 2.0 % |
| **Arachidic acid (C20)** | <= 2.0 % |
| **Eicosenoic acid (C20:1)** | <= 2.0 % |

Table 52: Technical data of Peceol™ released by the company Gattefossè (May 11, 2012)

The content of oleic acid as well as of linoleic and linolenic acid, which have unsaturated bonds in their lateral chains, can increase the sensitivity to oxidation reactions.
This is also attested by the values, listed in the table below, which are higher than those found for MCT oil (Paragraph 2.1).

| | |
|---|---|
| **Acid value** | <= 3.00 mgKOH/g |
| **Saponification value** | 150 to 175 mgKOH/g |
| **Iodine value** | 65.0 to 95.0 $gI_2$/100g |
| **Peroxide value** | <=12 $meqO_2$/Kg |

Table 53: Technical data of Peceol™ released by the manufacturer Gattefossè

Therefore, during formulation procedure, special precautions were taken into account. By following the handling instructions provided by Gattefossè, the heating step was performed in short intervals (e.g. ~30 seconds) and stirring the product between the intervals. Furthermore, the water bath was heated up to 50°C, hence inside the optimal range reported in Table 51, paying attention to reduce the influence of surrounding oxygen on Peceol™, by using a 50 ml Duran® bottle with a hole in the stopper, through which the glass stick was introduced for stirring.

In the literature, many case studies exist, where Peceol™ has been used as oily vehicle in self-emulsifying liquid formulations and as bioavailability enhancer for poorly soluble drugs [77].

In a particular case, novel lipid-based Amphotericin B (AmpB) oral formulations containing Peceol™ have shown to provide excellent drug solubilisation and drug stability in reproduced gastric and intestinal fluids [78]. Moreover, the co-administration of the AmpB with Peceol™ determine an increase of lymphatic transport, reducing the drug clearance due to hepatic first-pass metabolism [79].

The safety of use for Peceol™ is testified by available toxicological data and pharmaceutical products, already existing on the market [79].

# 3 Experiments and results

## 3.1 Operating procedure for the preparation

The aim of the master thesis was to develop a vitamin $D_3$ spray able to administer the required daily dosage for adults and kids suffering from hypocalcaemia. The formulation was defined taking into account the specifications provided by the company Fresenius Kabi Austria GmbH were the following (Table 54).

| |
|---|
| Formulation with 400 IU/Puff Vit $D_3$ |
| Puff a 120 µl |
| Daily dose 2 Puffs/day (800 IU/ days) |

Table 54: Specifications of Fresenius Kabi Austria GmbH for the dosage and dosing system

It was considered the conversion between the international unit (IU) and milligrams (mg). For vitamin $D_3$, 1 IU is the biological equivalent of 0.025µg and this means that 400 IU is equal to 10 µg ($=10^{-2}$ mg).

Since this amount has to be provided by on puff of 120 µl (=0.120 ml), the quantity of vitamin $D_3$ necessary to prepare a 100 ml solution is 8.3 mg. According to investigations previously conducted by the company, the vitamin $D_3$ undergoes a degradation until a value of 15% of the initial concentration in the first period after the manufacturing. Therefore, a production surcharge of the same value was counted in the amount of vitamin $D_3$.

In order to simplify the preparation, the concentrations weight over weight (w/w) were considered instead of weight over volume (w/v), assuming the density of final solution equal to 1g/ml.

In Table 55, the respective components and amounts of the formulation are listed.

| Components | Amounts |
|---|---|
| Vitamin $D_3$ | 8.3 mg |
| | Production surcharge: +15% =+ 1.245 mg |
| PECEOL™ , Gattefossè | 1% w/w |
| Tetrarome(R) Orange, Firmenich | 0.5% w/w |
| Medium chain triglycerides (MCT) | q.b. to 100 g |

Table 55: Components and corresponding amounts of vitamin $D_3$ spray formulation

The steps of preparation procedure were established by examining the physical state and the thermal behaviour of components (Table 56).

| Excipients | Crystallisation Temperature | Max heating | Flash Point |
|---|---|---|---|
| Peceol™ | ~ 30°C | 50-60° C | >200°C |
| Tetrarome(R) Orange | No data available | No data available | 44°C |
| MCT Oil | < -5°C | No data available | 235-260°C |

Table 56: Comparison of thermal behaviour of involved excipients

First, the emulsifier Peceol™ was weighed in a 50 ml Duran® bottle. Afterwards, the half of the necessary MCT oil was poured into the bottle already containing Peceol™, which was then placed in a water bath (Figure 23) heated up to 50°C, to dissolve Peceol™ crystals formed during storage. Due to oxidation sensitivity of the emulsifier (Paragraph 2.3), to minimize the interaction with oxygen, the stopper of the bottle had a hole, through which the solution was stirred by means of a glass stick for about 3 minutes, until getting a homogeneous solution.


Figure 23: Water bath, Memmert WB7

Before adding the flavour, the oily solution was left to stay for five minutes in order to bring it at room temperature and avoid the potential evaporation of Tetrarome(R) Orange at the moment of mixing. In the meantime, by working in partial absence of light, the vitamin $D_3$ was weighed separately and mixed with the remaining portion of MCT oil in a 200 ml glass Duran® bottle, which was additionally covered by silver paper to prevent the effect of filtering natural light.
Once the oily solution was cooled, the flavour was directly weighed into the same bottle containing already MCT oil and Peceol™. The mixing was then continued with the glass stick.
The obtained solution was hence poured into the bottle containing the vitamin $D_3$ and the other half of MCT oil, while stirring slowly again with a glass stick for thirty minutes to ensure an optimal mixing.
The resulting mixed solution appeared clear and with a light yellow colour (Figure 24) due to the used flavouring agent which also conferred the typical fruity smell.

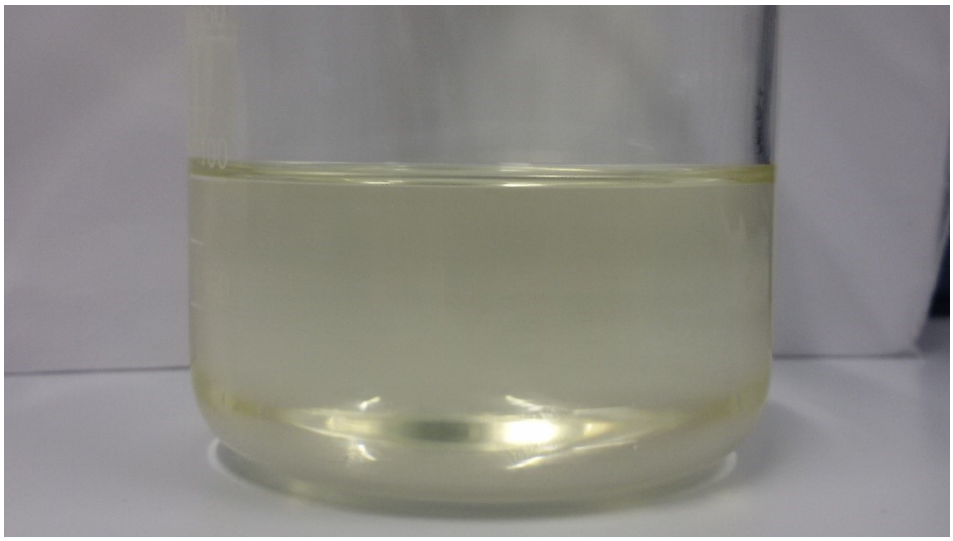


Figure 24: Solution resulting from the mixing of vitamin $D_3$, MCT oil, Peceol™ and Tetrarome(R) Orange

Afterwards, brown glass bottles of 20 ml were filled with 10 g oil and then closed by a stopper with a spray pump provided by Aptar Radolfzell GmbH (Figure 25).

Figure 25: Brown glass bottles (20 ml) with spray pump 120 µl thread and 100 mm rising pipe

## 3.2 Storage in climate cabinet at 25°C

The brown glass bottles were stored into a climate cabinet at 25°C and 60%RH immediately after the preparation. Monthly measurements of the vitamin $D_3$ content were performed by HPLC, in order to investigate the stability of the API in the formulation, together with other excipients.

## 3.3 Stability tests by RP-HPLC

### 3.3.1 Reversed phase chromatography

The *reversed phase* high-performance liquid chromatography (*RP-HPLC*) is a mechanism of separation, where the used *stationary phase* is less polar than the *mobile phase*. Therefore, it is the opposite of the normal-phase chromatography [80].
The hydrophobic stationary phase is constituted by an inert micro-particulate support of silica matrix, porous and insoluble[80]. It is bound to ligands, characterised by organic compounds with octyl ($C_8$) and octadecyl groups ($C_{18}$) [81]. The following table gives an overview of the most common stationary phases.

| **Stationary phase** | Decreasing hydrophobicity/ Increasing polarity ↓ | Increasing retention of non-polar analytes ↑ |
|---|---|---|
| $C_{18}$ | | |
| $C_8$ | | |
| $C_4$ | | |
| Cyano | | |
| Phenyl | | |
| Amino | | |

Table 57: Typical stationary phases for RP-HPLC [80]

The polar mobile phase is usually a mixture of aqueous and organic solvents, such as acetonitrile, methanol, and tetrahydrofuran (THF) [81]. As regards the elution step, it can be performed either in an *isocratic* way, meaning that the water-solvent composition is always the same during the separation process or using a *solution gradient*, with change in the water-solvent composition, aiming to decrease the polarity [81].

Figure 26: Agilent 1260 Infinity, diode array detector

The RP-HPLC for the analysis of vitamin $D_3$ spray was carried out by the *Agilent 1260 Infinity* (Figure 26), but using two different types of detectors for technical reasons: the diode array detector (DAD) for the first three months; the variable wavelength detector (VWD) for the remaining three months.
In the table below, the components of the chromatographic equipment are listed.

| **Components** | **Agilent 1260 Infinity DAD** | **Agilent 1260 Infinity VWD** |
|---|---|---|
| Pump | G5611A-1260 Bio Quat Pump | G13111C-1260 Quat Pump VL |
| Autosampler | G5667A-1260 Bio HiP ALS | G1329B-1260 ALS |
| Thermostat | G1330B-ALS Therm | G1330B-1290 Thermostat |
| Column Oven | G1316A-1260 TCC | G1316A-1260 TCC |
| Detector | G4212B-1260 DAD | G1314F-1260 VWD |

Table 58: Components of the HPLC equipment

### 3.3.2 Method of analysis

The analytical method was developed on the base of a method, provided by the company Fresenius Kabi Austria GmbH, but some changes were made in order to optimize the separation phase and shorten the method. Its description is shown in Table 59.

| | |
|---|---|
| **Column** | ZORBAX Eclipse Plus ($C_{18}$) 250x4.6mm (5µm) with guard column: 10x4 mm; |
| **Pressure** | 56 bar |
| **Flow rate** | 1.5ml/min |
| **Column temperature** | 35°C |
| **Injection volume** | 5 µl |
| **Eluent A** | 5 vol. Parts HAc solution (1%) + 5 vol. Parts THF + 90 vol. Parts ACN;<br>HAc solution (1%): 1 Part HAc (glacial) + 99 Parts water |
| **Eluent B** | 95 vol. Parts THF + 5 vol. Parts HAc 1% |
| **Solving compound** | 9 Parts 2-isopropanol (2-PrOH) + 1 Part n-Pentane |
| **Detector** | 1260 Infinity-DAD<br>1260 Infinity-VWD<br>Reference wavelenght: 264 nm; Other analysed wavelengths: 215, 230, 273 nm |
| **Retention time** | ~9.2 minutes (System II, STD) |

Table 59: Method for the stability analysis of vitamin $D_3$ spray

The separation process was conducted using the *solution gradient* operation way, where a gradient of 90% and 10% respectively between eluent A and eluent B was established (Table 60).

| **Solvent Composition** | | | |
|---|---|---|---|
| **Channel** | **Name 1** | **Used** | **Percent [%]** |
| A | Eluent A | Yes | 90.0 |
| B | Eluent B | Yes | 10.0 |
| C | Methanol | Yes | 0.0 |
| D | Water | Yes | 0.0 |

Table 60: Solvent composition of the method

The change of gradient during time is listed in Table 61.

| **Timetable** | | | | |
|---|---|---|---|---|
| **Time** | **A** | **B** | **C** | **D** |
| **[min]** | **[%]** | | | |
| 1.00 | 90.0 | 10.0 | 0.0 | 0.0 |
| 14.00 | 83.0 | 17.0 | 0.0 | 0.0 |
| 15.00 | 50.0 | 50.0 | 0.0 | 0.0 |
| 17.00 | 50.0 | 50.0 | 0.0 | 0.0 |
| 17.50 | 90.0 | 10.0 | 0.0 | 0.0 |

Table 61: Gradient change of the solvent composition over time

The solving compound mentioned in Table 59 was not involved in the elution phase, but it was adopted to prepare standards for the calibration and to solubilize the oily formulation, in order to promote then the partition of the hydrophobic vitamin $D_3$ between the stationary and mobile phase.

As concerns the standard solutions, the stock solution was prepared by weighing with precision of 0.1 mg an amount of vitamin $D_3$ reference substance (see Materials) between 34-35 mg, then solubilised into a 50 ml volumetric flask with solving compound.

From the stock, 1 ml was taken and dissolved by solving compound into a 20 ml volumetric flask, forming the first standard solution. The other four solutions were obtained by taking 10 ml of the previous solution and diluting it up to 20 ml with solving compound in a volumetric flask. Five standard solutions were prepared for each analysis.

According to the method, the spray formulation was tested by taking a sample amount in the range of 1.71-2.09 g, exactly weighed to 0.001g, by means of *EDOS Advanced Pipetting Station* (Figure 27, a) using pipette tips of the type *Combitips advanced®2.5 mL* (Figure 27,b).

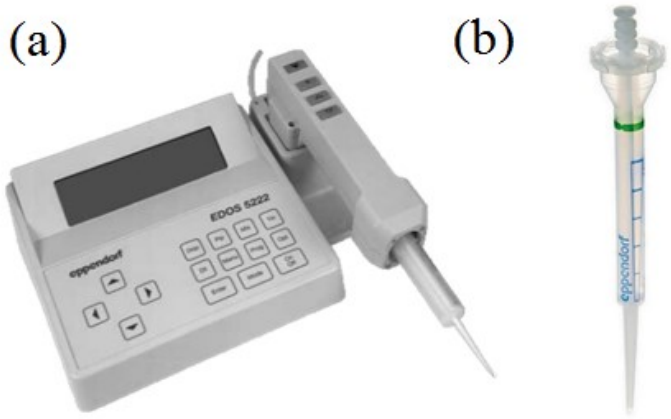



Figure 27: Instruments used for the weighing of vitamin $D_3$ spray samples:
(a) Eppendorf® EDOS® 5222 Advanced Pipetting Station;
(b) Eppendorf® Combitips advanced® 2.5 mL [82]

Afterwards, the sample amount was dissolved into a volumetric flask with the solving compound up to 10 ml and then distributed into amber vials.

As shown in Table 59, the injection volume was set at 5 µl and three consecutive injections were performed, both for the standards and the samples. The stop time for each run was 22.50 minutes.

### 3.3.3 Analysis of the absorption spectra

The RP-HPLC method of analysis, developed for testing the stability of formulation, was focused exclusively on the determination of the vitamin $D_3$ amount, the only API.
Therefore, in order to get a better separation of vitamin $D_3$'s peak, the analysis of absorption spectra for all formulation components was performed using an Implen Nanophotometer type 7122.
The instrument was calibrated before being used in the range between 200 nm and 800 nm for a pathlenght of 10 mm. Afterwards, samples were prepared by dilution with the solving compound (Paragraph 3.3.2), which was measured as a *blank*, whose resulting spectrum is shown in the figure below (Figure 28).

| Peak (nm) | Absorbance (A) |
|---|---|
| 217 | 0.070 |

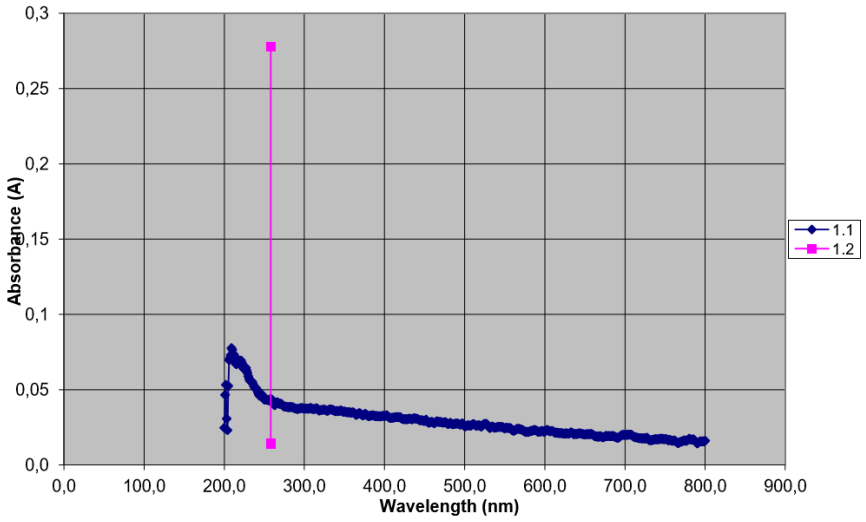



Figure 28: Absorption spectrum of the solving compound (blank) with n-pentane and isopropanol

Firstly, the lipophilic excipients, such as MCT oil and Peceol™, were measured. 1 ml of MCT oil, once diluted 1:20 with solving compound, was introduced by means of calibrated pipette into the cuvette, which was then placed inside the dedicated chamber. Figure 29 shows the resulting spectrum of MCT oil.

| Peak (nm) | Absorbance (A) |
|---|---|
| 231 | 2.127 |
| 273 | 0.153 |

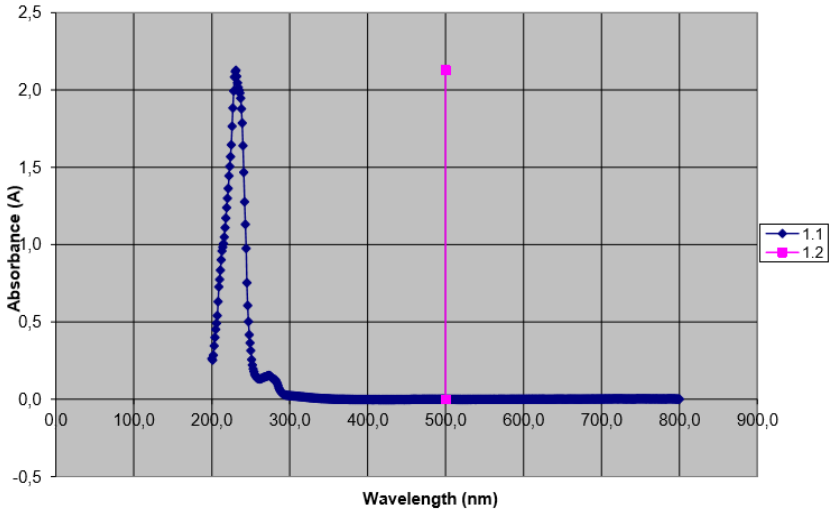



Figure 29: Absorption spectrum of MCT oil

As well as for the MCT oil also the Peceol™ was diluted before the measurement with solving compound, but at the ratio 1:50 and its spectrum is represented in the Figure 30.

| Peak (nm) | Absorbance (A) |
|---|---|
| 230 | 0.772 |
| 269 | 0.513 |

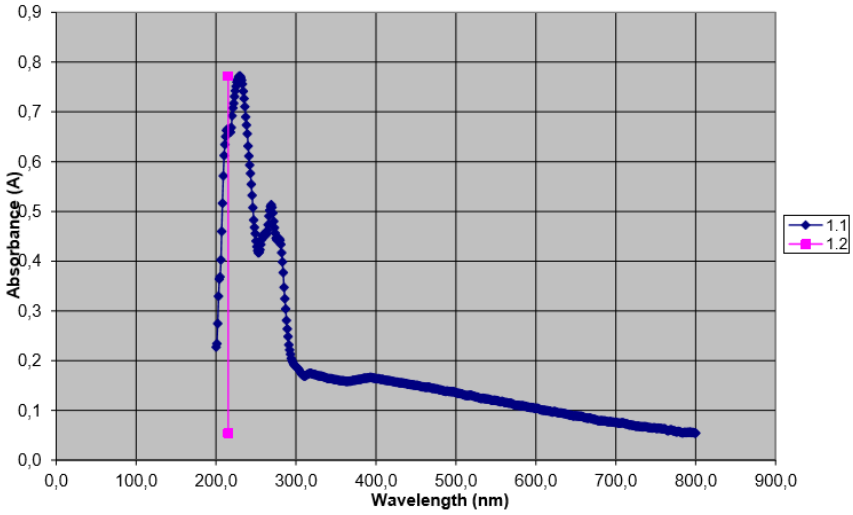



Figure 30: Absorption spectrum of Peceol™

Peceol™ showed a maximum at 230 nm, which is very close to that of MCT oil at 231 nm.

However, this result had no significant effect on the HPLC analysis, as shown in the following paragraphs, because the characteristic peak of vitamin $D_3$ was always separated from other excipients peaks. Before measuring the total formulation with vitamin $D_3$, a test was conducted on the solution containing MCT oil, Peceol™ and the flavour Tetrarome(R) Orange (Figure 31).

| Peak (nm) | Absorbance (A) |
|---|---|
| 217 | 1.222 |
| 230 | 2.205 |

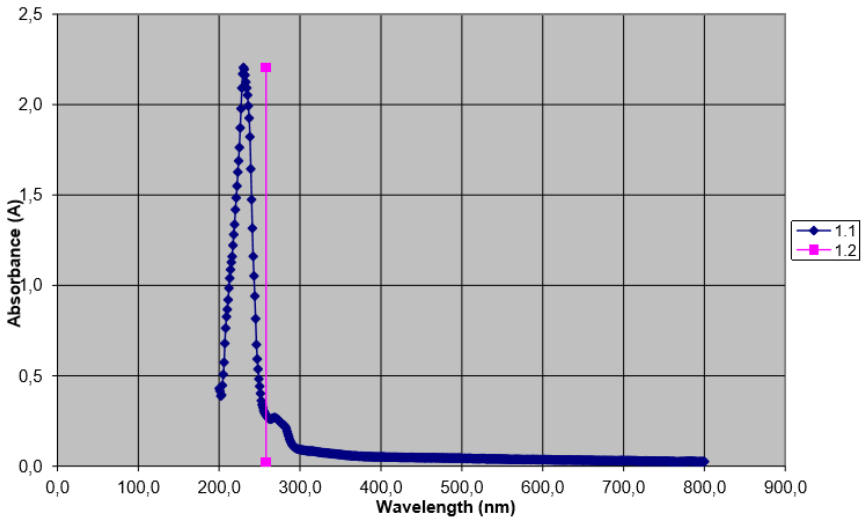



Figure 31: Absorption spectrum of the solution MCT oil, Peceol™ and Tetrarome(R) Orange

From this analysis, it resulted that the absorption spectrum of lipophilic excipients (MCT oil, Peceol™) and the flavour together had maximum values at 217 nm and 230 nm, as successively confirmed by testing the complete formulation (Figure 32).

The values indicated in Figure 32 were then used for the analytical investigation and in particular the 262 nm value was relevant for the quantitative detection of vitamin $D_3$. Referring to the analytical method of the company, this value was adopted to 264 nm instead of 262 nm.

| Peak (nm) | Absorbance (A) |
|---|---|
| 217 | 1.248 |
| 230 | 2.123 |
| 262 | 0.266 |

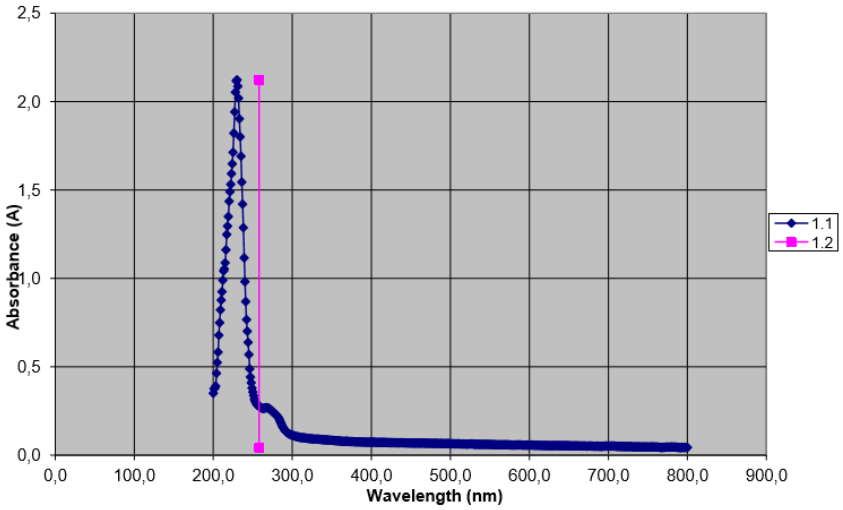



Figure 32: Absorption spectrum of the complete formulation with MCT oil, Peceol™, Tetrarome(R) Orange and Vitamin $D_3$

### 3.3.4 Monthly control of samples stored at 25°C, 60%RH

The chromatographic analysis was performed for samples of vitamin $D_3$ spray, deriving from two formulations, one prepared in July and the other one in September 2016. Therefore, it was possible to investigate the stability for six months in the case of the preparation of July and to compare it with the other one for the first four months.
Table 62 gives a report of the weighed amounts of components for both formulations.

| **Preparation July 2016** | **Preparation September 2016** |
|---|---|
| **Vitamin $D_3$ desired value (D.V.)** | |
| 8.3 mg<br><br>Over dosage: +15% = + 1.245 mg | |
| 9.545 mg | |

| **Preparation July 2016** | | | **Preparation September 2016** | |
|---|---|---|---|---|
| **Vitamin $D_3$ actual value (A.V.)** | | | | |
| 9.6 mg | | | 9.5 mg | |
| **D.V.** | **A.V.** | **Excipients** | **D.V.** | **A.V.** |
| 1 g | 1.0162g | PECEOL™, Gattefossè 1% w/w | 1 g | 1.0134g |
| 0.5 g | 0.5026 g | Tetrarome(R) Orange, Firmenich 0.5% w/w | 0.5 g | 0.5001g |
| 98.49 g | 98.49 g | Medium chain triglycerides (MCT) q.b. to 100 g | 98.49 g | 98.49 g |
| 100 | | Total weight (g) | 100 | |

Table 62: Overview of quantities weighed for each component

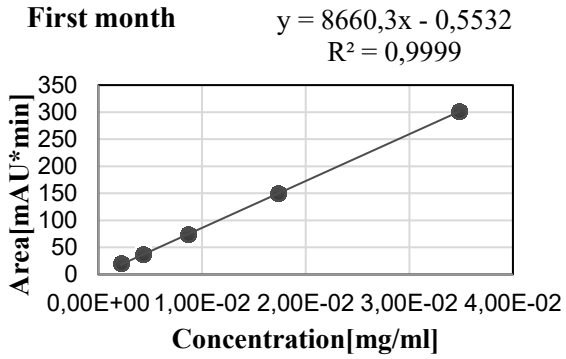
### 3.3.4.1 Quantitative determination of vitamin $D_3$

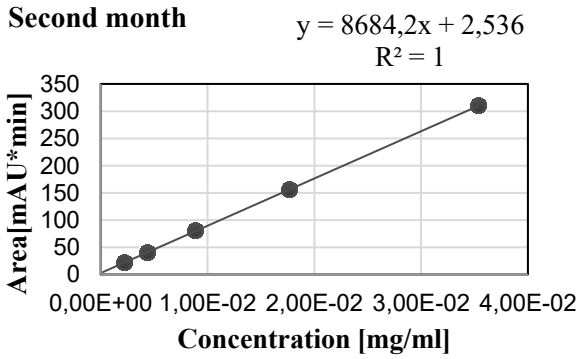
The vitamin $D_3$ content was investigated, in order to monitor a possible decomposition of the API occurred in the formulated spray during the storage period.
The calibration of the HPLC was performed each month, following the procedure explained in Paragraph 3.3.2. The weighed amounts for the stock solutions and the concentrations of relative standards are listed in Table 63.

| | Standards for calibration | | | | | |
|---|---|---|---|---|---|---|
| | First month | Second month | Third month | Fourth month | Fifth month | Sixth month |
| | Weighed vitamin $D_3$ for stock solution [mg] | | | | | |
| | 34.8 | 35.4 | 35.3 | 35.4 | 35.4 | 35.0 |
| **Concentrations [mg/ml]** | | | | | | |
| **STD 1** | 3,48E-02 | 3,54E-02 | 3,53E-02 | 3,54E-02 | 3,54E-02 | 3,50E-02 |
| **STD 2** | 1,74E-02 | 1,77E-02 | 1,77E-02 | 1,77E-02 | 1,77E-02 | 1,75E-02 |
| **STD 3** | 8,70E-03 | 8,85E-03 | 8,83E-03 | 8,85E-03 | 8,85E-03 | 8,76E-03 |
| **STD 4** | 4,35E-03 | 4,43E-03 | 4,41E-03 | 4,43E-03 | 4,42E-03 | 4,38E-03 |
| **STD 5** | 2,18E-03 | 2,21E-03 | 2,21E-03 | 2,21E-03 | 2,21E-03 | 2,19E-03 |

Table 63: Stock solutions and standards prepared monthly for the HPLC analysis

Next, a summary of calibration curves is given (Figure 33), resulting from the linear regression between the concentrations of above mentioned standard solutions and the measured, relative areas of vitamin $D_3$ peaks.



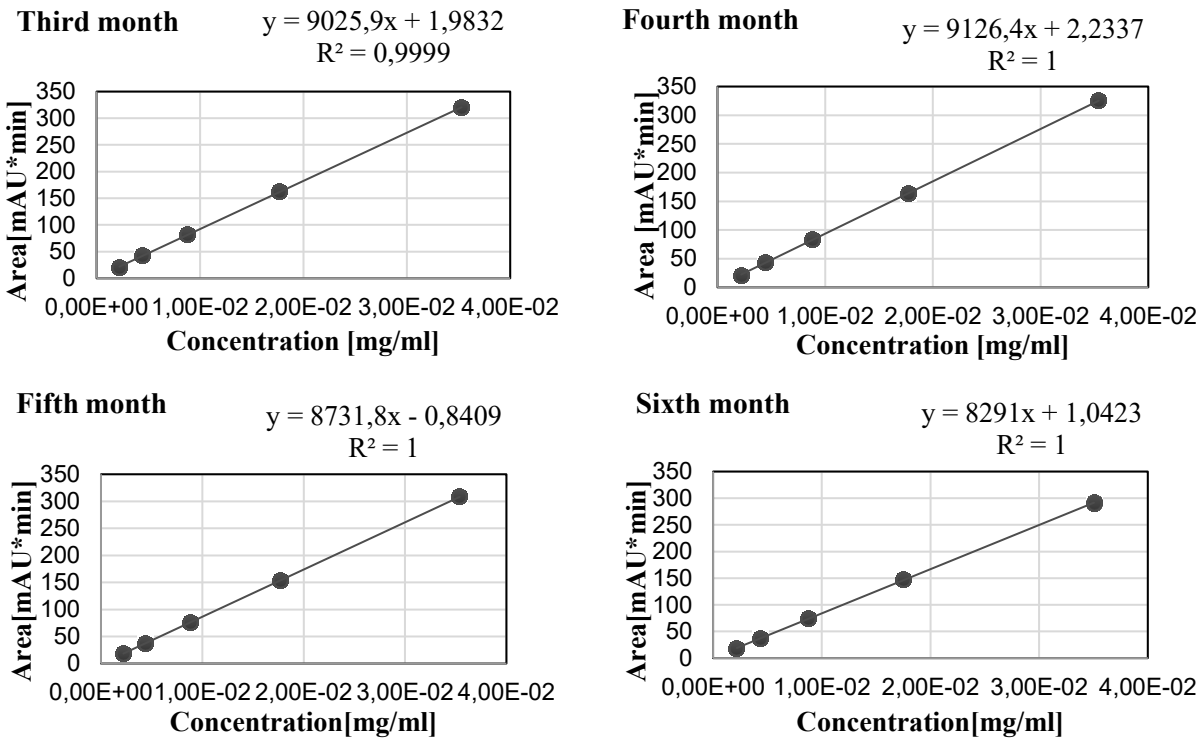



Figure 33: Summary of calibration curves

As it can be seen in Figure 33, the coefficient of determination $R^2$ had always a value around one, testifying that the regression line fitted well the real data points and hence ensuring the statistical goodness of the analysis.

For each formulation, the starting amount of vitamin $D_3$ was determined directly after manufacturing (Time:0) and then, in the following months, the recovery percentage respect to the initial weighed amount (Table 62) was determined, taking samples (Paragraph 3.3.2) of sprays, stored in the climate cabinet at 25°C and 60%RH.

The data of the analysis, related to the formulation prepared in July, were collected over six months and are shown in Table 64.

| **Formulation of July (9,6 mg)** | | | | |
|---|---|---|---|---|
| **Time** [Months] | **Weighed sample** [g] | **VitD$_3$ in 100 g** [mg] | **Recovery** [%] | **(X(VitD$_3$/100g)-XMean)^2** [mg^2] |
| 0 | 2,083 | 9,541 | 99% | 0,00056 |
| 1 | 1,8771 | 9,394 | 98% | 0,02916 |
| 2 | 1,8762 | 9,123 | 95% | 0,19540 |
| 3 | 1,8714<br>1,85 | 9,472 | 99% | 0,00871 |
| 4 | 1,861<br>1,8597 | 9,712 | 100% | 0,02169 |
| 5 | 1,7783<br>1,8819 | 9,847 | 103% | 0,07946 |
| 6 | 1,8622 | 9,866 | 103% | 0,09050 |
| **Mean of Vitamin D$_3$ in 100 g** | | [mg] | 9,565 | |
| **Sum of squared differences** | | [mg^2] | 0,425 | |
| **For n-1 data** | | | | |
| **Variance σ^2** | | [mg^2] | 0,070 | |
| **St. Deviation σ** | | [mg] | 0,266 | |

Table 64: Report of data collected over six months for the formulation of July

As it can be already seen in Table 64, the content of vitamin $D_3$ in the formulation presented a slight decrease for the first three months. However, the values oscillated almost in the same range and never exceeded 100% (9,6 mg) ± 5%.

From data, mean and standard deviation included in Table 64, it was possible to obtain the control chart of Figure 34. It allows evaluating and define the range of values, where the change of vitamin $D_3$ content can be considered acceptable in terms of quality.

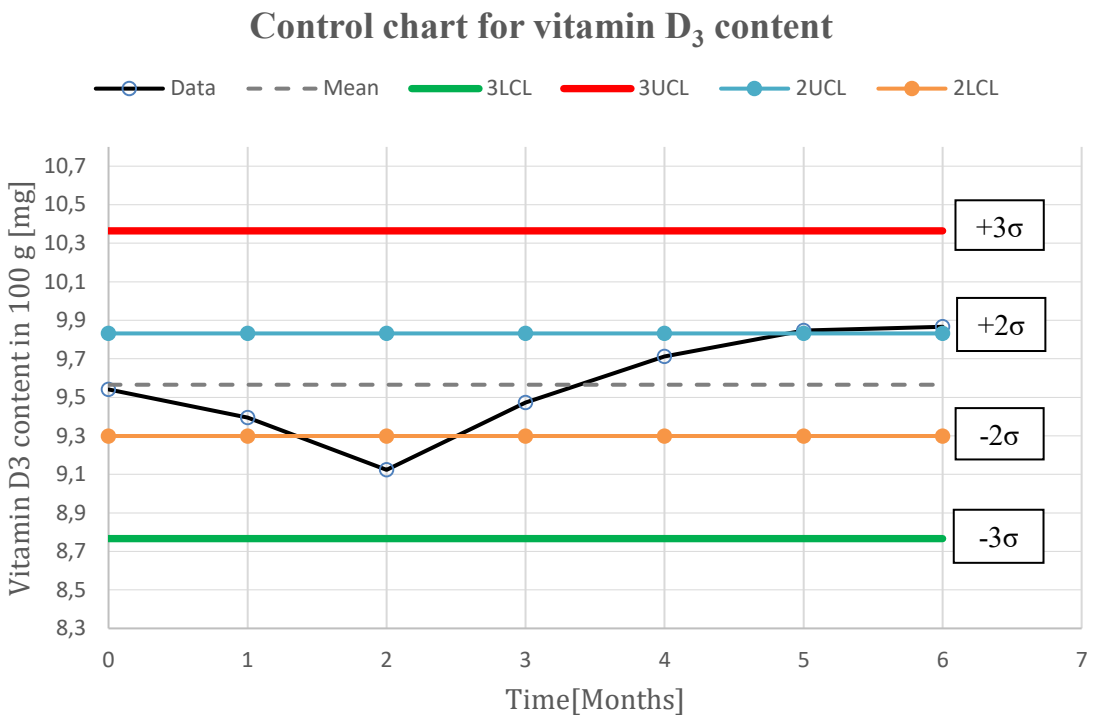


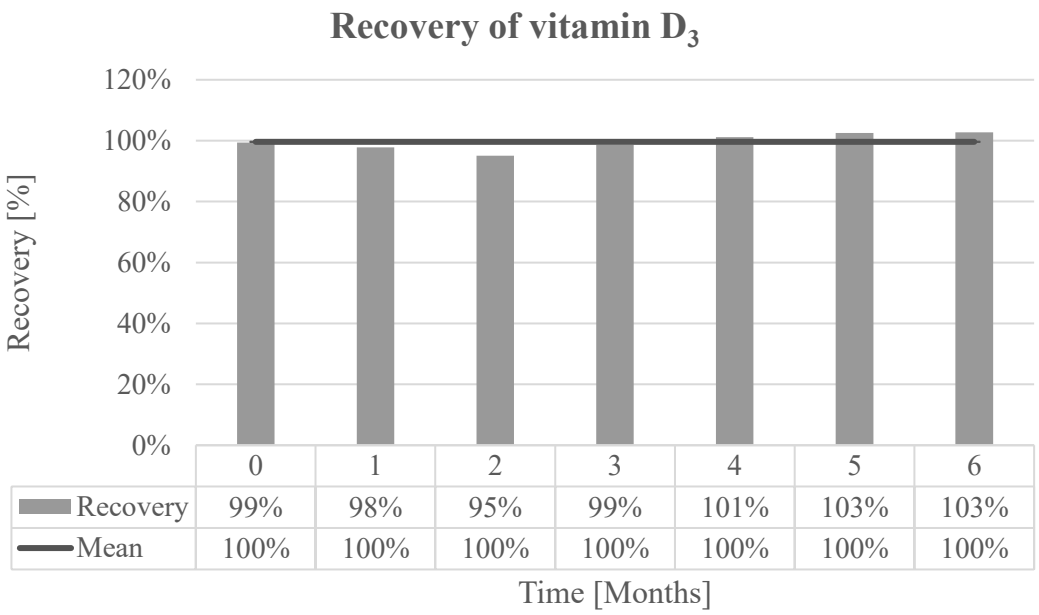

Figure 34: Variation of vitamin $D_3$ content in the formulation of July during six months

To this purpose, the upper control limit (UCL) and the lower control limit (LCL) indicate the highest and the lowest level of variability, detected for vitamin $D_3$ content within six months. The control limits represent the variation of data from the mean, considering two and three times standard deviation above and below it. They are obtained by the following calculations:

| **2σ** | **3σ** |
|---|---|
| 2UCL=Mean +2σ | 3UCL=Mean +3σ |
| 2LCL=Mean -2σ | 3LCL=Mean -2σ |

Observing the control chart, it results evident that vitamin $D_3$ was outside the 2LCL in the second month and exactly on the limit in the sixth month. Regarding the other months, it can be observed a slight fluctuation around the mean value.

The points out of range can be associated with the accuracy of the analytical instrument, which could have been compromised, e.g. because the column underwent an ageing process due to consecutive uses. Taking into account the 3UCL and 3LCL, no value was outside the limits and the content of vitamin $D_3$ showed a low variability, assuming always values close to each other and to the mean.




| | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Recovery | 99% | 98% | 95% | 99% | 101% | 103% | 103% |
| Mean | 100% | 100% | 100% | 100% | 100% | 100% | 100% |

Figure 35: Progress of recovery during the six months and relative mean

From the consideration of the mean for the monthly recovery of vitamin $D_3$ (Figure 35), it results that the developed formulation guarantees a good stability within six months. In fact, the calculated mean is equal to 100%. For the analytical deductions explained above, despite uncontrolled factors affected somehow the exact detection of vitamin $D_3$ amount, it can be inferred that the analytical method was good and the obtained data attested the stability of spray formulation.

In order to verify the reproducibility of the analysis, a comparison was conducted between the formulations prepared in July and in September, taking into account the vitamin $D_3$ content of the first four months (Table 65).

| **Weighed Vitamin $D_3$ [mg]** | **Formulation of July 9,6** | | **Formulation of September 9,5** | |
|---|---|---|---|---|
| **Time** [Months] | **$VitD_3$ in 100 g** [mg] | **(X($VitD_3$/100g)-Xmean)^2** [mg^2] | **VitD3 in 100 g** [mg] | **(X($VitD_3$/100g)-Xmean)^2** [mg^2] |
| 0 | 9,541 | 0,00012 | 9,4 | 0,01596 |
| 1 | 9,394 | 0,01839 | 9,362 | 0,02627 |
| 3 | 9,471 | 0,00338 | 9,683 | 0,02524 |
| 4 | 9,712 | 0,03328 | 9,653 | 0,01679 |
| **Mean [mg]** | 9,530 | | 9,524 | |
| **Sum of squared differences [mg^2]** | 0,055 | | 0,084 | |
| **For n-1 samples** | | | | |
| **Variance σ^2 [mg^2]** | 0,018 | | 0,028 | |
| **St.Deviation σ [mg]** | 0,135 | | 0,167 | |

Table 65: Data collected in the first four months for the two formulations

The data listed in Table 65 were graphically represented in Figure 36 and 37.

From Figure 36, it can be deduced that the variation of vitamin $D_3$ content showed a little fluctuation between the formulations, during the control period of four months.

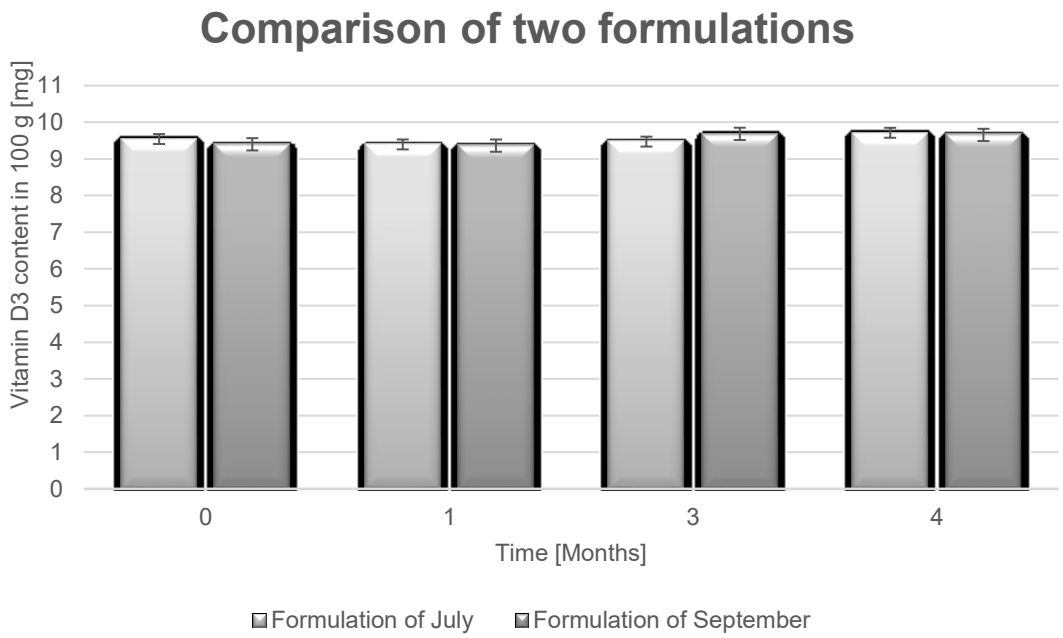



Figure 36: Comparison of the change of vitamin $D_3$ content in the first four months between the formulation of July and that of September

The variability of the content was further investigated considering the mean and the standard deviation (Table 65) of the data (Figure 37). In general, the variations were found to be not statistically significant.

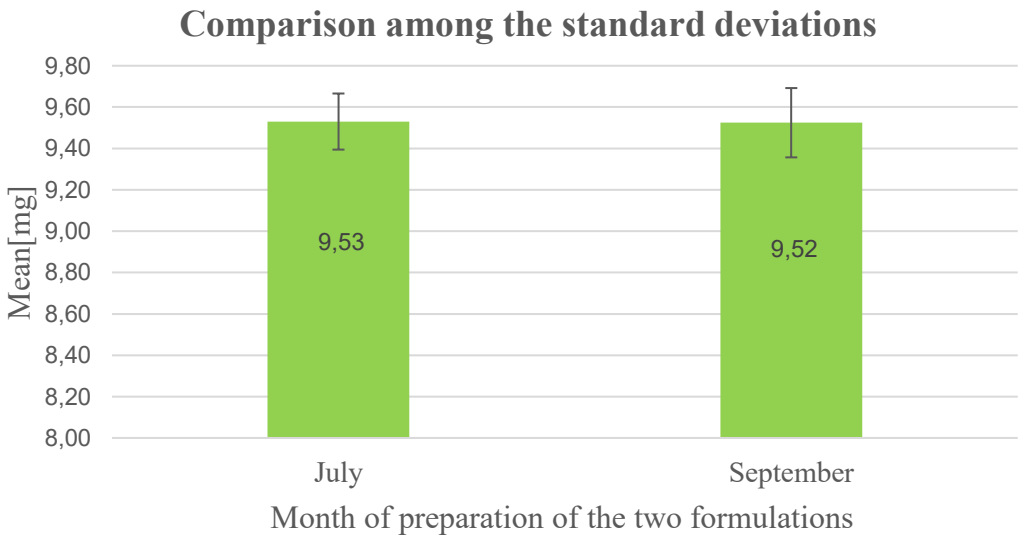



Figure 37: Comparison of standard deviations for the first four months

### 3.3.4.2 Qualitative evaluation of chromatograms

The evaluation of stability was also performed by considering the change in chromatograms, deriving from the analysis conducted during the six months.

No considerable variation of the peak aspect was detected for vitamin $D_3$ and no new peaks, able to absorb in the same region of vitamin $D_3$ and with similar retention time, appeared.

Therefore, only few chromatograms are shown below, in order to underline the apparent absence of decomposition and relative products, in the stored vitamin $D_3$ sprays.

The analysis of peaks was conducted for the lipophilic excipients, such as MCT oil and Peceol™, and the whole formulation. In order to get detectable peaks, MCT oil solution was prepared diluting 1 ml oil up to 20 ml with the solving compound, described in Paragraph 3.3.2.

Figure 38 shows the chromatogram resulting from MCT oil.

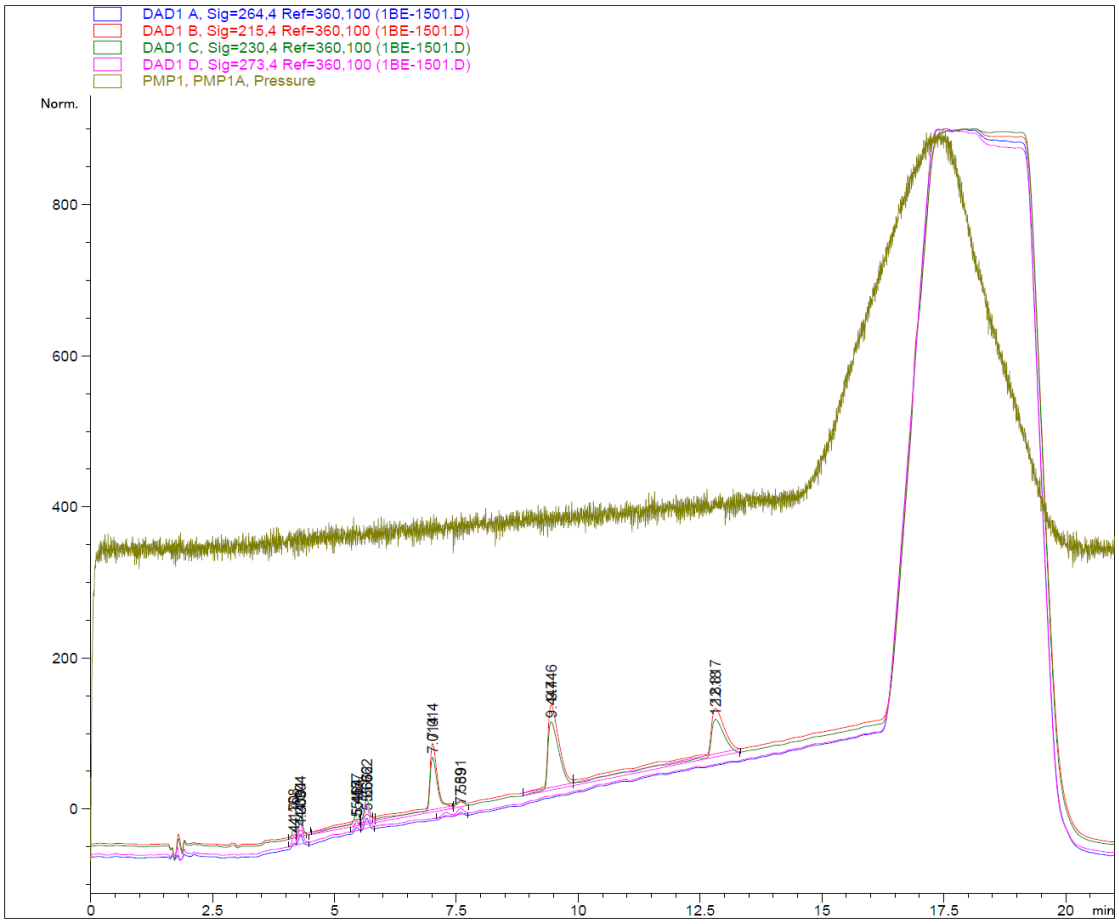



Figure 38: Chromatogram of MCT oil

The detection was performed by diode array detector (DAD) for four different wavelengths (215, 230, 264, 273 nm), which were found by the analysis of the absorption spectra (Paragraph 3.3.3) and thus considered useful for the identification of peaks.

As it is evident in Figure 38, the MCT oil mostly absorbs at 215 and 230 nm, for this reason it does not interfere with the peak of vitamin $D_3$.

As well as MCT oil, also 1 ml of Peceol™ was dissolved in the solving compound, by diluting 1:50.

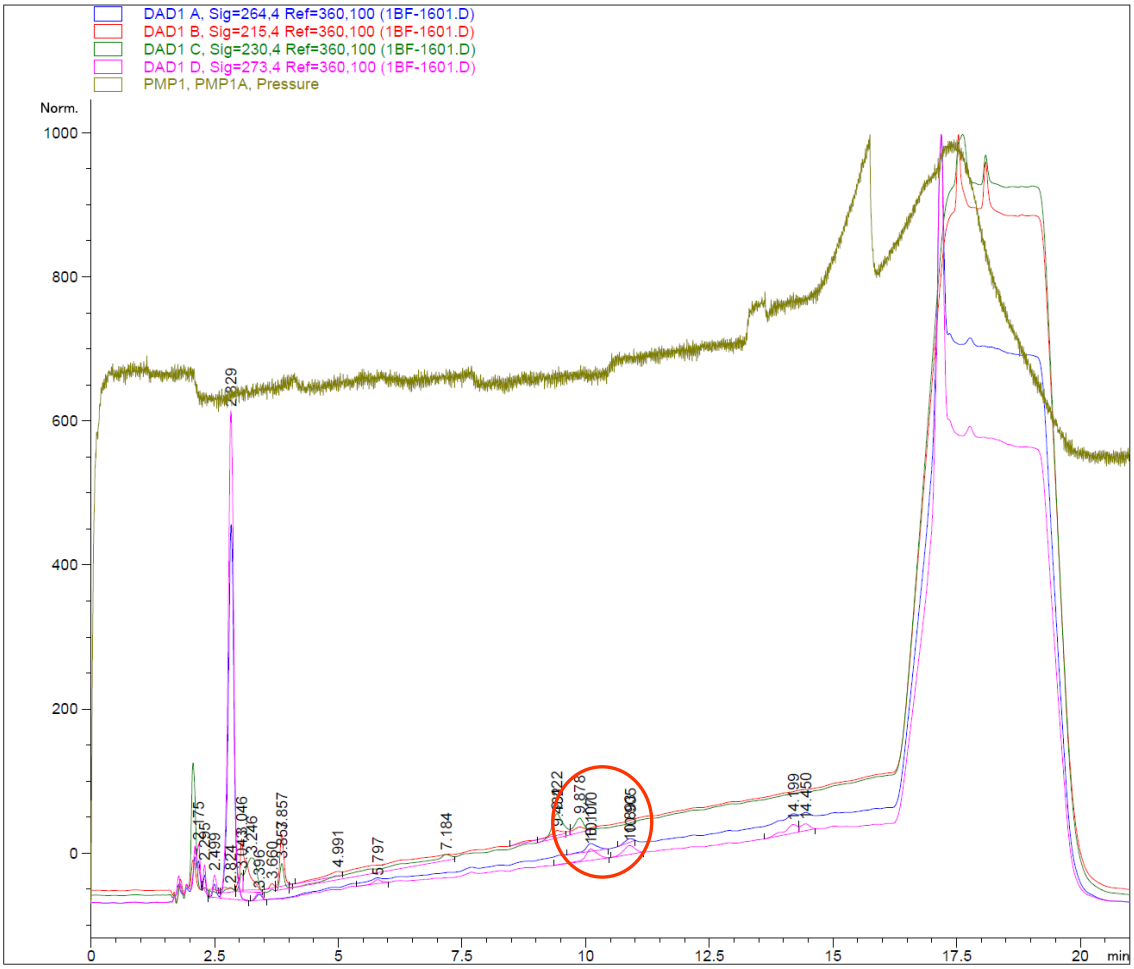



Figure 39: Chromatogram of Peceol™

As shown in Figure 39, the chromatogram of Peceol™, due to the different chemical composition, is more complex respect to MCT oil. However, like MCT oil, Peceol™ absorbs in a different region (215, 273 nm) than vitamin $D_3$ and the peaks can be identified.

Furthermore, the mixture of MCT oil and Peceol™ was analysed to understand, if possible mutual interactions and following changes could happen in the final formulation of vitamin $D_3$ spray.

Looking at Figures 39-40 (red circle), it is possible to notice some changes of the composition which can be correlated to a different concentration of components in the mixed solution, respect to the single one. Peaks which are present in the chromatogram of the single excipient are not lost, but they combine to each other, somehow by overlapping. The red circle in Figure 39 indicates two peaks, which can be found fused together in the chromatogram about the mixed solution, MCT oil and Peceol™ (Figure 40). Another difference is represented by the peak at 2.5 minutes, which is still detected, although its height is decreased.

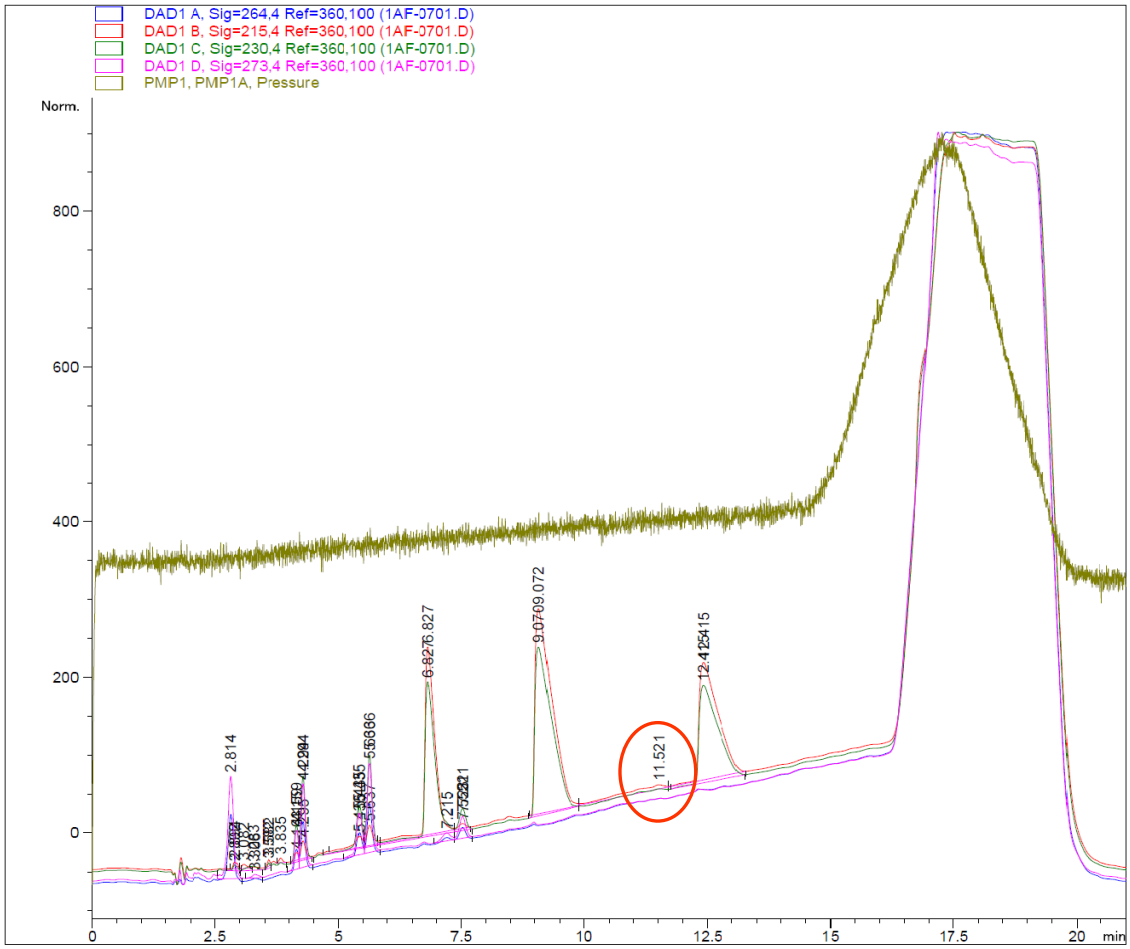



Figure 40: Chromatogram of solution containing MCT oil and Peceol™

After having analysed the lipophilic excipients, the chromatogram of the final formulation was considered in order to identify the vitamin $D_3$ peak and its change, as well as in order to distinguish the peaks belonging to other components.

The peak characteristic for vitamin $D_3$ was at 10.350 minutes and 264 nm, even though it was detected with similar retention time at 230 and 273 nm (Figure 41).

In Figure 41, the peaks corresponding to the components of formulation are indicated. It is clear that there is no interference with the peak of vitamin $D_3$, which is baseline separated from the others. In particular, after one month from the manufacturing, no evidence of decomposition appeared either by new peaks, or by changing peak characteristics.

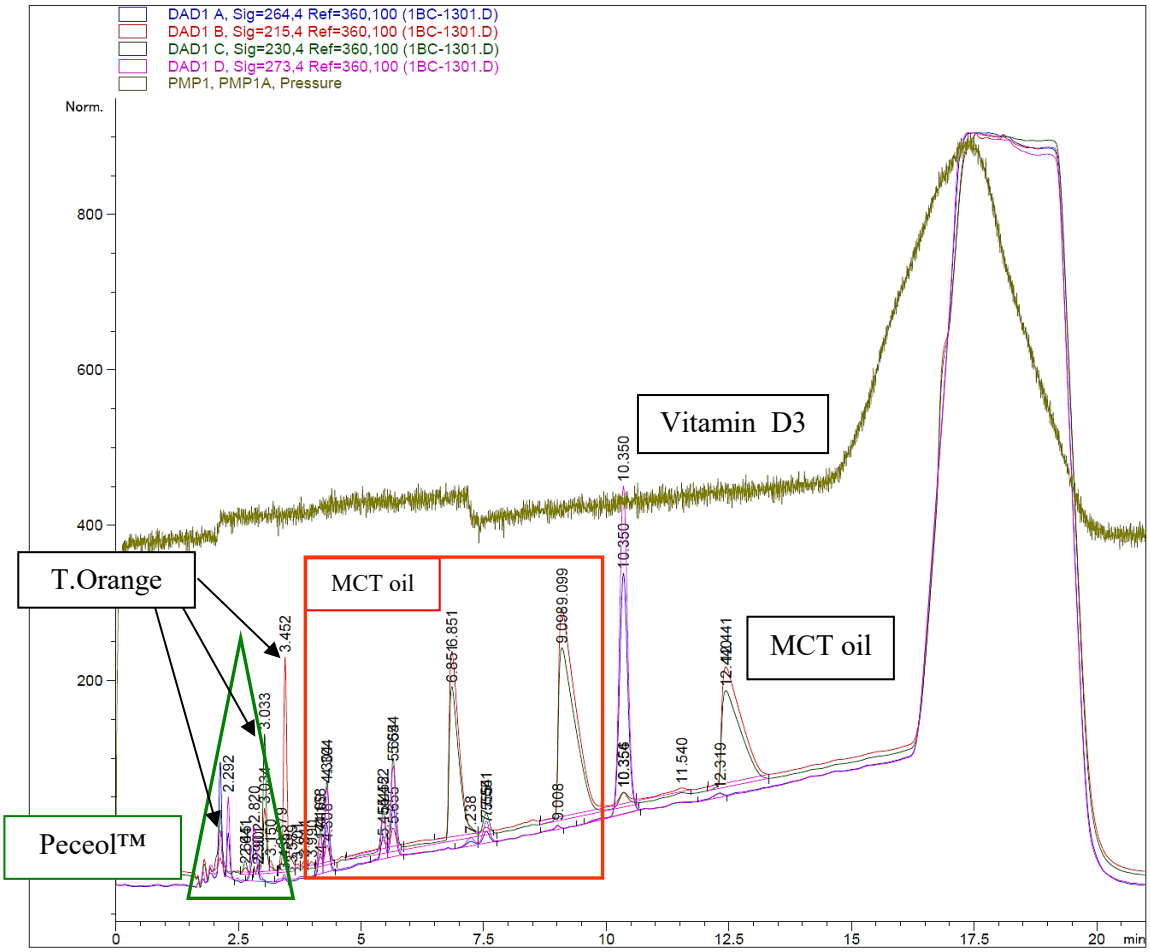



Figure 41: Chromatogram of the formulation with vitamin $D_3$, MCT oil, Peceol™ and Tetrarome (R) Orange (First month)

The chromatograms resulting from the monthly analysis did not show relevant differences, as it can be observed in Figure 42, about the analysis conducted after three months.

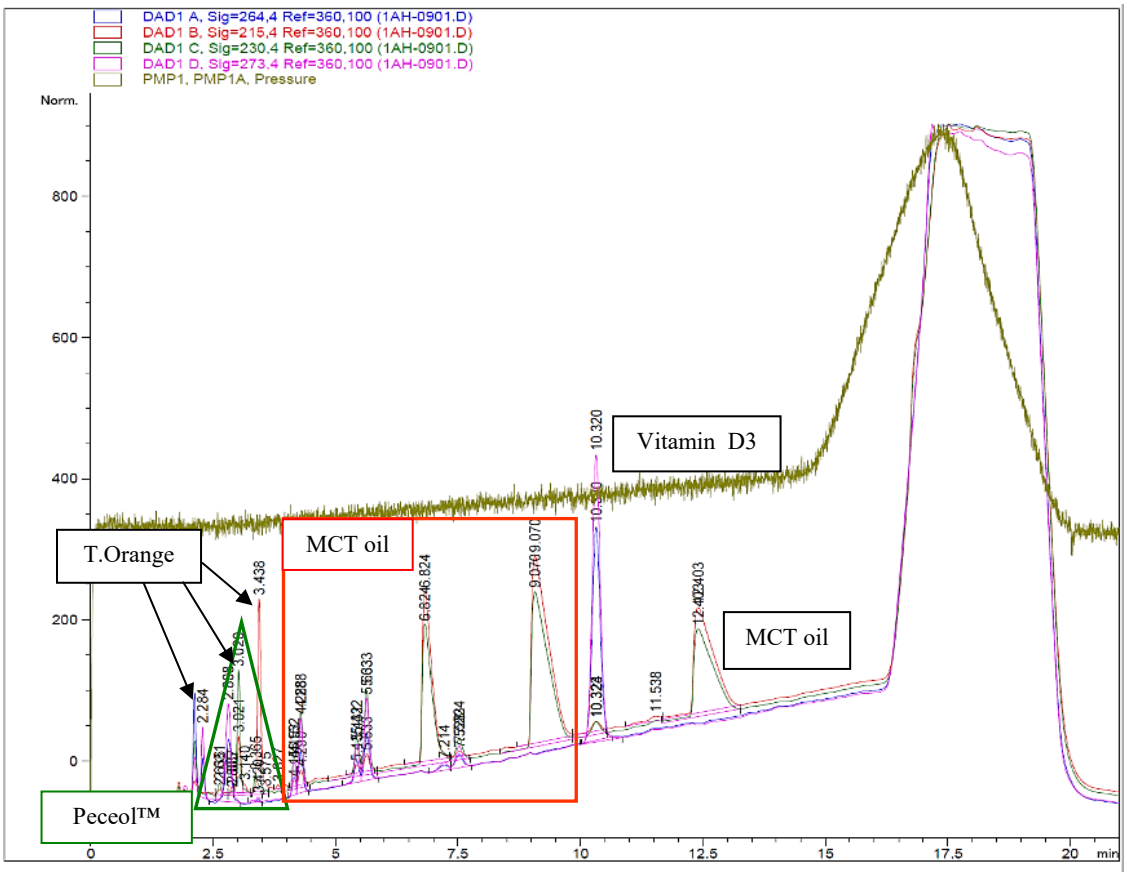



Figure 42: Chromatogram of the formulation with vitamin $D_3$, MCT oil, Peceol™ and Tetrarome (R) Orange (Third month)

Although in the first three months a slight reduction of vitamin $D_3$ contect was detected (Paragraph 3.3.4.1), it could not be distinguished any significant change in the chromatogram, indicating a likely decomposition.

## 3.3.5 Effect of light, high temperatures and oxygen on stability

The main degradation processes which the vitamin $D_3$ can undergo are thermal degradation, photo-degradation and oxidative degradation. Therefore, samples of the formulation were stressed by sun and UV light, at 60°C under bubbling oxygen, in order to evaluate the stability of vitamin $D_3$ and thus of the formulation, at critical conditions which can occur during the manufacturing process, at the time of administration or in case of wrong storage.

To perform the mentioned tests, the samples were introduced in glass test tubes and then placed in a sample concentrator of the type shown in the figure below (Figure 43).

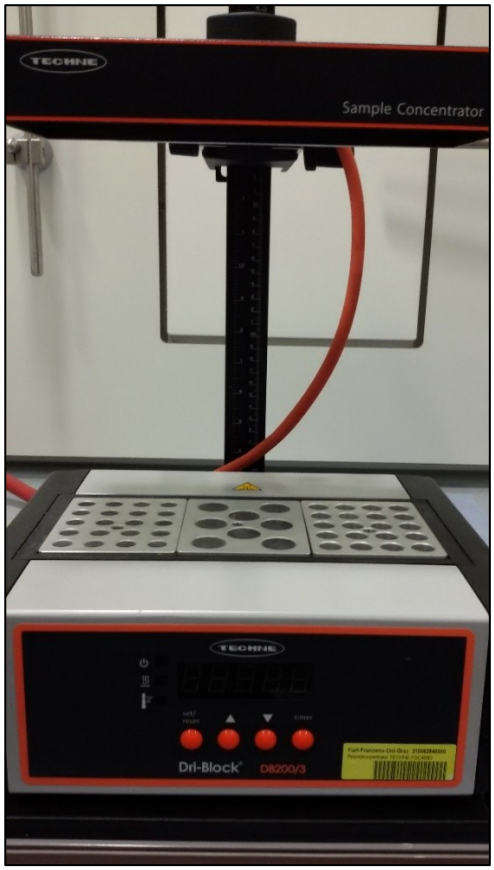



Figure 43: Sample concentrator Techne

The instrument was located in a room completely irradiated by sunlight. The glass test tubes with the samples were located in the holes of the heating top of the instrument and hollow, metal needles were inserted in the tubes, to allow the passage of the oxygen through the samples.

The first test was conducted by setting the heating temperature up to 60°C for 60 minutes, the oxygen pressure at 3 bar and leaving the samples in the lab under the widespread sunlight. The results are shown in the Table 66.

| Vitamin $D_3$ starting amount [mg/ 100 g] | Analysis at 60°C, for 60 minutes, with $O_2$ flow, under sunlight | | | | | |
|---|---|---|---|---|---|---|
| | RT | Standard deviation | Area | Standard deviation | Vitamin $D_3$ | Recovery of vitamin $D_3$ |
| | [min] | [%] | [mAU* min] | [%] | [mg/ml] | [%] |
| 9.36 | 10.44<br>10.44<br>10.43 | | 129.4<br>128.6<br>129.6 | | 1.50E-02 | 85.50 |
| | 10.44 | +/- 0.06 | 128.99 | +/- 0.36 | | |
| | Vitamin $D_3$ [mg/100g sample] | | | | 8.21 | |
| 9.39 | 10.45<br>10.44<br>10.44 | | 133.42<br>133.55<br>133.73 | | 1.55E-02 | 87.86 |
| | 10.44 | +/- 0.06 | 133.57 | +/- 0.12 | | |
| | Vitamin $D_3$ [mg/100g sample] | | | | 8.45 | |

Table 66: Results of the HPLC performed on samples in duplicate, stressed at 60°C, for 60 minutes under sunlight including oxygen treatment

As it can be seen in the Table 66, the effect of high temperature, oxygen and sunlight, caused a considerable reduction of vitamin $D_3$ in the formulation.

In the case of the first sample (9.36 mg) the resulting dosage (8.21 mg) was out of the 15% over dosage range. For the other sample (9.39 mg), the calculated amount (8.45 mg) was still in the production surcharge range and slightly above the therapeutic one (8.3 mg).

Probably, the formulation will never reach such extreme conditions used for this test, however the evaluation of the results obtained, allows making considerations concerning the manufacturing process or the daily handling of the drug by the patient (e.g. sunlight, oxygen).

The same purpose had the test performed using the UV lamp that was conducted on the samples previously stressed at high temperature, under oxygen and sunlight.

They were allocated next to an UV lamp, covered with a box and left for thirty minutes in order to maximize the effect of the light. The results are listed in the table below (Table 67).

| Vitamin $D_3$ starting amount [mg/ 100 g] | Analysis with UV lamp, 30 minutes | | | | | |
|---|---|---|---|---|---|---|
| | RT | Standard deviation | Area | Standard deviation | Vitamin $D_3$ | Recovery of vitamin $D_3$ |
| | [min] | [%] | [mA* min] | [%] | [mg/ml] | [%] |
| 9.36 | 10.53<br>10.49<br>10.48 | | 138.10<br>135.47<br>135.44 | | 1.58E-02 | 89.36 |
| | 10.50 | +/- 0.25 | 136.34 | +/- 1.12 | | |
| | Vitamin $D_3$ [mg/100g sample] | | | | 8.4 | |
| 9.39 | 10.49<br>10.54<br>10.57 | | 148.23<br>151.37<br>149.02 | | 1.73E-02 | 97.96 |
| | 10.53 | +/- 0.38 | 149.54 | +/- 1.09 | | |
| | Vitamin $D_3$ [mg/100g sample] | | | | 9.2 | |

Table 67: Results of the HPLC performed on samples in duplicate, stressed by UV lamp for 30 minutes

The results obtained from the UV test showed a recovery higher than expected. In fact, since the samples had been already stressed before being placed under the lamp, it should have been more reasonable to have a lower amount of vitamin $D_3$ because of photo-isomerisation, thermal- and photo-decomposition. The higher values of Table 67 are more probably related to the fact that vitamin $D_3$ by-products were formed with increased molar extinction coefficients. However, the analysis of this degradation products was not performed in this master thesis.

Figure 44 shows the principal degradation products of vitamin $D_3$ which are formed in the skin, but which can also be formed under influence of light, temperature and oxygen in drugs containing vitamin $D_3$.

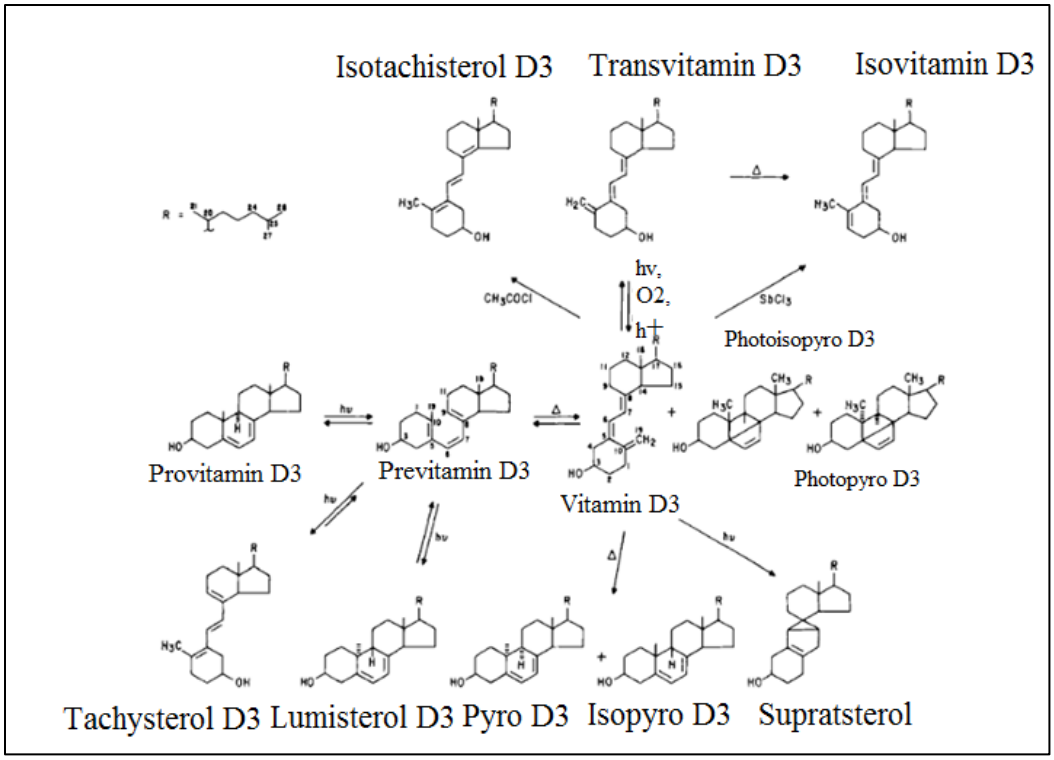



Figure 44: Photosynthesis of vitamin $D_3$ from 7-dehydrocholesterol (pro-$D_3$ or 7-DHC), photo-isomerization, photodegradation, of vitamin $D_3$ and photoisomers of previtamin $D_3$ [83]

The metabolism starts from the pre-vitamin $D_3$ resulting from the 7-dehydrocholesterol (pro-$D_3$ or 7-DHC) by absorption of UV–light (290-315 nm). Between pre-vitamin $D_3$ and vitamin $D_3$, obtained by photo-isomerization, it establishes an equilibrium, which is shifted towards the thermodynamically more stable form, the vitamin $D_3$.

If vitamin $D_3$ in the skin is exposed to sunlight prior to its transfer into the circulation by the vitamin-shifts binding protein (DBP), the triene system of vitamin $D_3$ structure will absorb solar radiation, releasing three major photoproducts, suprasterol 1, and suprasterol 2 and 5,6-trans-vitamin $D_3$, which can also result from oxidation [84].

As reported in the literature [26], among the above specified metabolites of vitamin $D_3$, the *trans- form* of vitamin $D_3$ has a similar mass spectrum, but its maximum absorption wave length is at 273 nm [26]. Regarding other metabolites, the absorption shifts to higher wavelengths, such as 280-290 for *tachysterol* and 300-312 nm for *supratserol* [26]. Therefore, it is possible that at extreme conditions, unknown decomposition products can derive from chemical reactions between the API and lipophilic excipients or between the excipients. In this case, the analysis of formulation by mass spectroscopy could be useful, to identify the chemical structure of metabolites.

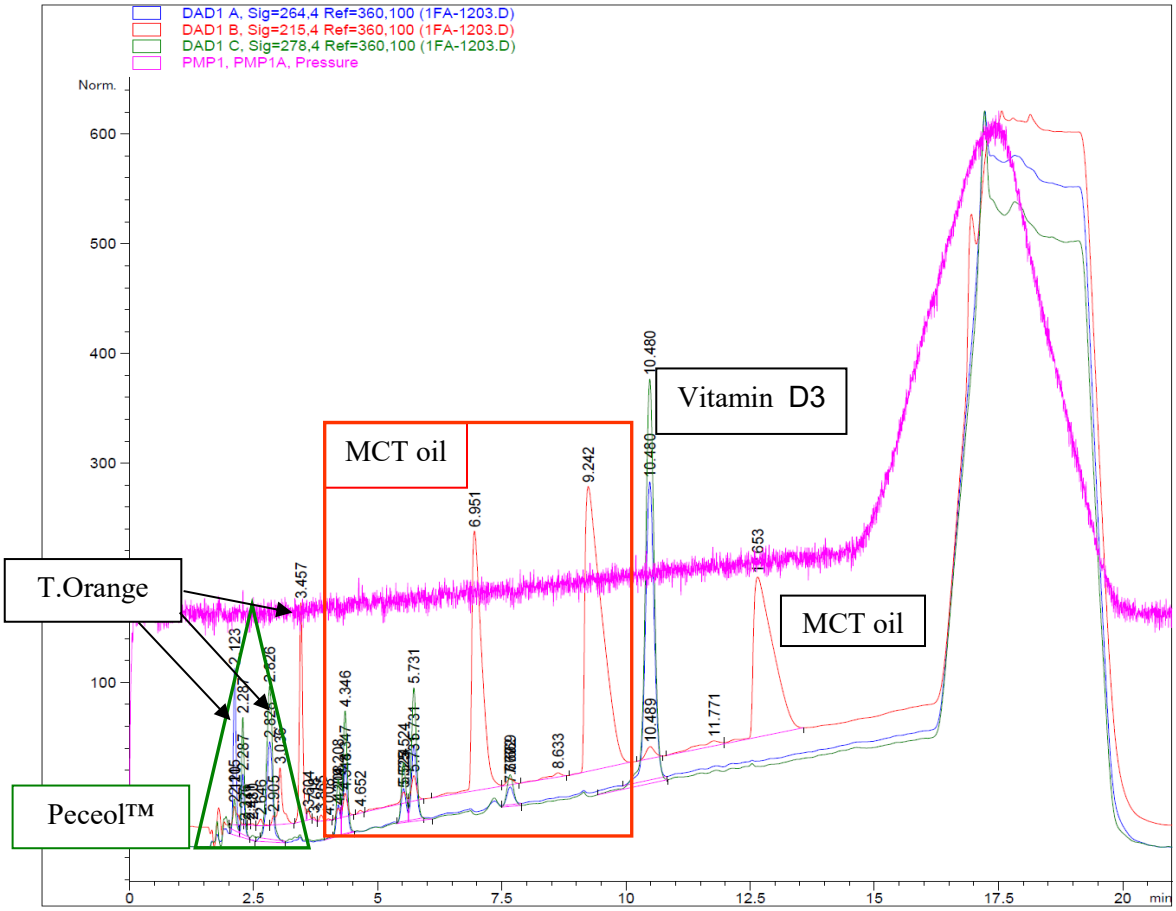



Figure 45: Chromatogram of vitamin $D_3$ spray after being stressed at 60°C, with oxygen pressure of 3 bar, under sunlight and UV lamp

Figure 45 presents the chromatogram of vitamin $D_3$ spray, stressed by temperature at 60°C, oxygen flow at pressure 3 bar, sunlight and UV lamp.

Despite the assumptions made about the by-products of vitamin $D_3$, observing the chromatogram, there is no evident sign of decomposition, since it did not occur either a change of vitamin $D_3$ peak or the appearance of new peaks.
The only slight difference which can be noted in respect to the original chromatogram (Figure 41) is that the peak of vitamin $D_3$ is thinner and decreases in height. Therefore, HPLC combined with mass spectroscopy should be applied to analyse further degradation products.

## 3.4 Viscosity measurement

The viscosity determination of vitamin $D_3$ spray was conducted, in order to ensure the functionality of the spray pump and the correct intake by each puff.
In fact, in case of a spray formulation, a low-viscous fluid is advantageous for the mechanic action of the spray pump, since it reduces the risk of sticking and guarantees the optimal flowing of the solution.
The viscosity measurement was performed using an *Anton Paar MRC 301* Rheometer (Figure 46).

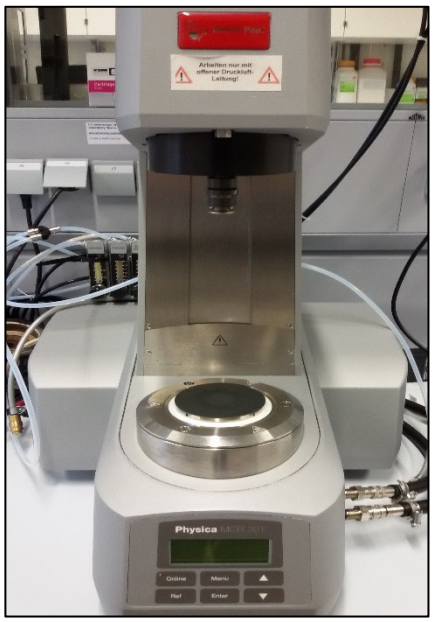


Figure 46: Anton Paar MRC 301 Rheometer

The working principle used in this case for the viscosity measurement consisted in the rotation, at different velocities, of a conical shaped tool over a sample, placed on a plate [85]. The viscosity was then calculated by the instrument through the ratio between the shear stresses [mPa] and the shear rate [1/sec]. The instrument parameters used are described in the table below (Table 68).

| | |
|---|---|
| **Measurement mode** | Rotation CSR (controlled shear rate) |
| **Measurement tools** | CP 50 (cone/plate) |
| **Temperature** | 25 °C |

Table 68: Instrument parameters for dynamic viscosity [mPa*s] determination

The MCT oil was tested separately to compare the result to that obtained for the formulation and estimate the possible differences.

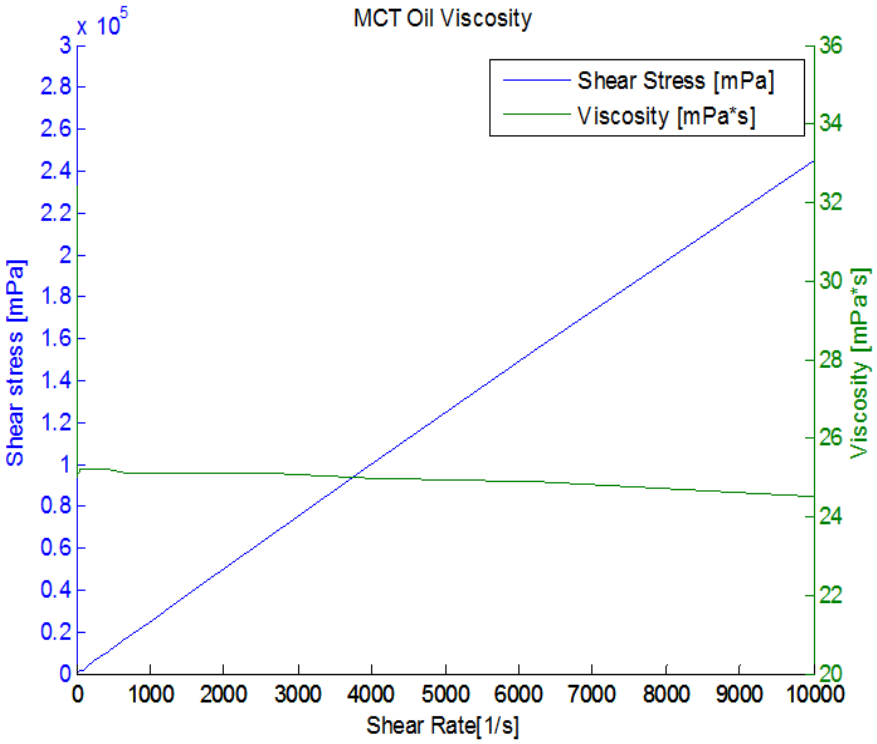



Figure 47: Viscosity of MCT oil dependent on shear stress

Figure 47 shows how the MCT oil viscosity (green line) decreased slightly at high shear stresses (blue line) and shear rate. However, there was no sharp change in the flow behaviour (Figure 48).

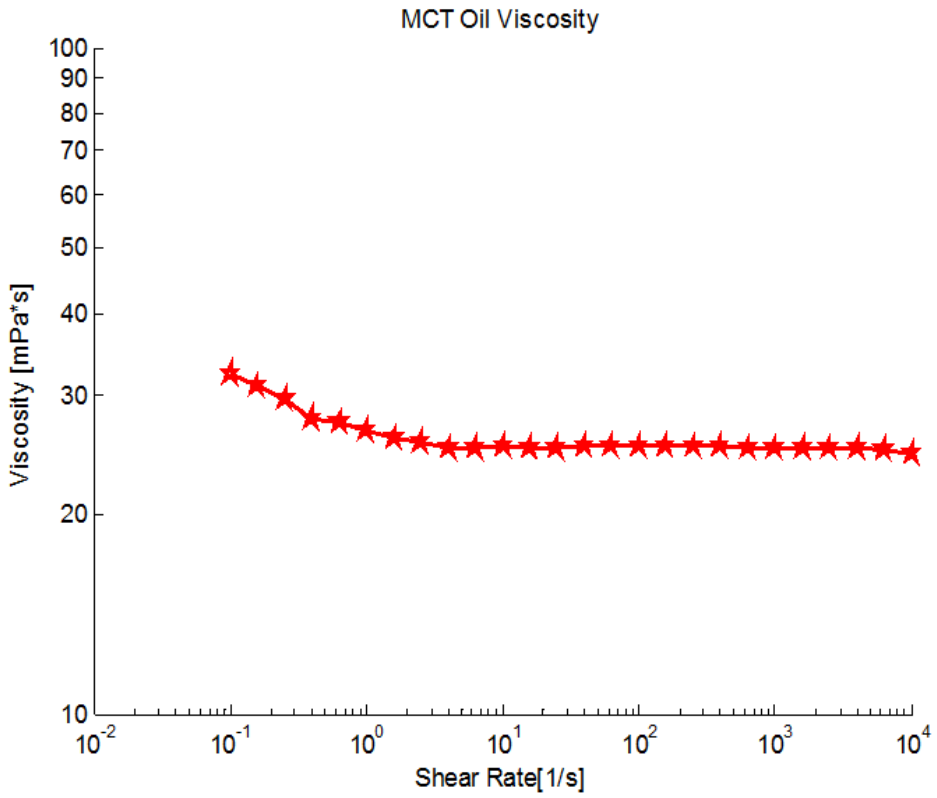



Figure 48: Flow behaviour and viscosity of MCT oil

After an initial phase where the oil behaviour seemed to be affected by the shear rate's change, the dynamic viscosity continued in a constant way as a Newtonian fluid and its value was stable at 25 mPa*s.

Since the MCT oil is the principal component of the formulation in terms of amount, it was expected that the behaviour of vitamin $D_3$ spray was nearly identical. This was confirmed by the analysis, whose result is shown in Figure 49.

It is evident the similarity to the MCT oil. The only difference can be found in a slight but not significant decrease of the viscosity from 25 to 24 mPa*s, but the Newtonian-like behaviour is preserved.

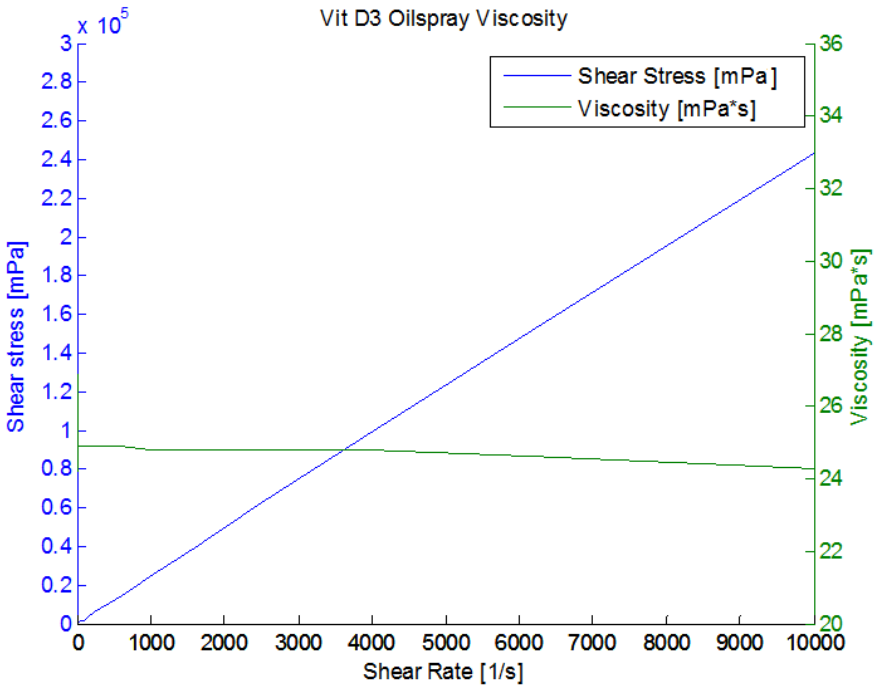



Figure 49: Viscosity of vitamin $D_3$ formulation dependent on shear stress

The viscosity remains constant while increasing the shear rates (Figure 50).

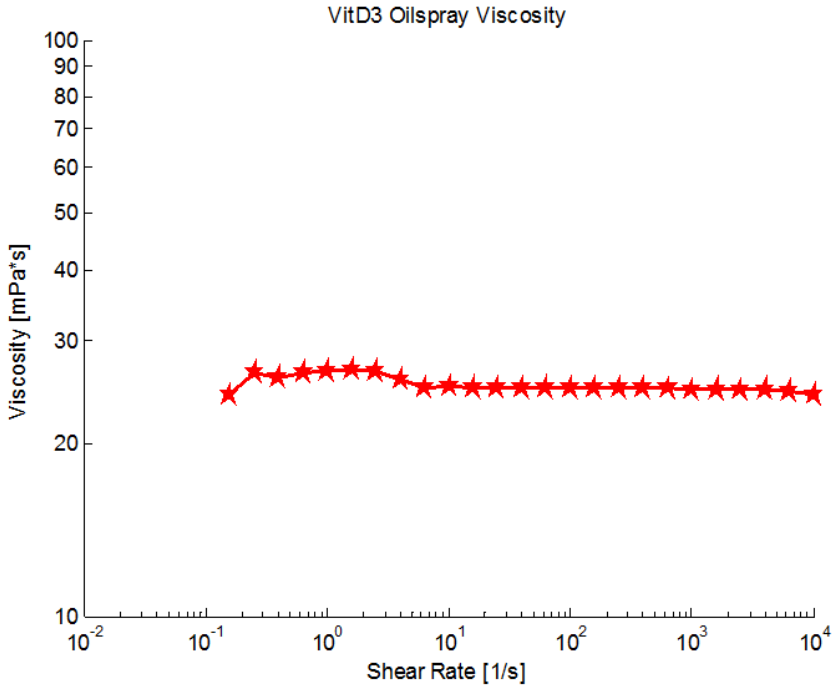



Figure 50: Flow behaviour and viscosity of vitamin $D_3$ formulation

## 3.5 Density measurements

The density is considered as the viscosity another significant quality parameter. In fact, it gives insight into the condition of pharmaceutical raw materials, e.g. media, which are present in the production process, as well as into the finished medicine.
This makes possible to intervene quickly and decide whether the production and the final product are within the defined specifications for continuing the process and proving the quality of the product.
The density measurement was performed by an *Anton Paar digital densitymeter DMA 35* (Figure 51), which operates according to the law of harmonic oscillation, related to the oscillating U-tube principle [86].


Figure 51: Anton Paar digital densitymeter DMA 35

The measuring cell (Figure 52) is represented by an oscillator, which is a hollow U-shaped borosilicate glass tube, able to contain around 0.7 ml of the sample [86]. The tube is made of double walls, which delimit an internal space with a gas, characterised by a high coefficient of thermal conductivity [86]. In the same space, there is a platinum resistance thermometer which is responsible for the temperature measurement of the fluid [86].

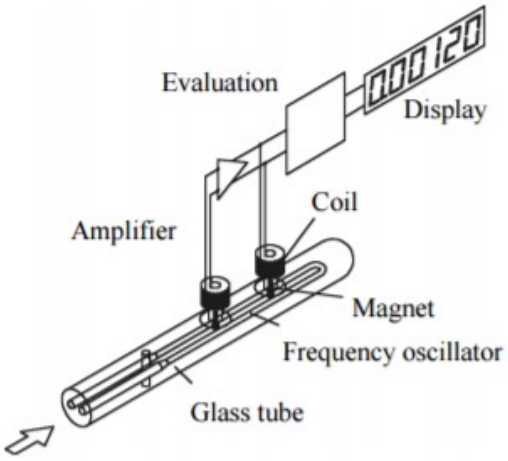



Figure 52: Measurement cell of an oscillation-type density meters [86]

The instrument includes an electronic system, causing the vibration of the tube at its characteristic frequency, which changes depending on the density of the sample. The sample is charged into the U-tube, on which an electromagnetic force acts. The frequency and the duration of vibration of the tube filled with the sample are measured, thus determining the density value of the sample [86].

The density obtained for the MCT oil was 940 Kg/m3 at 20°C, which is within the range found in literature of 930-960 Kg/m3 [87]. Exactly the same value at the same testing temperature, was detected for the vitamin $D_3$ formulation.

This was expected, since the MCT oil is the component which predominates in quantity and influences the physical properties of the whole formulation as already described.

## 3.6 Refractive index measurement

The refractive index is a parameter easy to determine, which allows characterising the analysed substance by ensuring its physical identity.

The refractive index was calculated by means of the *Refractometer ABBE-REF 1* (Figure 53).

Figure 53: Refractometer ABBE-REF 1, PCE GmbH

A schematic diagram of the optical system is shown below in Figure 54.

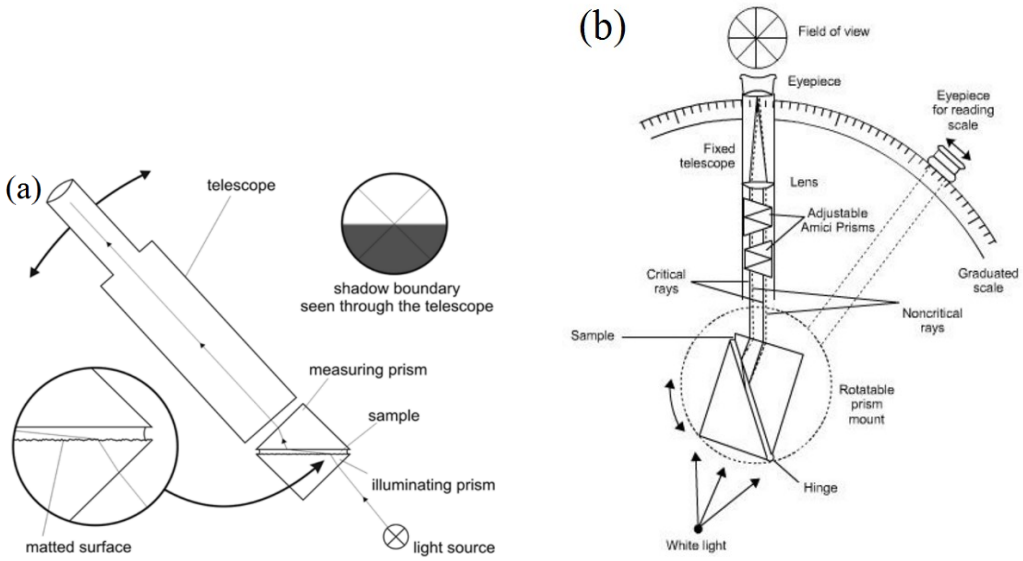


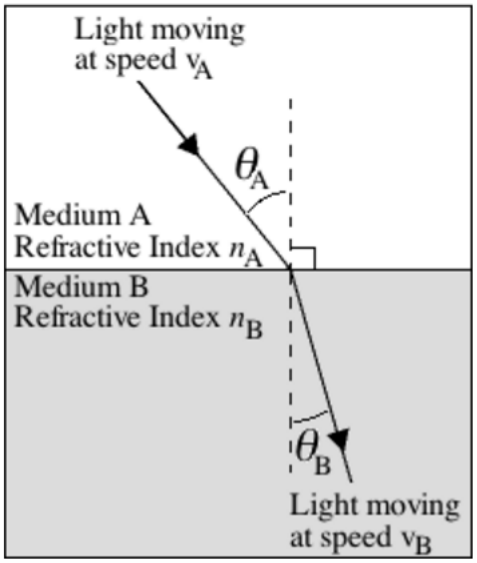

Figure 54: Abbe type critical angle refractometer [88, 89]:(a) External overview; (b) Detail of internal components

The sample was placed between two prisms as a thin layer (~0.1mm) (Figure 54-(a),(b)): an upper prism (measuring prism), which rotates according to the trajectory indicated by dotted lines in Figure 54, b, and a lower one (illuminating prism) connected to the upper [89].

As shown in Figure 54, the light passes through the illuminating prism (rough surface) and reaches first the sample and then the upper measuring prism (smooth surface), where the light is refracted at critical angle [88].

Figure 55 describes how the light can be refracted (e.g. bent from its original path) by passing from a transparent medium into an another, where it acquires a different speed [90].

The refractive index, R.I., is a measure of how much the speed of light is reduced in the medium and is determined by measuring the change in the direction of the incident radiation as it passes from medium A (the air in the specific case) to the medium B (the sample) [90, 91]. Figure 55 shows that the light ($v_A$) in medium A moves faster than ($v_B$) in medium B [85].




$$\frac{v_A}{v_B} = \frac{\sin \theta_A}{\sin \theta_B} = \frac{n_B}{n_A}$$

Figure 55: Behaviour of the crossing light with the angle of incidence (θA) and the angle of refraction (θB) [90]

Medium, temperature and wavelength have an influence on the speed of light in a medium specified [91]. The type of light applied to measure the refractive index of a sample is the monochromatic one [91]. The sodium D-line wavelength of 589 nm is the most used and the corresponding refractive index ($n_D$) is commonly measured at temperature 20 °C or 25 °C [91]. Regarding the developed formulation, the refractive index was measured for MCT oil and the complete formulation. The resulting value for MCT oil at 24.4°C was 1.4475 and hence it is included in the range 1.440-1.452 as reported in the U.S. Pharmacopeia [92]. By performing the measurement for the complete formulation, the outcome was the same as for the MCT oil alone.

## 3.7 Thermal analysis of vitamin $D_3$ spray by DSC

The differential scanning calorimetry (DSC) was used to perform the thermal analysis of samples. The working principle of this instrument consists in measuring the changes of energy that happen during heating, cooling or while keeping the sample in isothermal state. The changes are then correlated to the temperature at which they take place [93].
At each energy change occurring in the sample, the thermal transitions can be quantitatively determined and hence, the material can be characterized according to melting, crystallisation profiles, glass transitions and other more complex events (e.g. oxidation behaviour) [93].
In the specific case of thesis work, the instrument used to conduct the thermal analysis for the whole formulation and the lipophilic excipients was the *DSC 204 F1 Phoenix®* by Netzsch (Figure 56).


Figure 56: DSC 204 F1 Phoenix® by Netzsch

The DSC measures the heat flow, which is the flow of energy into or out of the sample, at a certain temperature or time [93]. The typical unit to express the heat flow is mW, which corresponds to mJ/s, i.e. the flow of energy in unit time [93].

The system described by the Figure 57, represents a DSC measuring cell.
The furnace is the principal component containing two locations, one for the sample and the other for a reference pan, which are placed on sensors, connected to thermocouples [94].

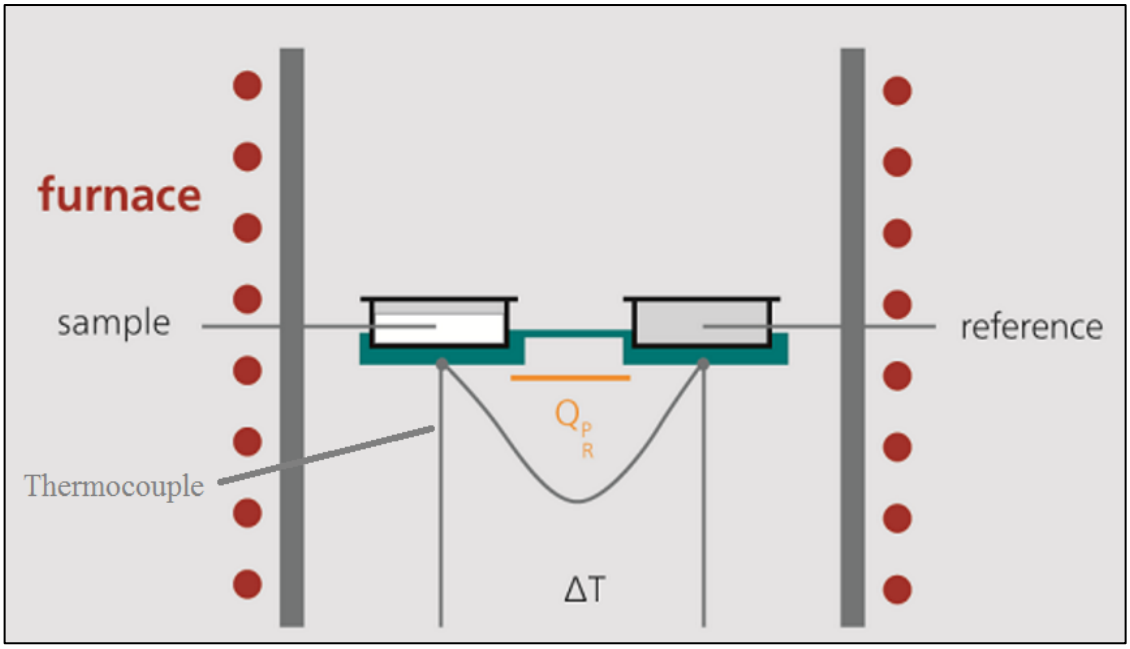



Figure 57: Schematic diagram of DSC measuring cell [94]

The thermocouples allow recording the temperature difference between the sample and reference side, thus producing the corresponding signal, which is the result of DSC analysis [94].
During heating, the sample generally heats slower (TP, red line) than the reference, usually an empty pan (TR, green line), as shown in Figure 58 [94]. By heating at a constant heating rate, the sample (red line) and reference (green line) have similar behaviour, until the sample starts to melt at time, t1 (Figure 58, a).

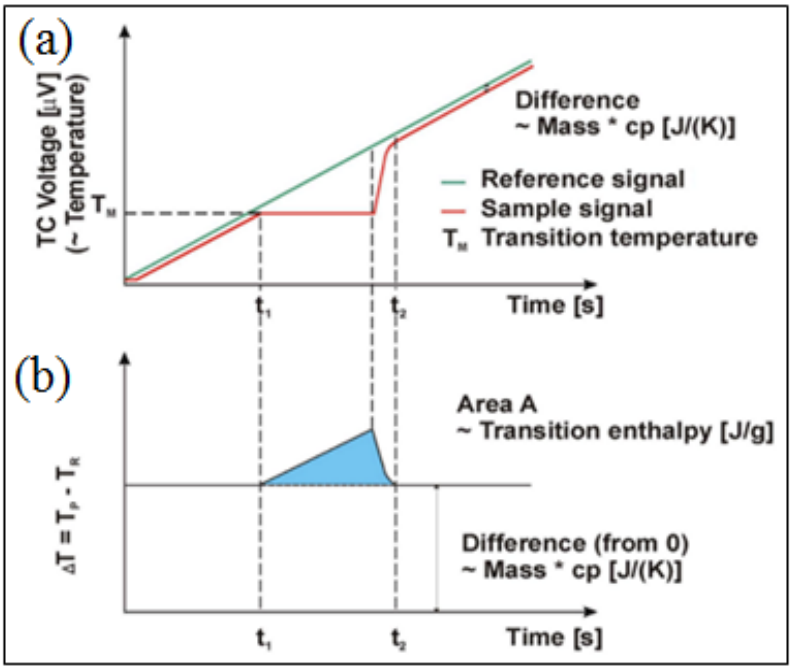



Figure 58: Schematic diagram of melting according to DSC: (a) course of the temperature during melting; (b) transition process curve, given from the temperature difference between reference and sample

In the case shown above, the temperature of the sample remains constant during melting, while that of the reference continues to increase linearly [94]. Once melting is concluded, the sample temperature increases again and regains the linear behaviour at time t2 [94].
In Figure 58(b), the signal coming from the difference (ΔT) of the two temperature lines is represented by the blue peak, which relates to the endothermic melting process [94].
According to the setup of the software, the reference temperature can be subtracted from the sample temperature or vice versa, therefore the resulting peak heads upward or downward [94]. The heat amount related to the transition (enthalpy in mW/mg or J/g), which arises from the temperature difference, is given from the peak area [94].

### 3.7.1 Melting and crystallisation behaviour

The melting behaviour as well as the crystallisation of MCT oil, PECEOL™ and the whole formulation were analysed in order to compare them between each other and hence determine the stability of the vitamin $D_3$ spray, during thermal transitions.
The procedure used for melting/crystallisation analysis was the same for all samples and the steps are listed in the table below (Table 69).

| **Sample (11 mg)** | **Temperature steps** |
|---|---|
| | **Initial step** |
| MCT oil<br>PECEOL™<br>Vitamin $D_3$ formulation | 1. 20°C as starting temperature |
| | 2. Heating up to 80°C at rate 10°K/min |
| | 3. Isotherm at 80°C held for 5 minutes<br>(in order to promote dissolution of crystal nuclei) |
| | **I Crystallisation** |
| | 4. Cooling down to -60°C at rate 10°K/min |
| | 5. Isotherm at -60°C held for 5 minutes |
| | **I Melting** |
| | 6. Heating up to 80°C at rate 10°K/min |
| | 7. Isotherm at 80°C held for 5 minutes |
| | **II Crystallisation** |
| | 8. Cooling down to -60°C at rate 10°K/min |
| | 9. Isotherm at -60°C held for 5 minutes |
| | **II Melting** |
| | 10. Heating up to 80°C at rate 10°K/min |
| | 11. Isotherm at 80°C held for 5 minutes |

| | **Final step** |
|---|---|
| | 12. Cooling down to 20°C |
| | 13. Stand-by at 20°C |

Table 69: Temperature steps applied for the melting and crystallisation analysis

The sample preparation was performed carefully by weighing the amount of ~ 11 mg into an aluminium pan, with a perforated lid, in order to allow that vapours, formed during temperature transition, could leave the pan.

### 3.7.1.1 Investigation of the crystallisation behaviour

The thermal profile of fats and oils is affected by their principal constituents, the triacylglycerol (TAG), which determine the appearance of characteristic peaks during the crystallisation and the melting phase [95].

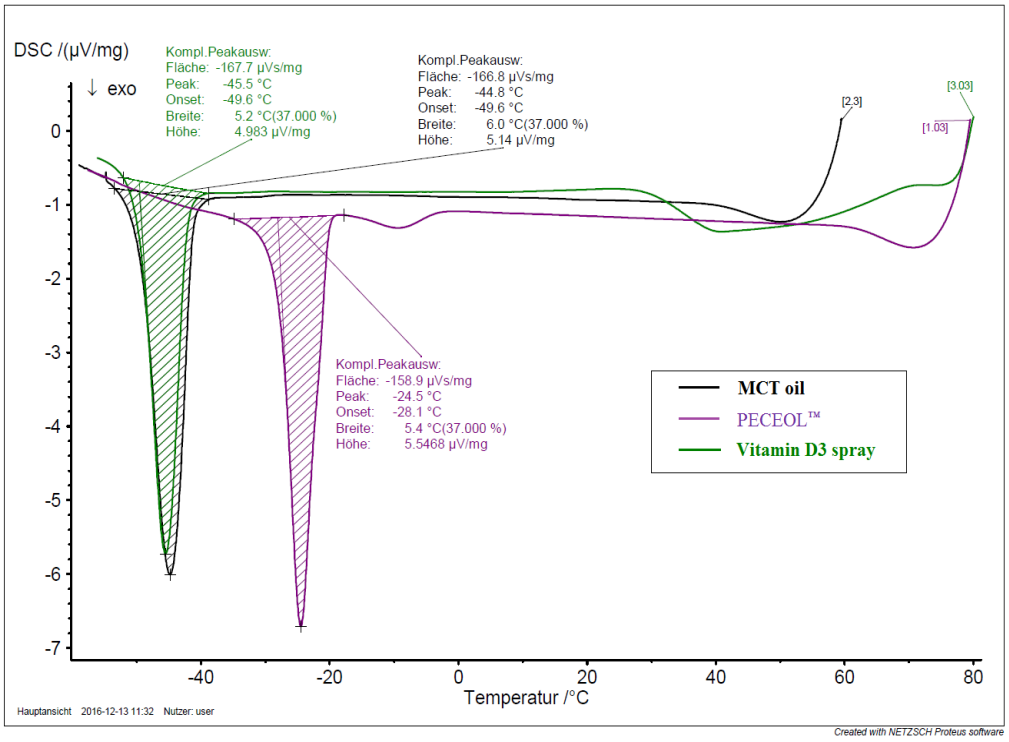



Figure 59: First time crystallisation: cooling down to -60°C at rate 10°K/min

Figure 59 shows that each analysed sample gave a unique distinct peak: the black for the MCT oil, the violet for PECEOL™ and the green for the whole formulation with vitamin $D_3$. In particular, it can be observed the peak-overlapping of MCT oil and the formulation.
It may be related to the fact that MCT oil is the principal component of the formulation and influences in a certain way the thermal behaviour of other components, prevailing on them.
The crystallisation profile resulting from the second cooling step did not show any significant changes, neither in the position or breadth of the peaks (Figure 60) nor in the characteristics parameters (Table 70).

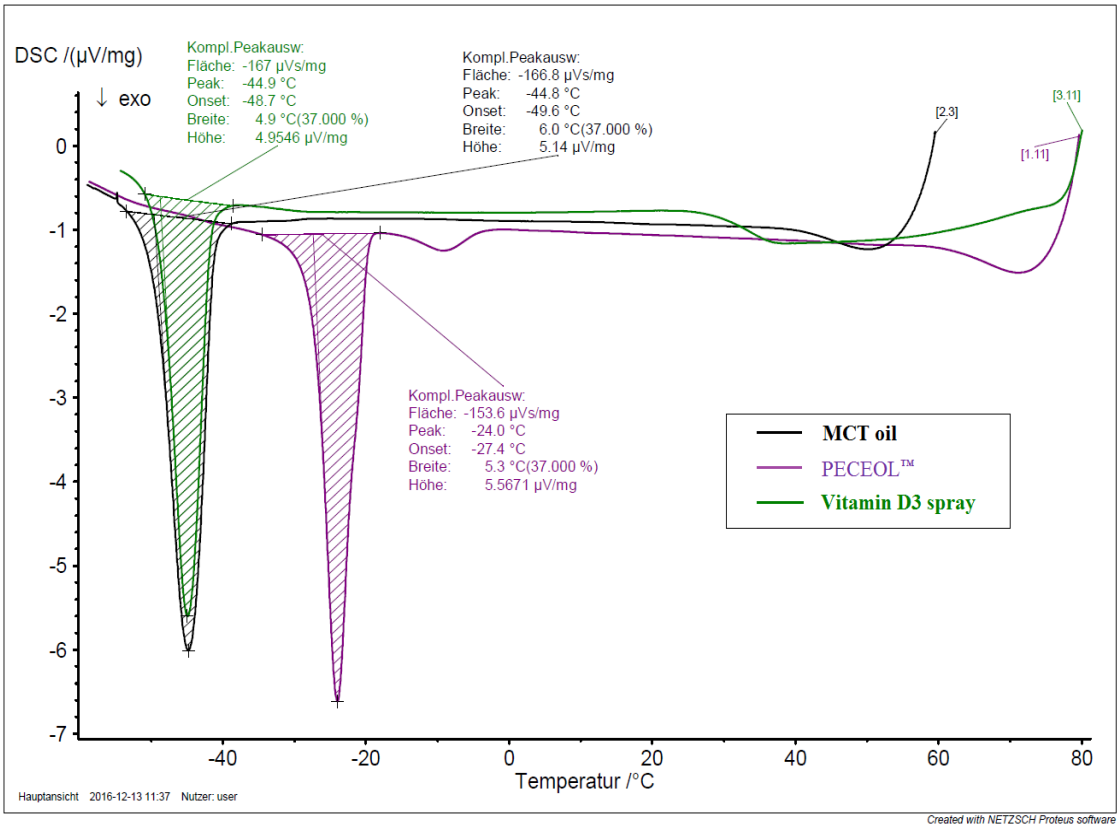



Figure 60: Second time crystallisation: cooling down to -60°C at rate 10°K/min

Table 70 shows a comparison of the results coming from the analysis of peaks. It can be seen that the values of first and second crystallisation were very similar to each other and in the case of MCT oil (the red marked values) were exactly the same.

| | Area [μVs/mg] | Peak [°C] | Onset [°C] | Width[3] [°C; %] | Height [μV/mg] |
|---|---|---|---|---|---|
| **MCT oil** | | | | | |
| First crystallisation | -166.8 | -44.8 | -49.6 | 6°C (37%) | 5.14 |
| Second crystallisation | -166.8 | -44.8 | -49.6 | 6°C (37%) | 5.14 |
| **PECEOL™** | | | | | |
| First crystallisation | -158.9 | -24.5 | -28.1 | 5.4°C (37%) | 5.5468 |
| Second crystallisation | -153.6 | -24 | -27.4 | 5.3°C (37%) | 5.5671 |
| **Vitamin $D_3$ spray** | | | | | |
| First crystallisation | -167.7 | -45.5 | -49.6 | 5.4°C (37%) | 4.983 |
| Second crystallisation | -167 | -44.9 | -48.7 | 5.3°C (37%) | 4.9546 |

Table 70: Parameters resulting from the analysis of crystallisation peaks

Investigating more detailed the crystallisation behaviour of the samples, it was noted that the percentage of a certain sample crystallized at a given temperature was nearly the same, between the first (Figure 61) and second (Figure 62) crystallisation step.

[3] The width as percentage, indicates the peak height respect to the selected baseline.

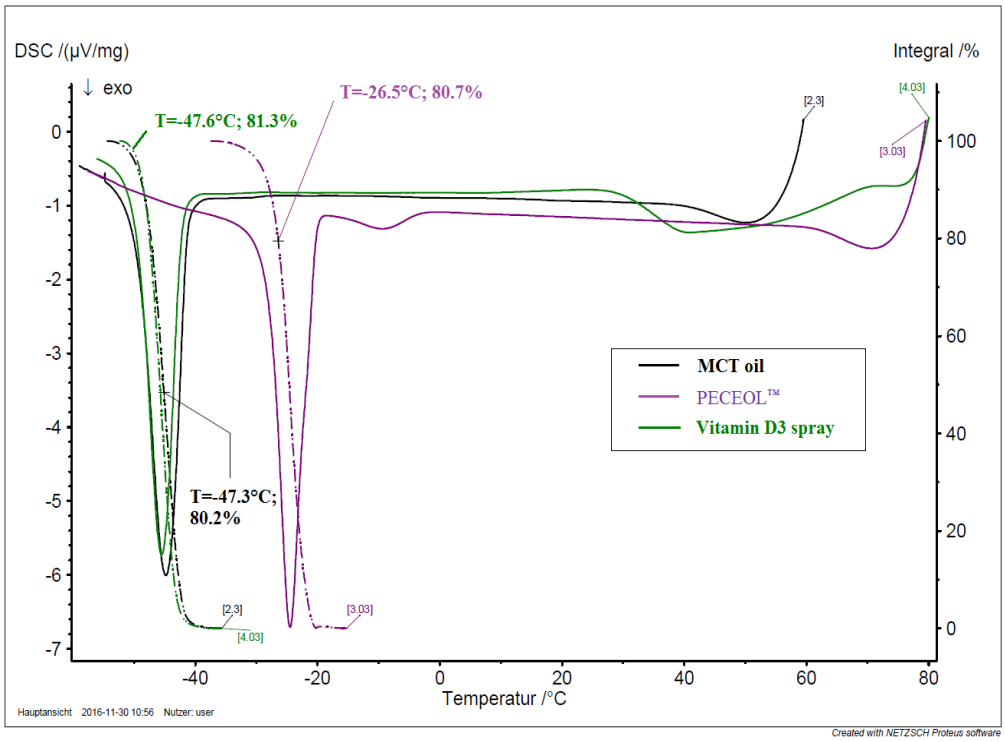



Figure 61: Integral values of the crystallisation profile for the first cooling step

Figure 62 shows the comparison for the second crystallisation.

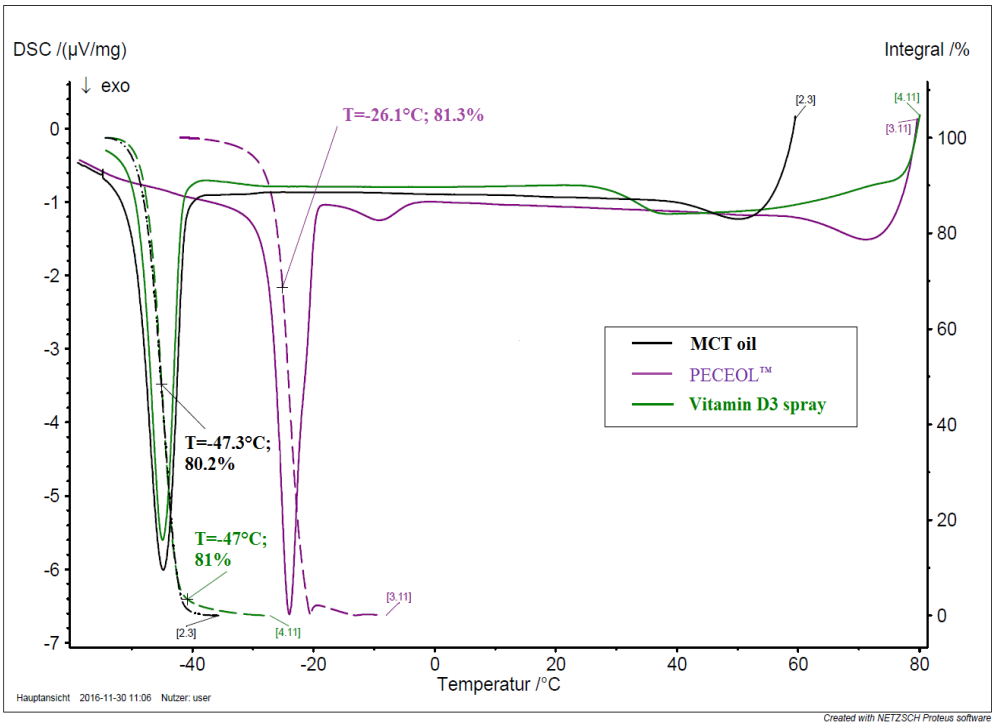



Figure 62: Integral values of the crystallisation profile for the second cooling step

In order to get a better overview of similarities, the temperature and the integral in percentage for each sample are listed in the table below (Table 71).

| | **Temperature[°C]** | **Integral[%]** |
|---|---|---|
| **MCT oil** | | |
| First crystallisation | -47.3 | 80.2 |
| Second crystallisation | -47.3 | 80.2 |
| **PECEOL™** | | |
| First crystallisation | -26.5 | 80.7 |
| Second crystallisation | -26.1 | 81.3 |
| **Vitamin $D_3$ spray** | | |
| First crystallisation | -47.6 | 81.3 |
| Second crystallisation | -47 | 81 |

Table 71: Parameters resulting from the integration of crystallisation peaks

As shown in the table above, the 80.2% of the MCT oil had crystallized at -47.3°C both during the first and the second cooling step. The 80.7% of PECEOL™ had crystallized at -26.5°C during the first cooling step and the 81.3% at -26.1°C during the second one.
As concerns the vitamin $D_3$ spray, the 81.3% had crystallized at -47.6°C during the first cooling and 81% at-47°C during the second cooling.
It is evident from the results of the peak analysis and from the integration that no sharp change occurs during two consecutive cooling phases and this reproducibility testifies the stability of single components as well as of the whole formulation, when the samples undergo thermal modifications.

### 3.7.1.2 Investigation of the melting behaviour

The content of TAG (triacylglycerol) in the considered fat samples is responsible for the endotherms profile and as in the case of PECEOL™, it can determine the splitting of the curve in more than one peak. The reason for this peculiar behaviour is to attribute mainly to the following reasons:

- specific features of TAG: the highly saturated TAG melt at high temperatures than the highly unsaturated TAG [95];
- melting-recrystallisation of the TAG crystallites and the successive melting; it is due to the *polymorphic* characteristic of fatty acids [95].

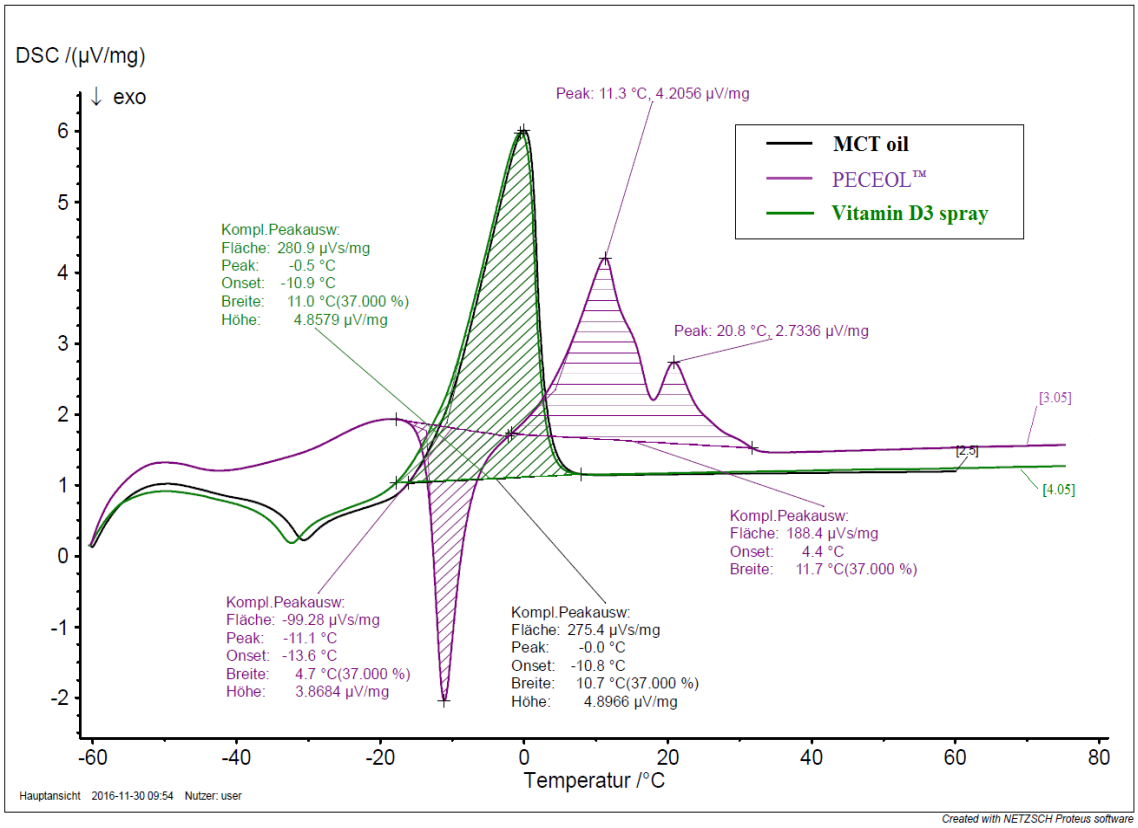



Figure 63: First heating step according to the specific temperature range of Table 69

As shown in the Figure 63, the profile of MCT oil had no multiple endotherms, as characteristic for oil substances [95], but a melting signal characterized by one distinct peak.

This appeared in the temperature range between -20°C and 20°C and overlapped the melting peak of the formulation with vitamin $D_3$. As already explained for the crystallisation analysis, the MCT oil, as the principal component of the formulation, had an influence on the thermal behaviour of the formulation, because its physical properties were dominant on those of the other components.

The profile of PECEOL™ differs from that of MCT oil and vitamin $D_3$ formulation, because of its content of unsaturated acids and polymorphic structures.

In fact, as shown in the Figure 63, the exothermic violet peak in the range between -20°-0°C indicates that a crystallisation occurred, while the melting curve is a multiple endotherm with two distinct peaks, one at 11.3°C, with corresponding height of 4.2056 μV/mg and the other at 20.8°C, with height equal to 2.7336 μV/mg.

Due to the polymorphism phenomenon [95], after the initial heating step, during which the thermal history of the sample is erased, the melting of the less thermally-stable TAG polymorph occurred, while the other TAG underwent a rearrangement and recrystallisation (the exothermic violet peak), forming a more stable polymorph, whose melting point shifted towards higher temperatures (the violet multiple endotherm).

In order to test the influence of excipients on the whole formulation, the melting phase, as the crystallisation, was repeated two times.

The second melting phase (Figure 64) gave for PECEOL™ a similar bell-shaped multiple endotherm, with a slight difference in the peaks temperature, which was 11.2°C for the first and 21.3°C for the second, as well as for the peak height, respectively corresponding to 4.1308 μV/mg and to 2.5301 μV/mg.

The melting peak of vitamin $D_3$ spray overlaps, as in the case of crystallisation (Paragraph 3.7.1.1), the peak of MCT oil, for the reasons already discussed.

Moreover, looking at the Figure 64, it results that the oily excipients, such as PECEOL™ and MCT oil, as well as the formulation, do not show any evident change, as concerns the peak shape, its position and its breadth.

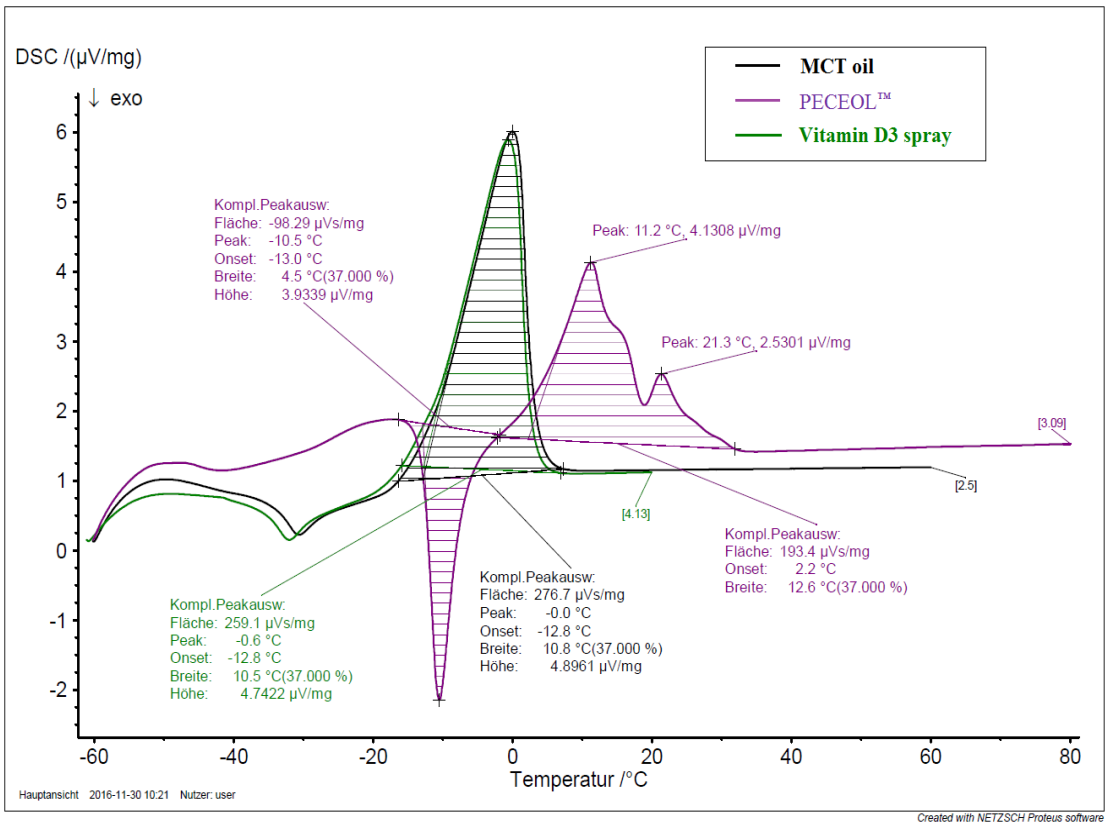



Figure 64: Second heating step according to the specific temperature range of Table 69

By performing a comparative analysis between the first and second melting step according to peak's parameters, there is no evidence of sensible variations. A detailed overview of the respective values are listed in the table below.

| | Area [µVs/mg] | Peak [°C] | Onset [°C] | Width [°C; %] | Height [µV/mg] |
|---|---|---|---|---|---|
| **MCT oil** | | | | | |
| First melting | 275.4 | 0 | -10.8 | 10.7 (37%) | 4.8966 |
| Second melting | 276.7 | 0 | -12.8 | 10.8 (37%) | 4.8961 |

| **PECEOL™** | | | | | |
|---|---|---|---|---|---|
| First melting | | | | | |
| 1°Peak | 188.4 | 11.3 | 4.4 | 11.7 (37%) | 4.2056 |
| 2°Peak | | 20.8 | | | 2.7336 |
| Second melting | | | | | |
| 1°Peak | 193.4 | 11.2 | 2.2 | 12.6 (37%) | 4.1308 |
| 2°Peak | | 21.3 | | | 2.5301 |
| Recrystallisation | | | | | |
| First melting | -99.28 | -11.1 | -13.6 | 4.7 (37%) | 4.8966 |
| Second melting | -98.29 | -10.5 | -13 | 4.5 (37%) | 4.8966 |
| **Vitamin $D_3$ spray** | | | | | |
| First melting | 280.9 | -0.5 | -10.9 | 11 (37%) | 3.8684 |
| Second melting | 259.1 | -0.6 | -12.8 | 10.5 (37%) | 3.9339 |

Table 72: Parameters resulting from the analysis of melting peaks

This was probably related to the rearrangement of PECEOL™ chemical structure after the first melting into a looser structure, which began to melt at lower temperatures, therefore the onset decreased. The values of recrystallisation for the first and second melting remained almost unchanged.

Looking at the values of vitamin $D_3$ spray, a considerable variation was observed for the area, which was higher for the first melting (280.9 µVs/mg) than for the second (259.1 µVs/mg). Since the area under the curve is proportional to the enthalpy of the thermal change, the higher value of the first step indicates a more energetic event respect to the second one. This is also confirmed by the onset temperatures, which were respectively -10.9 °C and -12.8°C, indicating that the first melting step required a higher temperature than the second one.

In fact, as well as for PECEOL™, the structure could have been remodelled after the first melting, yielding a molecular organisation more unstable and therefore able to melt at lower temperatures.

Except for a few differences, the melting behaviour of emulsifier, MCT oil and vitamin $D_3$ formulation were very similar, which demonstrates their stability at induced thermal changes. Furthermore, the comparison between the two melting steps was performed by considering the percentage of sample melted at a certain temperature and noting the possible differences (Figure 65). The dashed lines in Figure 65 show the resulting integral of the melting curve.

Looking at PECEOL™ profile, where two distinct peaks were distinguished, the partial area was calculated in order to get a better evaluation of the integral.

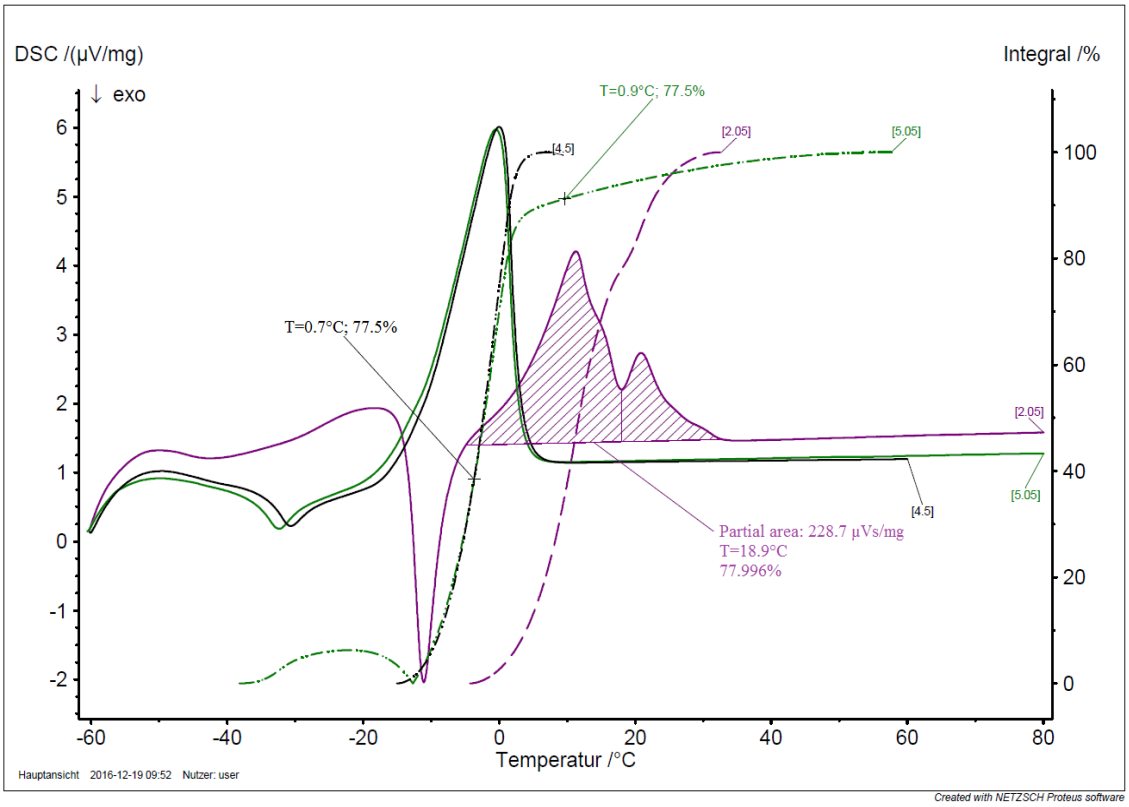



Figure 65: Integral values of the melting profile for the first melting step

Figure 66 represents the second melting step and the dashed lines as well as the partial area are indicated.

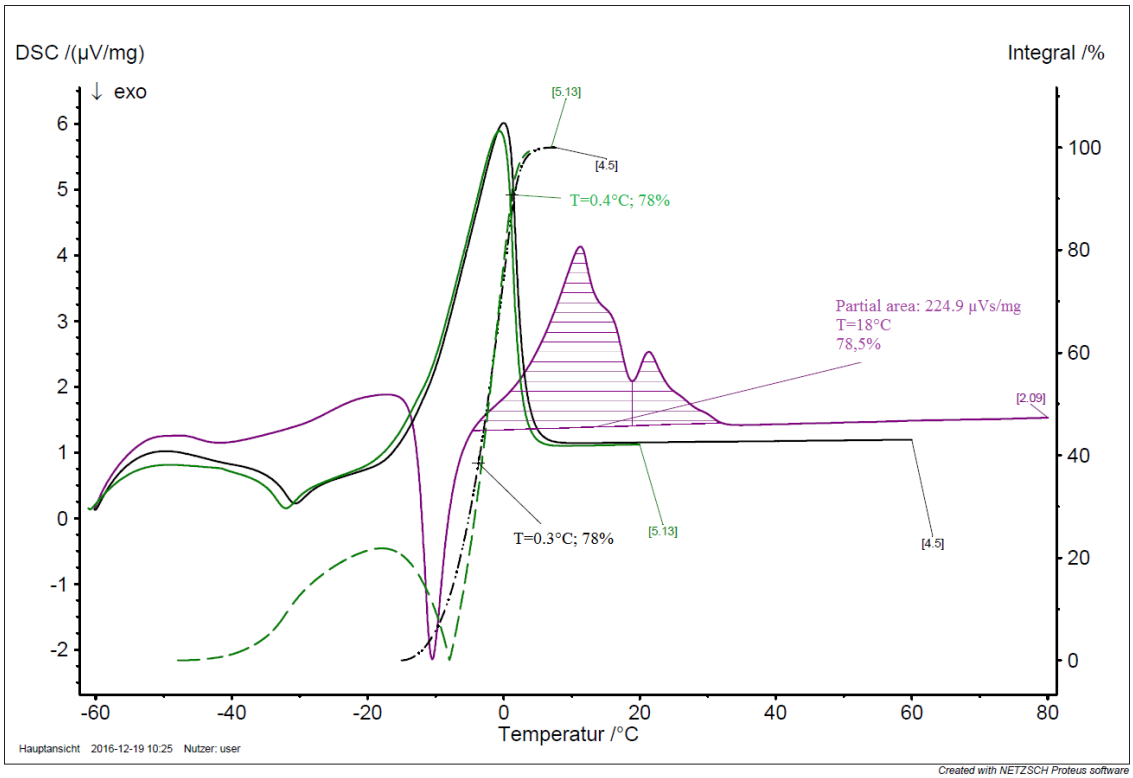



Figure 66: Integral values of the melting profile for the second melting step

The combination of the data resulting from the integral analysis of the first melting and those of the second melting yields the following table, which gives an overview of differences and similarities.

| | Temperature[°C] | Integral[%] |
|---|---|---|
| **MCT oil** | | |
| First melting | 0.7 | 77.5 |
| Second melting | 0.3 | 78 |

| **PECEOL™** | | |
|---|---|---|
| First melting | 18.9 | 78 |
| Second melting | 18 | 78.5 |
| **Vitamin $D_3$ spray** | | |
| First melting | 0.9 | 77.5 |
| Second melting | 0.4 | 78 |

Table 73: Parameters resulting from the integration of melting peaks

As shown in Table 73, during the first step, the 77.5 % of MCT oil had already melted at 0.7°C and during the second one, the 78% of it had melted at 0.3°C. Therefore, nearly the same percentage of MCT oil had melted at a lower temperature in the second melting.
This could be due to a rearrangement of the molecules in the first phase, which promoted the thermal transition at low temperatures.
As well as for the MCT oil, a slight temperature decrease was observed for the PECEOL™, which had melted for the 78% already at 18.9°C in the first step and for the 78.5% at 18°C in the second step.
Similar to MCT oil, which is the principal component of the whole formulation, the percentage of vitamin $D_3$ spray, melted during the two melting steps, was comparable, as well as the corresponding temperatures.
The data analysis, presented before, testifies as, the excipients, as well as the whole formulation preserve the same physical characteristics, during repeated processes of crystallisation and melting, thus confirming the stability of the spray to thermal transitions.

### 3.7.2 Oxidation kinetics: evaluation according to Arrhenius

External factors (e.g. light in terms of UV radiation, temperature, atmospheric oxygen, atmospheric impurities) can cause the aging of the formulation, influencing the smell, the taste and bringing to the formation of toxic components. The chemical aging is principally due to oxidation, therefore the determination of oxidation stability is important to evaluate the shelf-life [96].

In the specific case of the developed formulation, two components such as MCT oil and PECEOL™ are composed of esters of fatty acids, which are sensitive to the risk of oxidation, through free radical chain reactions, characterized by the steps described in the Figure 67 below.

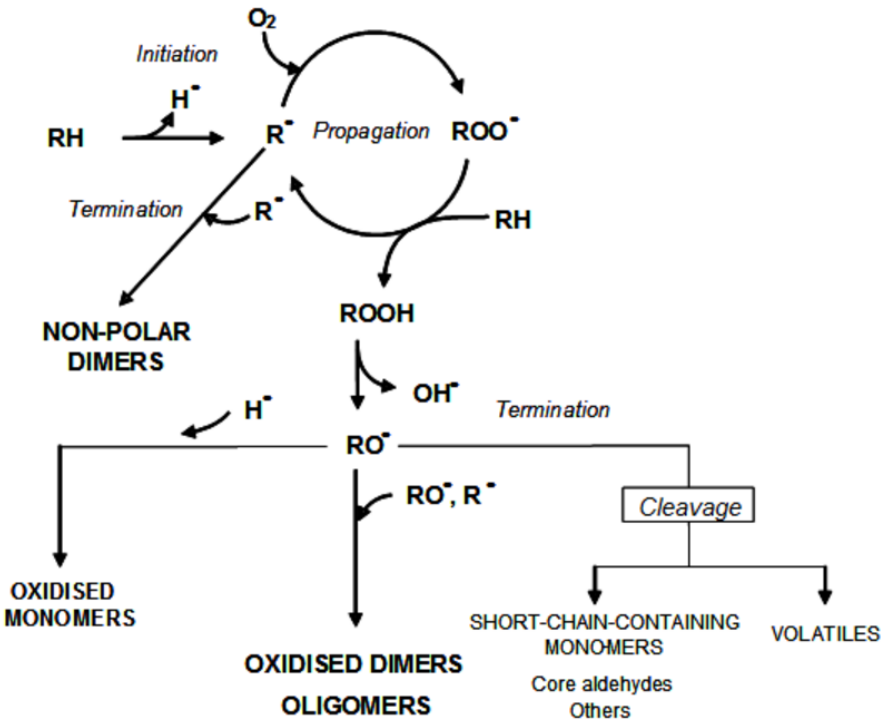



Figure 67: Scheme of oxidation process [97]

As shown in the scheme above, the oxidation reaction starts from a triacylglycerol (RH), which loses a *hydrogen radical* ($H^-$) in the initiation stage, changing into an *alkyl radical* ($R^-$) [97]. Since the hydrogen radical can be easily released by unsaturated fatty acids [97], PECEOL™ is more unstable than MCT oil, which has no unsaturated bonds in its structure.

In the following propagation step, the alkyl radical links an oxygen molecule, releasing *peroxyl radicals* ($ROO^{\cdot}$), which react with other triacylglycerols, becoming *hydroperoxides* (ROOH) [97].

In the end, in the termination stage, radicals formed by the *hydroperoxides* react with each other, producing species which are stable and non-radical, such as aldehydes, ketones, alcohols and hydrocarbons, responsible for the rancid smell, produced by oxidised fats [97].

The DSC allows the analysis of oxidation stability via determination of the oxidation induction temperature / oxidation induction time (OIT). There are two different methods available: the dynamic and isothermal OIT. In the dynamic technique, the sample is heated up to a certain temperature, using constant heating rate and specific oxidising conditions, until the reaction begins [96]. In the isothermal oxidation test (IOT), as shown in the Figure 68, there is a first heating step (e.g.190-200 °C), during which the sample is surrounded by inert gas (e.g. nitrogen) and kept at a constant temperature for some minutes in order to establish equilibrium [96]. Afterwards, the exposition to air or atmospheric oxygen occurs. The oxidation induction time (OIT) is the time between the first contact with oxygen and the beginning of oxidation [96]. After the OIT, the exothermic profile, characteristic of the oxidation process, appears.

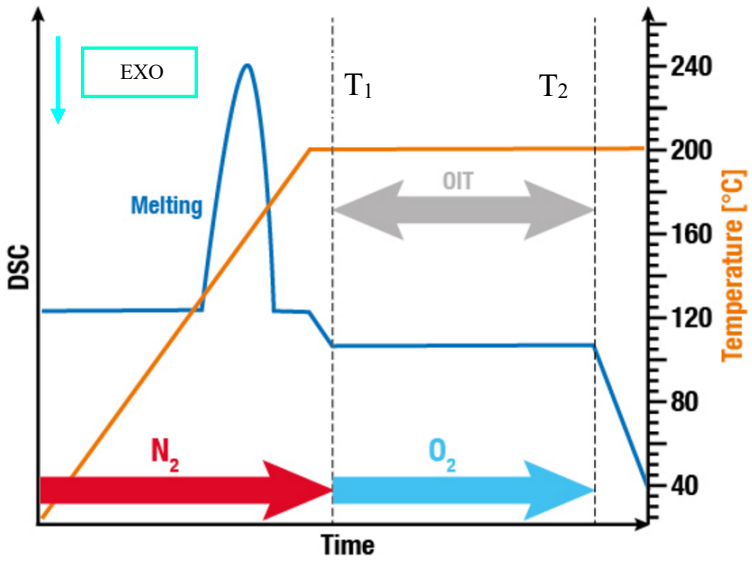



Figure 68: Schematic diagram of the isothermal OIT test [98]

The steepness of the decreasing profile can be more or less sharp. In fact, it depends on the temperature and hence it will be less pronounced at low temperatures and more at higher temperatures.

In the analysis performed for the thesis work, the method of the isothermal oxidation test was used and the temperature setup was chosen according to the thermal stability of single components, present in the formulation. In addition, instead of pure oxygen air was used

As suggested in some literature [99], a smaller amount of sample (~5mg) than the melting/crystallisation studies is used, because in this way the surface area in contact with air is larger, yielding a stronger oxidation effect. The sample was placed in an aluminium pan, which was previously perforated in order to allow the passage of air.

The stability study was performed for vitamin $D_3$, PECEOL™, MCT oil, the newly developed spray and the previous oily-drops, trade formulation.

The oxidation reaction can be considered as an *apparent first-order reaction*, because the amount of sample which reacts with oxygen is very low compared to the high concentration of oxygen in the air [99]. Therefore, the oxygen concentration is taken as constant, since its consumption is negligible, while the change of sample amount is the only to be linearly correlated to the reaction rate.

The reaction and the corresponding rate constant can be generally defined as follows:

$$\text{Oxygen} + \text{Sample} \xrightarrow{k} \text{Oxidation products}$$

$$r = k * C_{O_2}^n * C_{Sample}^n \qquad \text{Equation 3}$$

Where r is the reaction rate, k is the rate constant, $C_{O_2}$represents the oxygen concentration, $C_{Sample}$ the sample concentration and n the order of reaction.

Since the degradation rate (reaction rate) is proportional to sample concentration, the rate law is described by the following equation:

$$r = -\frac{d[Sample]}{dt} = k * [Sample] \qquad \text{Equation 4}$$

The n term, exponent of the sample term, assumes the value of one and the concentration of oxygen is combined together with the rate constant k, to form the new $k=k'*[O_2]$.

The samples were tested at different temperatures, in order to perform the evaluation of oxidation kinetics, by determining the activation energy ($E_a$), using the following Arrhenius equation:

$$lnk = lnA - \frac{E_a}{RT} \quad \text{Equation 5}$$

$$k = Ae^{-\frac{E_a}{RT}} \quad \text{Equation 6}$$

Where $E_a$ is the activation energy (kJ/mol), T the absolute temperature (°K), A pre-exponential factor ($min^{-1}$) and R the gas constant (8.3143 J/mol/°K).
In the specific case of the oxidation reaction, the k is directly proportional to the inverse of the oxidation time [100]:

$$ln\frac{1}{OIT} = lnA - \left(\frac{E_a}{R}\right)\frac{1}{T} \quad \text{Equation 7}$$

Graphically, the OIT is the point of intersection between the extrapolated baseline and the tangent line of the exothermic curve, as indicated in the figure below.

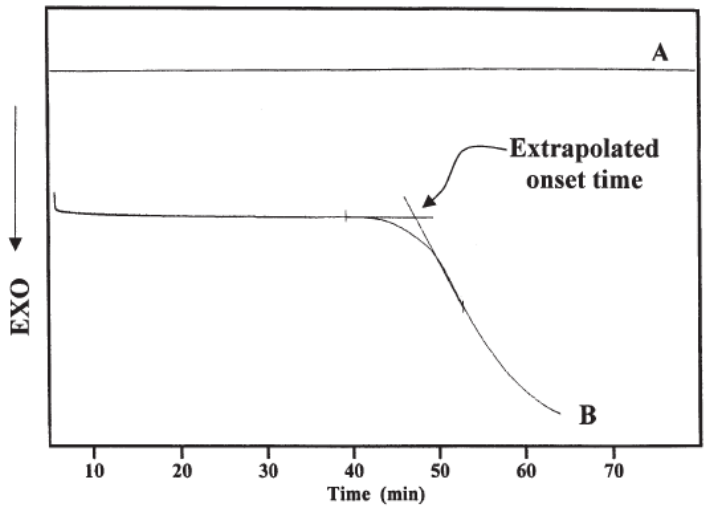



Figure 69: Example of oxidation curve: (A) Isothermal with nitrogen flow in the chamber; (B) Isothermal with air flow [99]

$E_a$ indicates the energy necessary to overcome the energetic barrier, which prevents the reaction to start. The frequency factor A, refers to the frequency of collisions among the molecules, which occurs in the correct orientation during a reaction [101]. These values are obtained respectively from the slope and the intercept of the line, resulting from the correlation between the tested temperatures and the inverse of the relative oxidation induction times (1/OIT).

### 3.7.2.1 Analysis of vitamin $D_3$ API

The sample of vitamin $D_3$ (5 mg) was heated up to 87°C, 125°C and 150°C under nitrogen (20 ml/min) as protective gas. Once reached the chosen temperature, it was kept constant for three minutes, always holding the nitrogen flux. Afterwards, the nitrogen was stopped and only pure air began to come into the sample chamber.

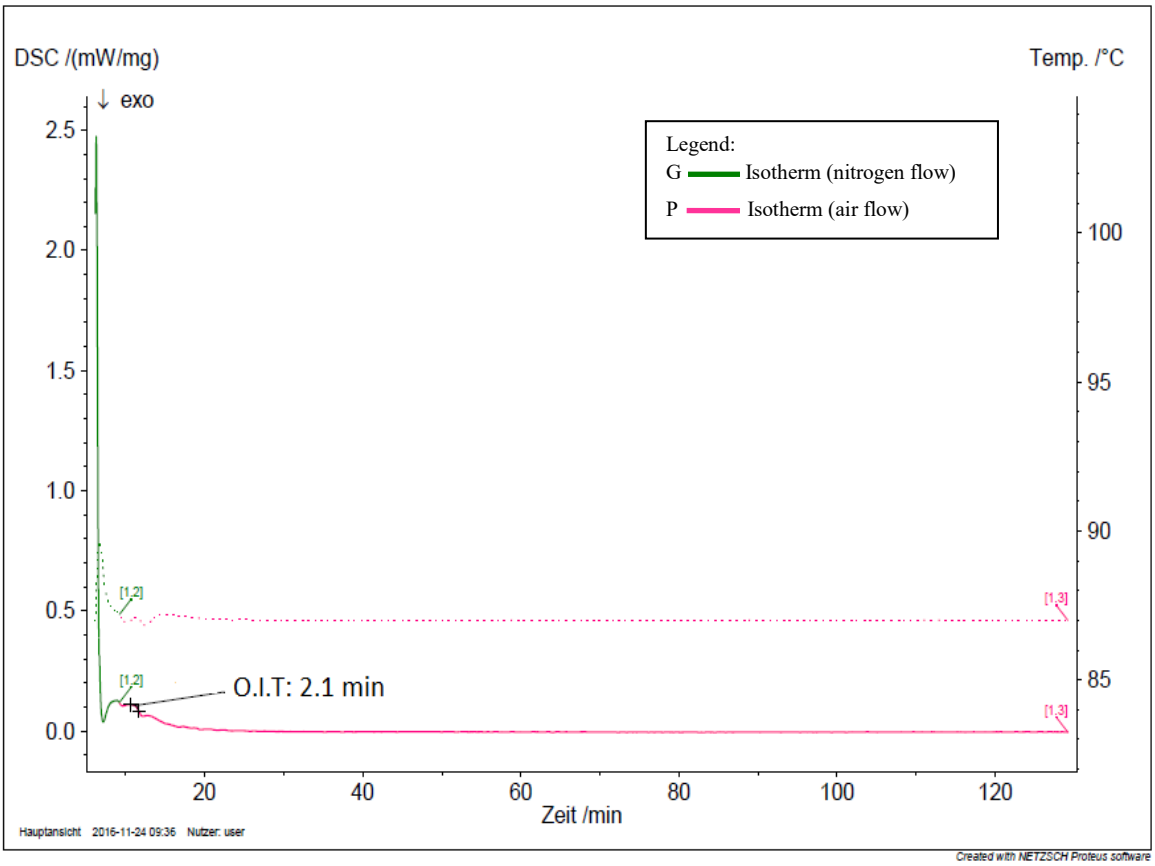



Figure 70: Oxidation behaviour of vitamin $D_3$ at 87 °C

Figure 70 describes what happened when vitamin $D_3$ was heated up to 87 °C and exposed to air at this temperature for two hours (pink segment), after three minutes of isothermal state under nitrogen flow (green segment). The OIT was calculated to be of 2.1 minutes. The result of the heating up to 125 °C is shown in Figure 71.

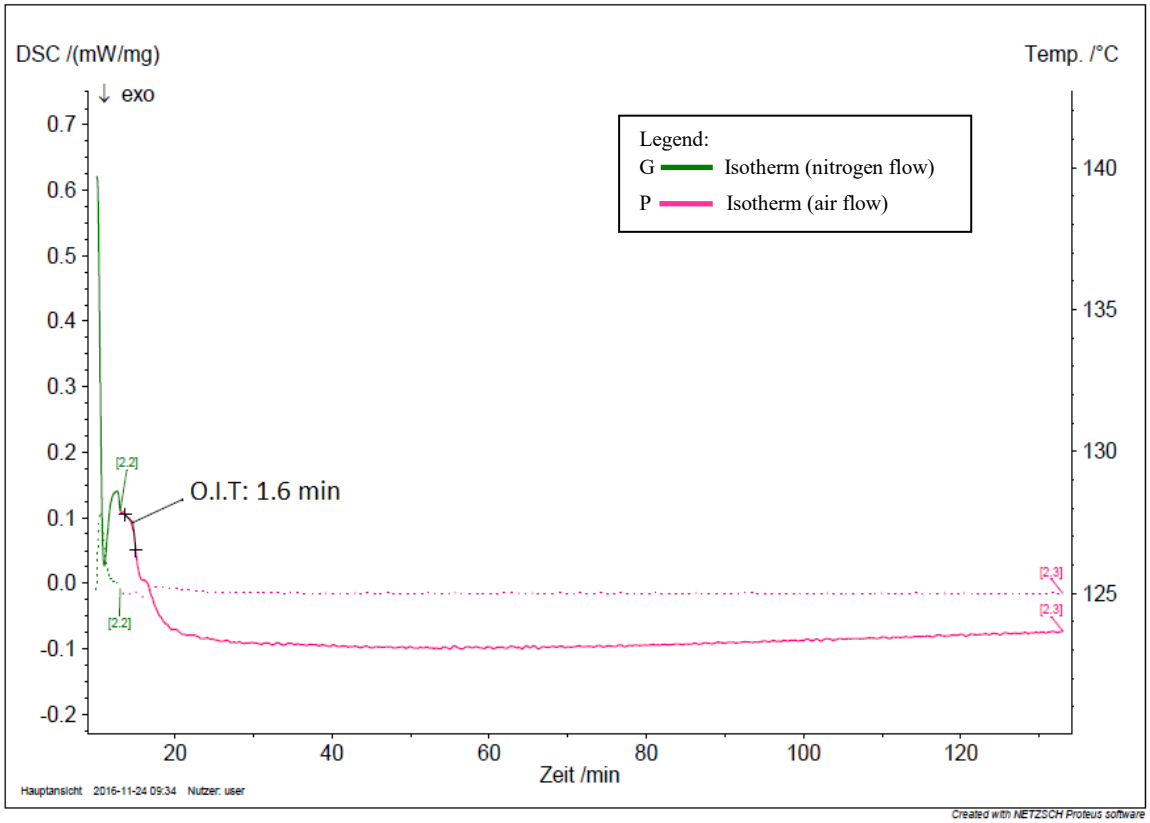



Figure 71: Oxidation behaviour of vitamin $D_3$ at 125 °C

The OIT calculated was 1.6 minutes, which is lower than before, because, as expected, at higher temperature and under air flow, the time necessary to trigger the oxidation is less. As last test, vitamin $D_3$ was heated up to 150 °C and exposed to air, at this temperature for one hour and a half. The OIT decreased, as assumed, to 1.3 minutes (Figure 72).

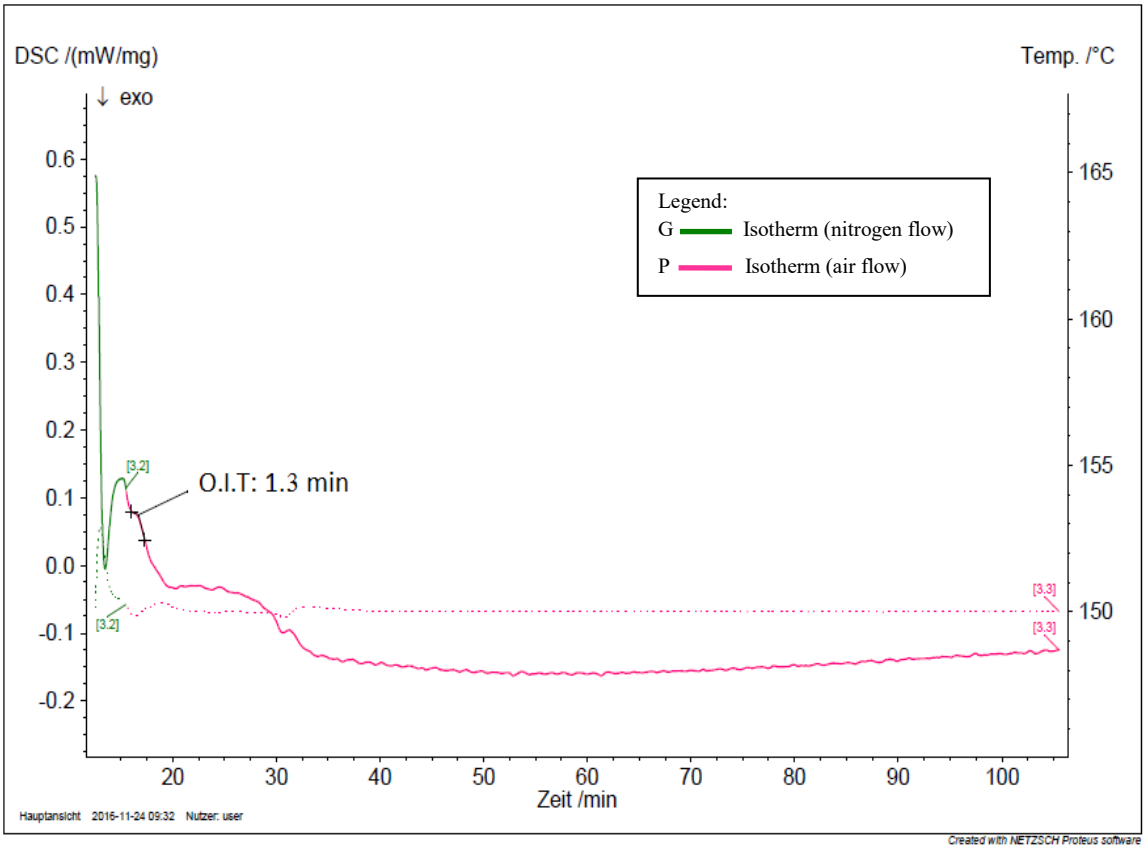



Figure 72: Oxidation behaviour of vitamin $D_3$ at 150 °C

The inverse of the OITs calculated to the relative temperatures, represents the corresponding oxidation rate constant k and by means of the modified Arrhenius equation (Equation 7) the effect on the lipid oxidation was investigated. The following table is a report of parameters used for the kinetics evaluation.

| **DSC Data** | | **Parameters of Arrhenius equation** | | | |
|---|---|---|---|---|---|
| T | OIT | T | 1/T | k=1/OIT | lnk |
| [°C] | [min] | [°K] | [1/°K] | [min-1] | [min-1] |
| 87 | 2,1 | 360,15 | 0,00277 | 0,47619 | -0,74194 |
| 125 | 1,6 | 398,15 | 0,00251 | 0,625 | -0,47 |
| 150 | 1,3 | 423,15 | 0,00236 | 0,76923 | -0,26236 |

Table 74: Data resulting from the DSC analysis of vitamin $D_3$, converted in parameters of Arrhenius equation

The calculated least squares linear regression, resulting from the parameters of Table 74, is shown in the figure below. The coefficient of determination $R^2$ was 0.9929, which testified a good statistical reliability of the analysis.

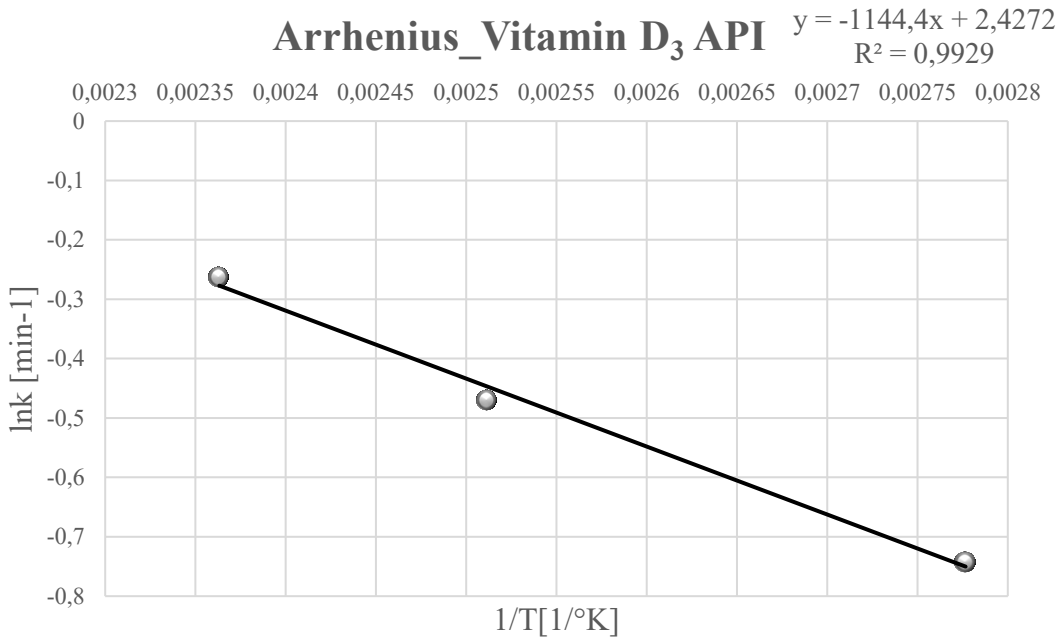



Figure 73: Least squares linear regression according to Arrhenius equation for vitamin $D_3$

From the intercept and the slope of the linear regression, the frequency factor A and the activation energy $E_a$ were respectively calculated. The outcomes are listed in the table below.

| **Slope** | | | **Intercept** | | |
|---|---|---|---|---|---|
| **$E_a$/R** | 1144,4 | K° | **lnA** | 2,427 | [$min^{-1}$] |
| **R** | 0,0083143 | KJ/mol*K° | | | |
| **$E_a$** | 9,515 | KJ/mol | **A** | 11,327 | [$min^{-1}$] |

Table 75: Activation energy Ea and frequency factor A for the oxidation reaction of vitamin $D_3$

The calculation of the activation energy $E_a$ is useful to find the rate constant k at different temperatures and evaluate the stability of vitamin $D_3$, e.g. during the manufacturing procedure, the storage time. Moreover, the comparison of the $E_a$ values, resulting from the analysis of excipients and the whole formulation, contributes to estimating the stability of the spray.
As expected, the value of $E_a$ for the oxidation reaction of vitamin $D_3$ was not so high, because its conjugated double bonds are very sensitive to oxygen linkage.

### 3.7.2.2 Analysis of MCT oil

The sample of MCT oil (~ 5mg ) was heated up to 70° C, 80°C and 90°C using nitrogen flow at 20 ml/min as protective gas. As well as for the vitamin $D_3$, after 3 minutes of isothermal state at the tested temperature, the nitrogen was turned off and only air was let to pass through the measuring chamber for 45 minutes.

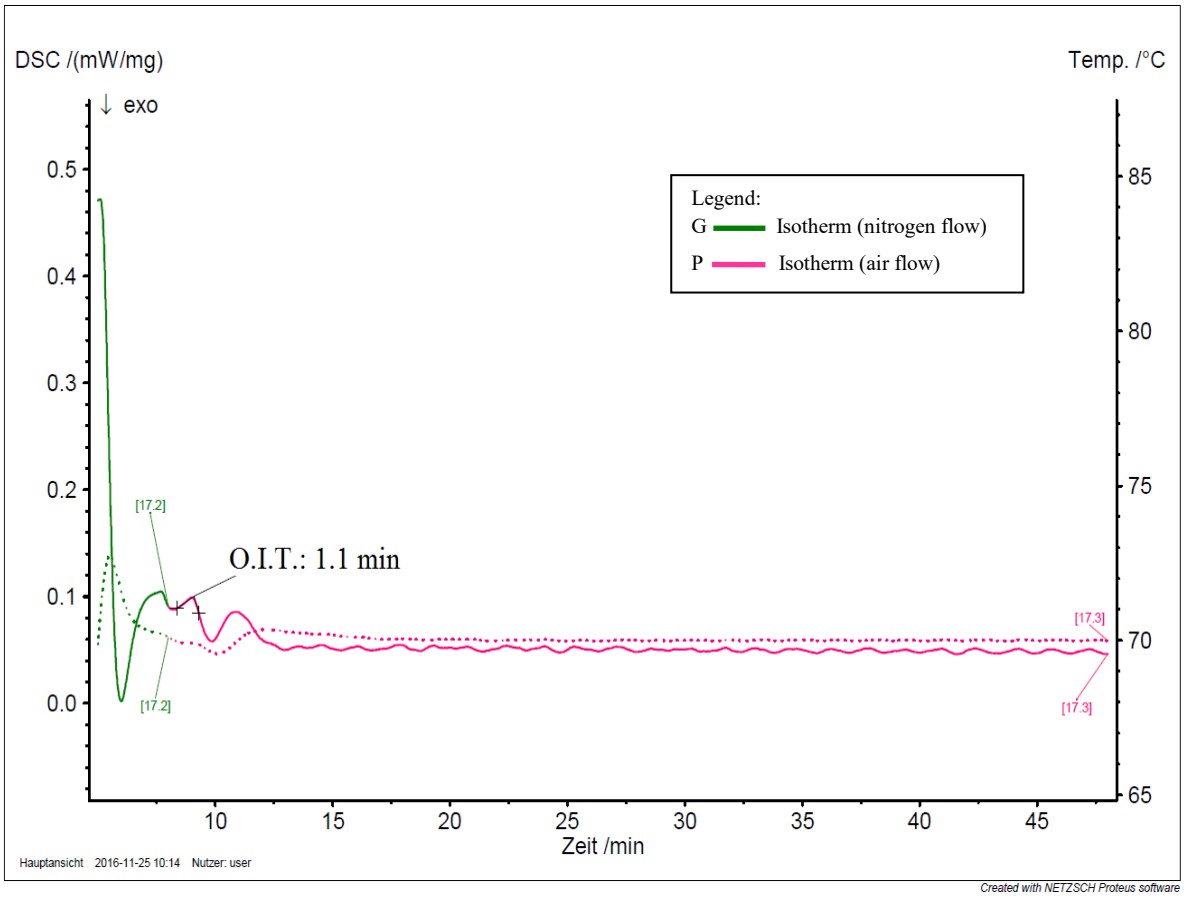



Figure 74: Oxidation behaviour of vitamin $D_3$ at 70 °C

Figure 74 shows that at 70°C, the oxidation of MCT oil started after 1.1 minutes from the incoming of air flow. As indicated by the legend, the green segment of the curve corresponds to the isothermal with nitrogen flow, while the pink segment is the oxidation curve.
By rising the temperature to 80°C, as expected, the OIT decreased to 0.9 minutes and the steepness of the exothermic curve already became more pronounced respect to the curve at 70°C, indicating that the decomposition was rather advanced (Figure 75).

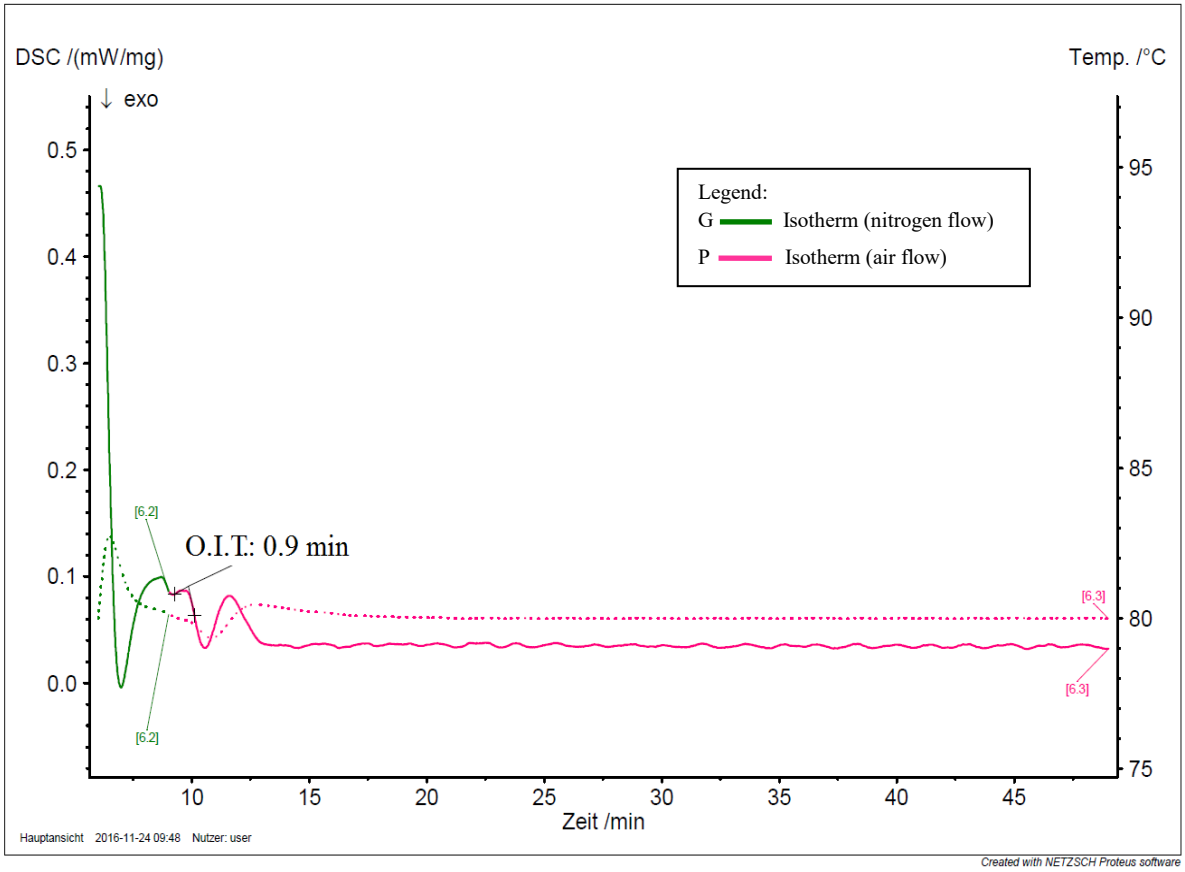



Figure 75: Oxidation behaviour of vitamin $D_3$ at 80 °C

Looking at Figure 75 and 76, it can be observed that the shape of the oxidation curve did not show big differences between 80°C and 90°C, just the OIT changed to 0.8 minutes.

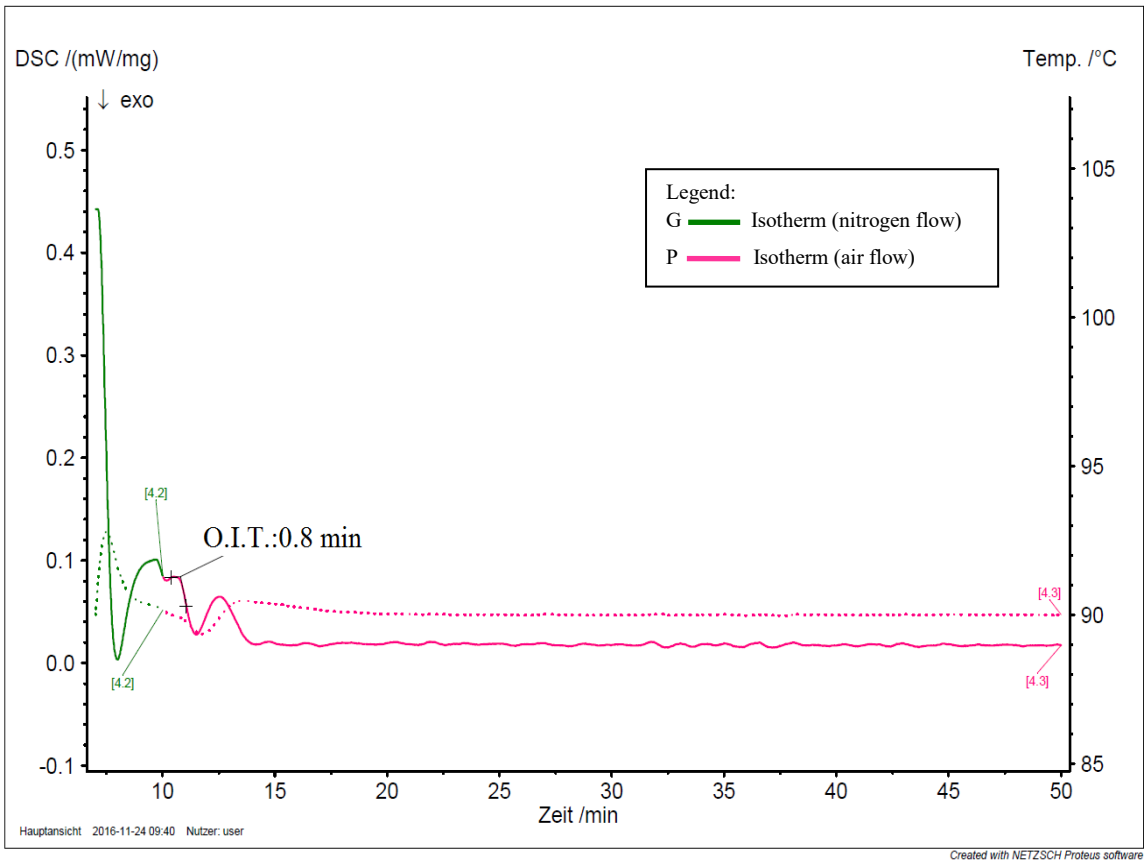



Figure 76: Oxidation behaviour of vitamin $D_3$ at 90 °C

As for vitamin $D_3$, the Arrhenius equation can be applied to evaluate the oxidation kinetics of MCT oil, which is considerably stable to the oxidation, because of its high content of saturated fatty acids. In the table below, the DSC data and the corresponding conversion to get the Arrhenius parameters are listed.

| **DSC Data** | | **Parameters of Arrhenius equation** | | | |
|---|---|---|---|---|---|
| T | OIT | T | 1/T | k | lnk |
| [°C] | [min] | [°K] | [min-1] | [min-1] | [min-1] |
| 70 | 1,1 | 343,15 | 0,00291 | 0,90909 | -0,09531 |
| 80 | 0,9 | 353,15 | 0,00283 | 1,11111 | 0,10536 |
| 90 | 0,8 | 363,15 | 0,00275 | 1,25 | 0,22314 |

Table 76: Data resulting from the DSC analysis of MCT oil, converted in parameters of Arrhenius equation

The computed least squares linear regression is presented in Figure 77.

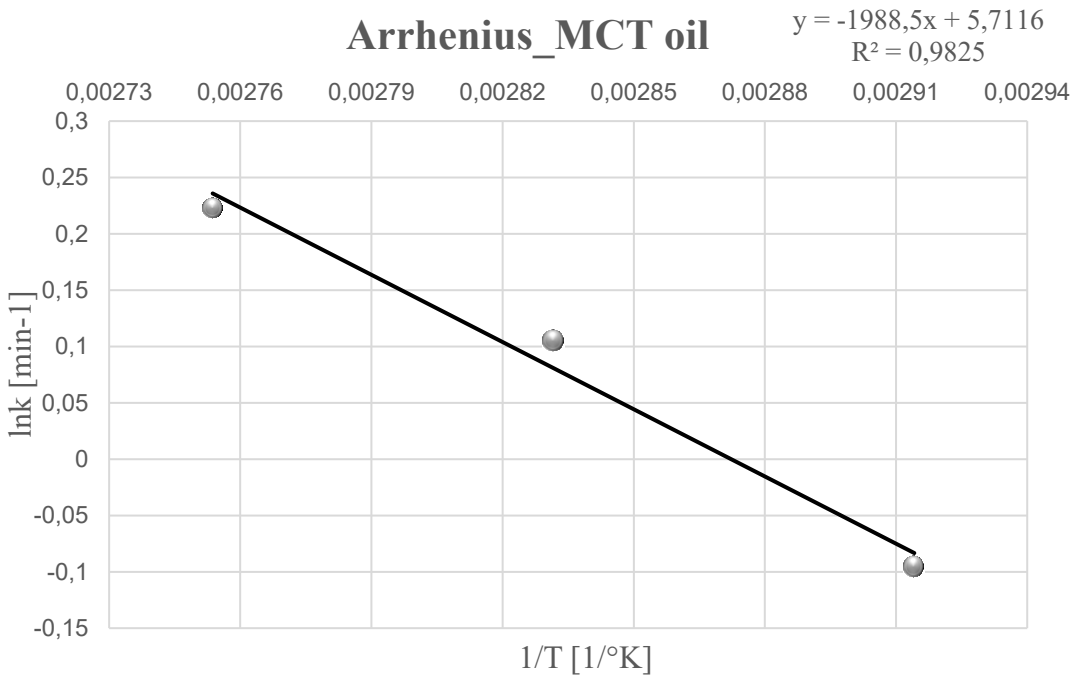



Figure 77: Least squares linear regression according to Arrhenius equation for MCT oil

Table 77 is a report of values found for $E_a$ and A, deriving respectively from the slope and the intercept of the regression line. The value of $R^2$ 0.9825 is lower than that found for vitamin $D_3$, but it testified anyway a reasonable statistical accuracy.

| **Slope** | | | **Intercept** | | |
|---|---|---|---|---|---|
| **$E_a$/R** | 1988,5 | K° | **lnA** | 5,711 | [$min^{-1}$] |
| **R** | 0,008314 | KJ/mol*K° | | | |
| **$E_a$** | 16,532 | KJ/mol | **A** | 302,354 | [$min^{-1}$] |

Table 77: Activation energy ($E_a$) and frequency factor (A) for the oxidation reaction of MCT

As it can be seen in Table 77, in comparison to vitamin $D_3$, the activation energy resulted to be higher, because the MCT oil is not so sensitive to the oxygen like vitamin $D_3$. This is due to the absence of double bonds, which, in case of unsaturated fatty acids, are responsible for promoting the linkage of oxygen molecules. From the conducted analysis, it results that MCT oil was affecting neither the storage nor the manufacturing process, because of the high energy required to initiate the oxidation reaction.

### 3.7.2.3 Analysis of PECEOL™

The same procedure as the vitamin $D_3$ and MCT oil was applied for the oxidation stability test of PECEOL™. The sample weight was always around 5 mg and the tests were performed at 40°C, 50°C and 60°C.

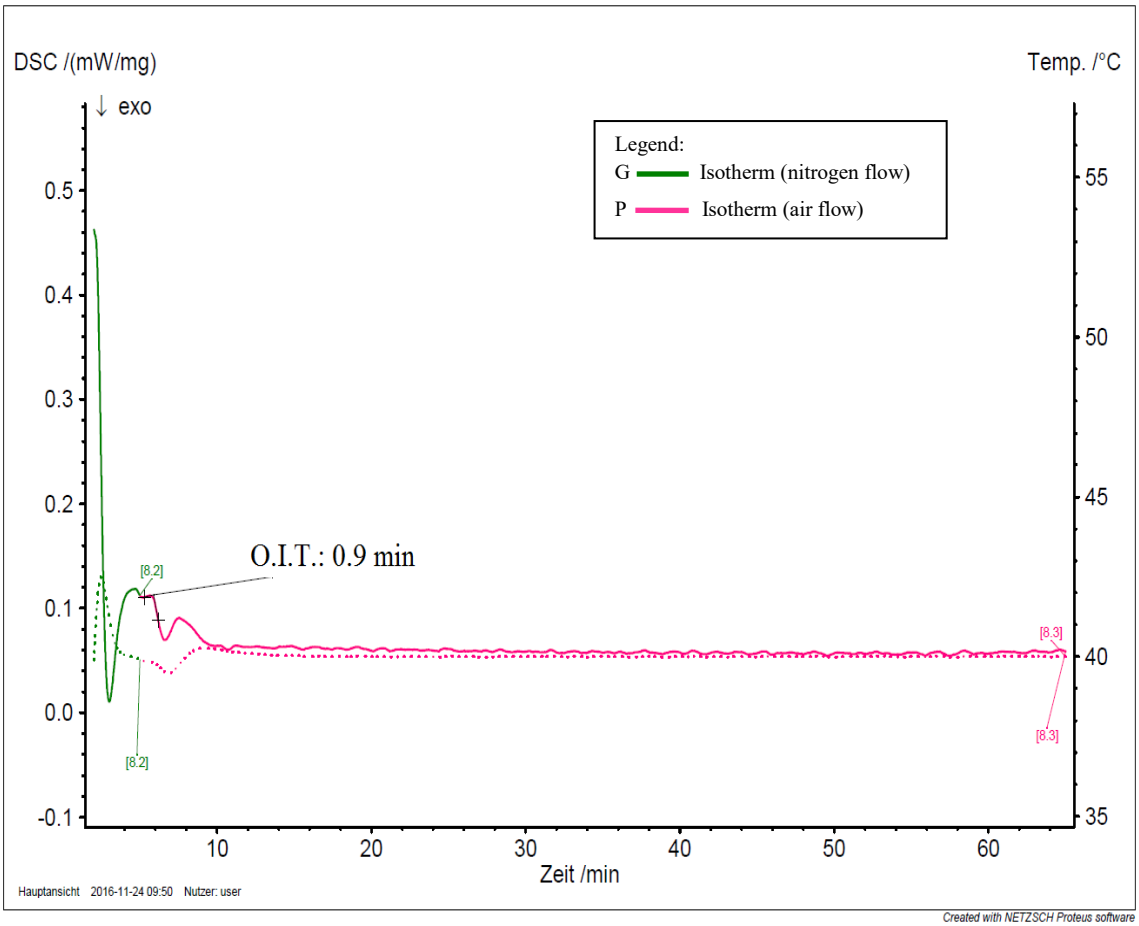



Figure 78: Oxidation behaviour of PECEOL™ at 40 °C

The temperature chosen were lower than in the previous test (e.g. vitamin $D_3$, MCT oil), because already at 70°C, the emulsifier's decomposition takes place and the curve is misshapen, not allowing the calculation of the OIT.

Figure 78 describes what happened, when PECEOL™ was heated up to 40 °C and exposed to air at this temperature for one hour. The resulting OIT was 0.9 minutes. By heating up to 50 °C and exposing the sample to air at this temperature for one hour and a half, it was expected, as for vitamin $D_3$ and MCT oil, that the OIT decreased (Figure 79).

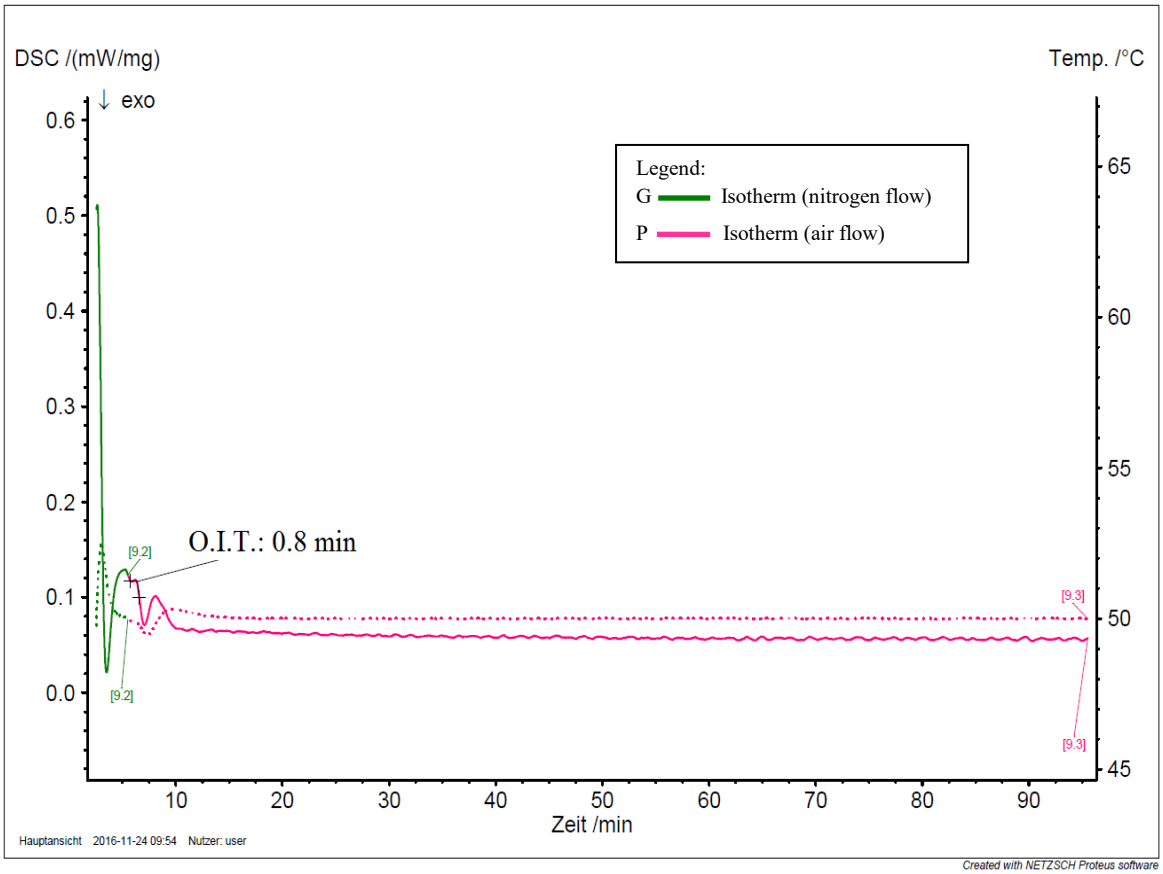



Figure 79: Oxidation behaviour of PECEOL™ at 50 °C

As it is shown in Figure 80, when PECEOL™ was kept under air flow at the temperature of 60 °C, the OIT assumed the value of 0.7 minutes.

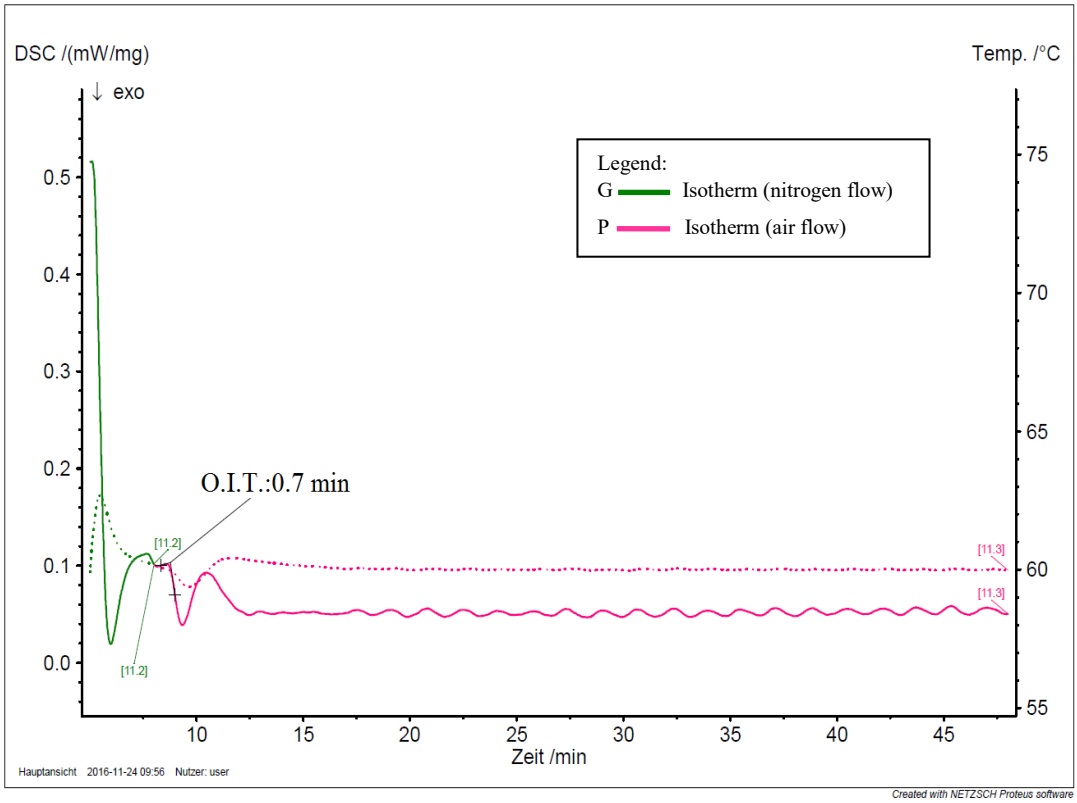



Figure 80: Oxidation behaviour of PECEOL™ at 60 °C

The difference with the MCT oil is already evident by looking at the OITs, which were rather similar, but in case of PECEOL™ had been detected at lower temperatures.
This proved the higher sensitivity of the emulsifier to the oxygen, principally due to the content of unsaturated fatty acids. The analysis of DSC data, using the Arrhenius equation, showed more the differences more detailed (Table 78).

| **DSC Data** | | **Parameters of Arrhenius equation** | | | |
|---|---|---|---|---|---|
| T | OIT | T | 1/T | k | lnk |
| [°C] | [min] | [°K] | [min-1] | [min-1] | [min-1] |
| 40 | 0,9 | 313,15 | 0,00319 | 1,11111 | 0,10536 |
| 50 | 0,8 | 323,15 | 0,00309 | 1,25 | 0,22314 |
| 60 | 0,7 | 333,15 | 0,00300 | 1,42857 | 0,35667 |

Table 78: Data resulting from the DSC analysis of PECEOL™, converted in parameters of Arrhenius equation

As for the other samples previously discussed, the least squares linear regression was calculated (Figure 81) and the value of $R^2$ equal to 0.9971 indicated the good representativeness both of the collected data and the relative interpretation by Arrhenius.

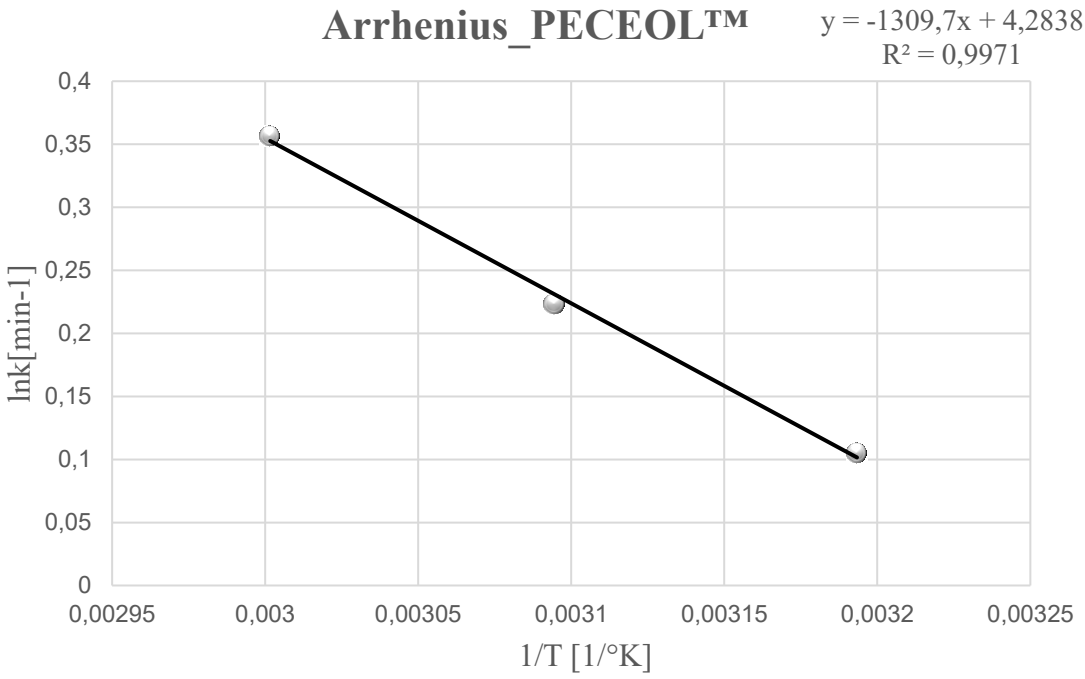



Figure 81: Least squares linear regression according to Arrhenius equation for PECEOL™

| **Slope** | | | **Intercept** | | |
|---|---|---|---|---|---|
| **$E_a/R$** | 1309,7 | K° | **lnA** | 4,283 | [$min^{-1}$] |
| **R** | 0,008314 | KJ/mol*K° | | | |
| **$E_a$** | 10,889 | KJ/mol | **A** | 72,515 | [$min^{-1}$] |

Table 79: Activation energy $E_a$ and frequency factor A for the oxidation reaction of PECEOL™

Looking at Table 79, the activation energy resulting from the analysis was lower than that of MCT oil (16, 53299 KJ/mol) but higher than that of vitamin $D_3$ (9,515 KJ/mol).

PECEOL™, together with vitamin $D_3$, can be considered the principal components, which can determine oxidation of the formulation. However, the low concentration of emulsifier (1%w/w) used reduces the probability of rancidity.

### 3.7.2.4 Analysis of the vitamin $D_3$ spray

The oxidation behaviour of single components has influence over the whole formulation, which will have anyway an own oxidation kinetics depending on the amount of the considered components. The amount of sample use for the formulation analysis was 5 mg and the test was performed at 60°C, 70°C and 80°C.

The oxidation behaviour of vitamin $D_3$ spray resulting at 60°C is shown in Figure 82 and the calculated OIT was of 1.5 minutes.

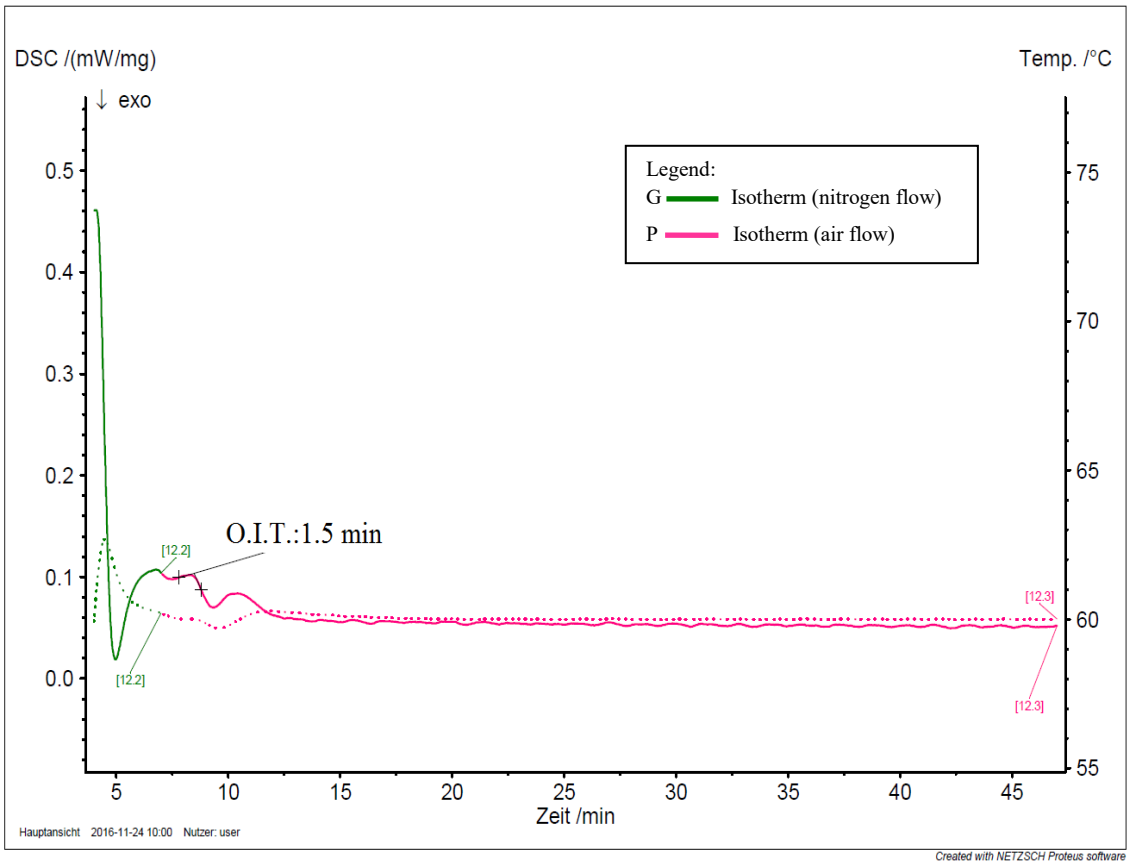



Figure 82: Oxidation behaviour of vitamin $D_3$ spray at 60 °C

By increasing the temperature, as already seen for the previous tests, the OIT decreased to 1.4 minutes at 70°C (Figure 83) and 1.3 minutes at 80°C (Figure 84).

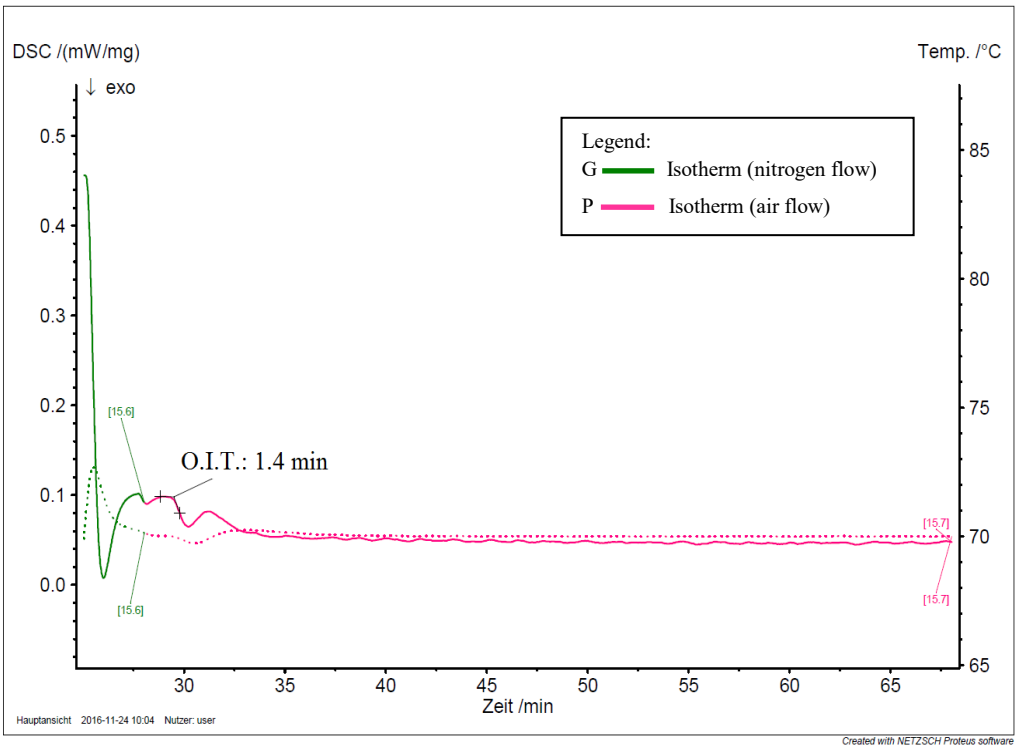



Figure 83: Oxidation behaviour of vitamin $D_3$ spray at 70 °C

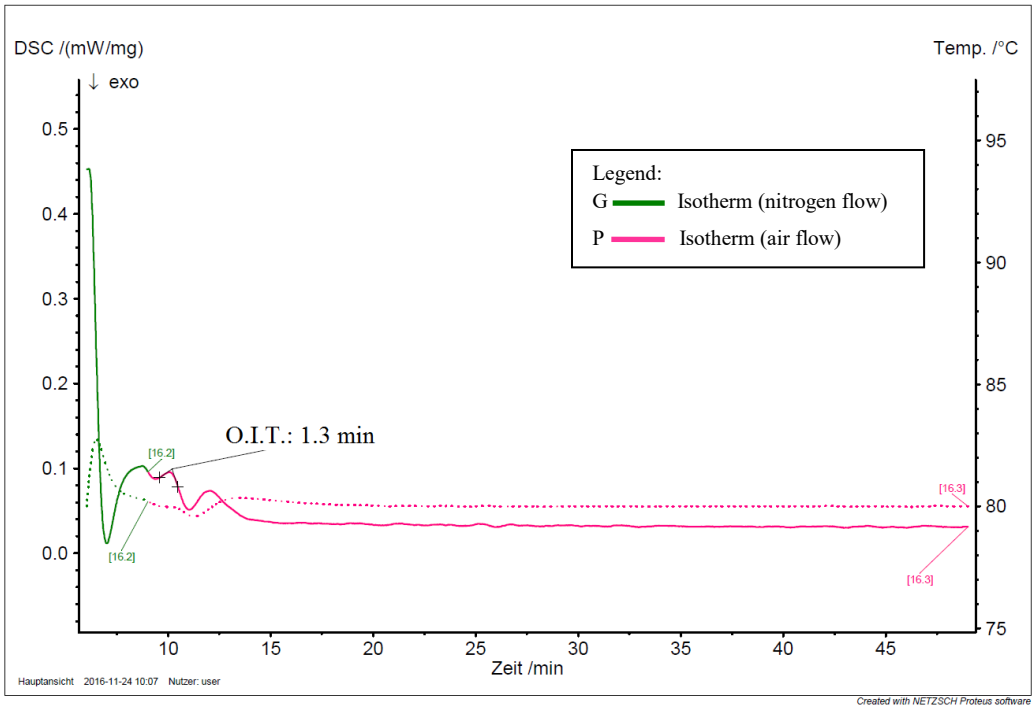



Figure 84: Oxidation behaviour of vitamin $D_3$ spray at 80 °C

The DSC data were converted into parameters useful for the evaluation according to Arrhenius (Table 80).

| **DSC Data** | | **Parameters of Arrhenius equation** | | | |
|---|---|---|---|---|---|
| T | OIT | T | 1/T | k | lnk |
| [°C] | [min] | [°K] | [min-1] | [min-1] | [min-1] |
| 60 | 1,5 | 333,15 | 0,00300 | 0,66666 | -0,40547 |
| 70 | 1,4 | 343,15 | 0,00291 | 0,71428 | -0,33647 |
| 80 | 1,3 | 353,15 | 0,00283 | 0,76923 | -0,26236 |

Table 80: Data resulting from the DSC analysis of vitamin $D_3$ spray, converted in parameters of Arrhenius equation

The resulting least squares linear regression was obtained by plotting the lnk against 1/T (Figure 85).

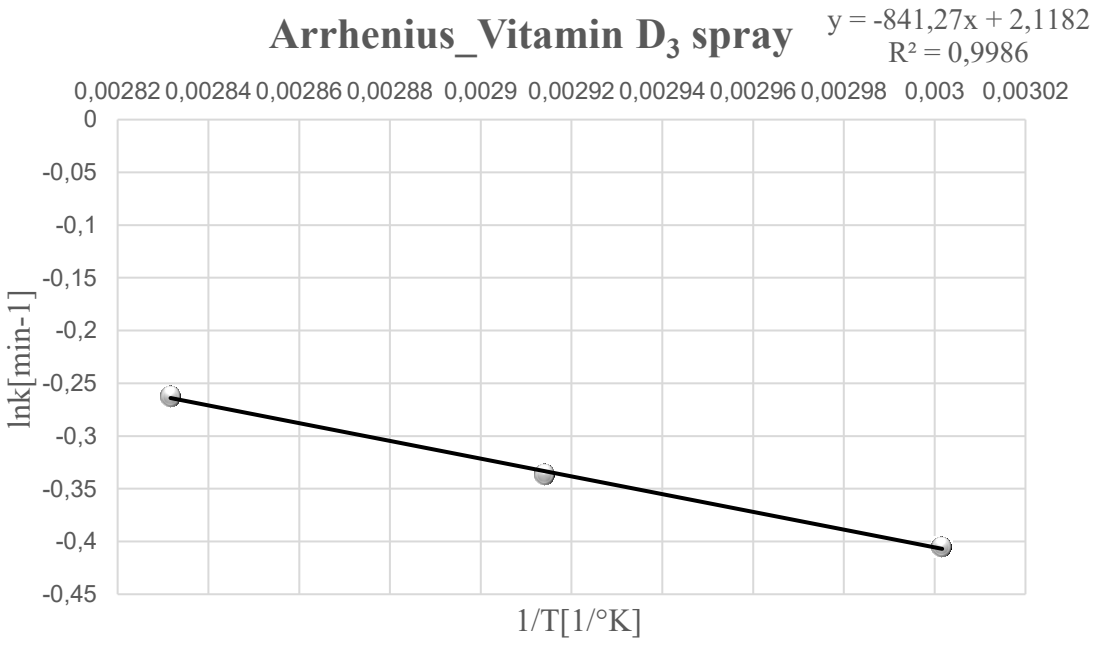



Figure 85: Least squares linear regression according to Arrhenius equation for vitamin $D_3$ spray

Since the value $R^2$ was very close to one, the fitting was considered statically reliable. Therefore, from the slope and the intercept of the linear regression, the activation energy and the frequency factor of the oxidation reaction were respectively derived (Table 81).

| **Slope** | | | **Intercept** | | |
|---|---|---|---|---|---|
| **$E_a/R$** | 841,27 | K° | **lnA** | 2,118 | [$min^{-1}$] |
| **R** | 0,008314 | KJ/mol*K° | | | |
| **$E_a$** | 6,994 | KJ/mol | **A** | 8,316 | [$min^{-1}$] |

Table 81: Activation energy $E_a$ and frequency factor A for the oxidation reaction of vitamin $D_3$ spray

The calculated activation energy, necessary to start the oxidation reaction in the vitamin $D_3$ spray, was lower than that required for the vitamin $D_3$, the MCT oil and PECEOL™.
In fact, as expected, the combination of the pure API with excipients (MCT oil, PECEOL™, flavour) increased the oxidation probability.

### 3.7.2.5 Analysis of the OLEOVIT™

The isothermal oxidation test by DSC was finally performed also for OLEOVIT™, in order to compare the kinetics with the new spray formulation.

A small sample (~5mg) was taken and the analysis was conducted at 70°C, 80°C and 90°C, by following the same procedure described in the previous paragraphs.

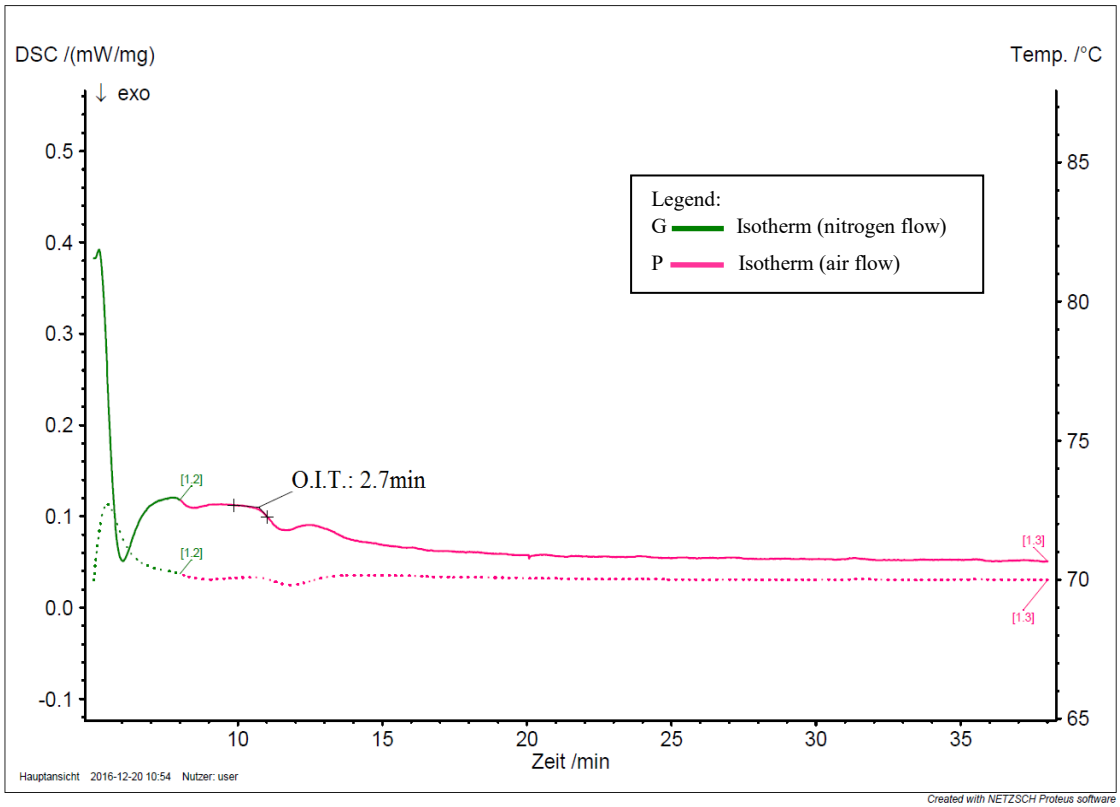



Figure 86: Oxidation behaviour of OLEOVIT™ at 70 °C

As it is indicated in Figure 86, at 70°C the OIT of OLEOVIT™ gave a higher value (2.7 minutes) than that of vitamin $D_3$ spray (1.4 minutes) at the same temperature. By increasing the temperature, the reduction of OIT occurred, as for other tests (Figure 87).

A remarkable difference between the two formulations came also out from the analysis at 80°C, because the calculated OIT for OLEOVIT™ was of 1.8 minutes, while the one for vitamin $D_3$ spray was 1.3 minutes.

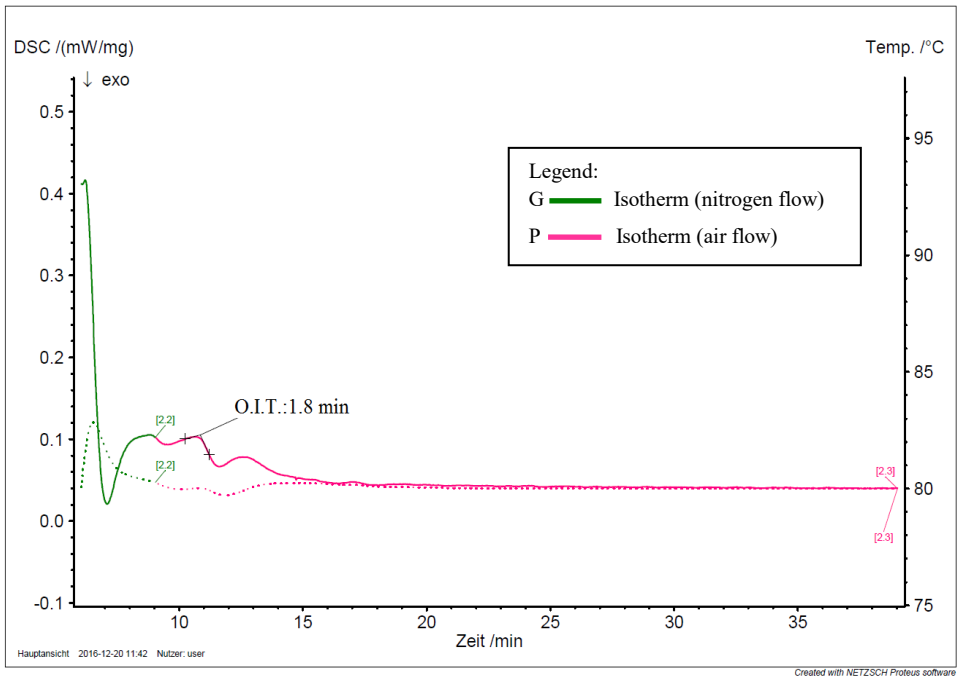



Figure 87: Oxidation behaviour of OLEOVIT$^{TM}$ at 80 °C

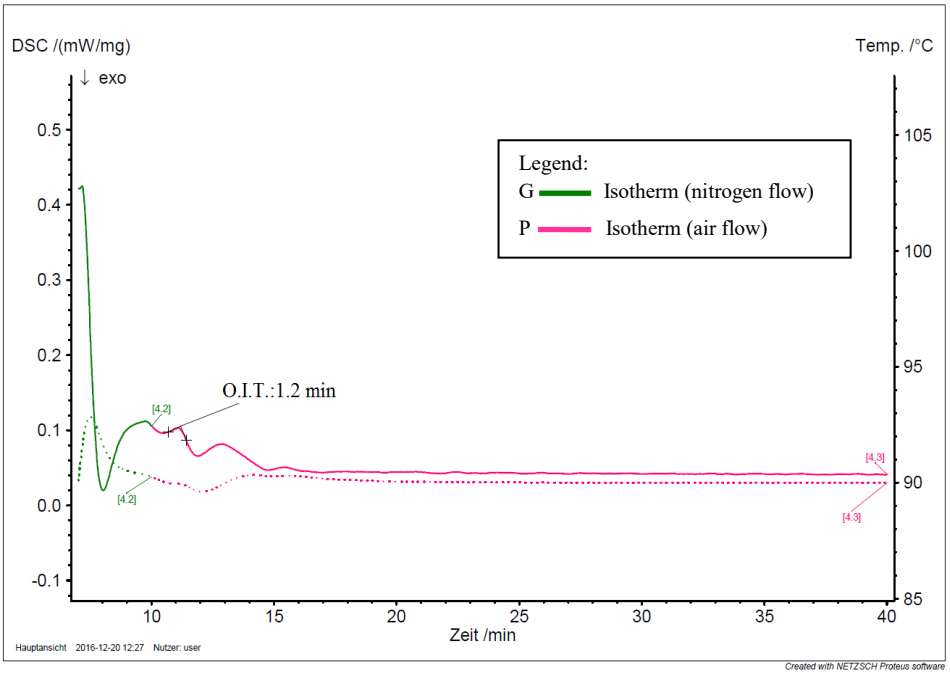



Figure 88: Oxidation behaviour of OLEOVIT$^{TM}$ at 90 °C

The measurement of OIT at 90°C for vitamin $D_3$ spray gave no reasonable results, because the heating step under nitrogen had already caused the sample decomposition, making then impossible to obtain a noticeable curve, to define the OIT.

However, the test was carried out for OLEOVIT™ and Figure 88 shows the result. As for vitamin $D_3$ spray, the temperatures and the relative OIT, where transformed into Arrhenius parameters, in order to conduct the investigation of kinetics (Table 82).

| **DSC Data** | | **Parameters of Arrhenius equation** | | | |
|---|---|---|---|---|---|
| T | OIT | T | 1/T | k | lnk |
| [°C] | [min] | [°K] | [min-1] | [min-1] | [min-1] |
| 70 | 2,7 | 343,15 | 0,00291 | 0,37037 | -0,99325 |
| 80 | 1,8 | 353,15 | 0,00283 | 0,55555 | -0,58779 |
| 90 | 1,2 | 363,15 | 0,00275 | 0,83333 | -0,18232 |

Table 82: Data resulting from the DSC analysis of OLEOVIT™, converted in parameters of Arrhenius equation

By plotting the lnk against 1/T, the least squares linear regression, shown in Figure 89, was obtained.

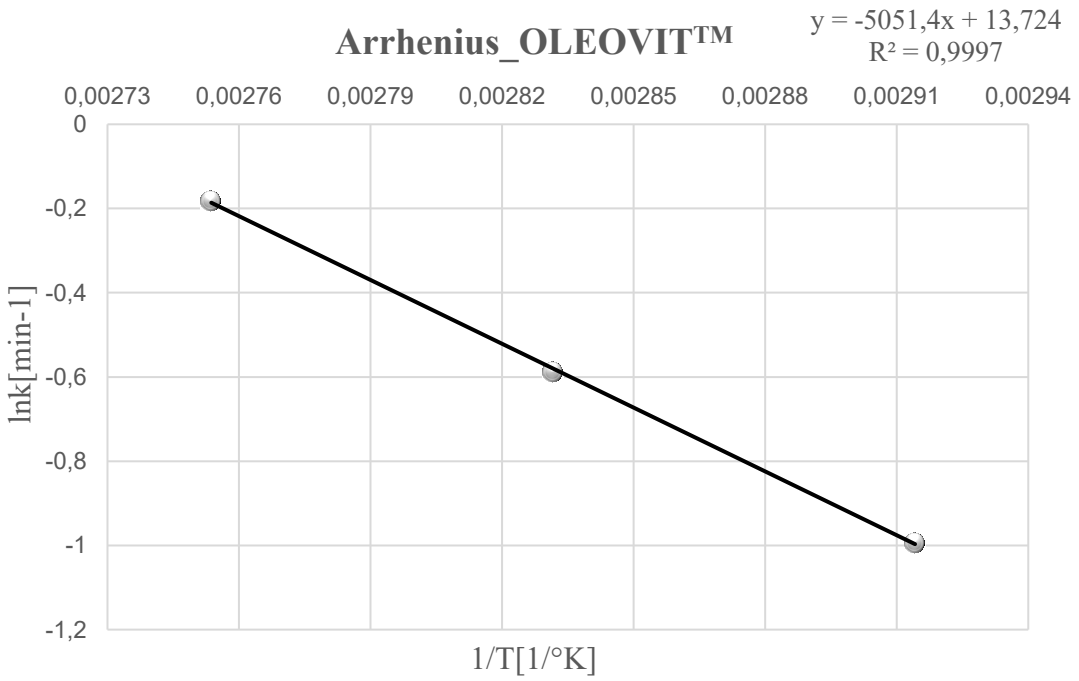



Figure 89: Least squares linear regression according to Arrhenius equation for OLEOVIT™

Given the accuracy of the linear regression ($R^2$=0,997), the slope and the intercept were used to calculate respectively the activation energy $E_a$ and the frequency factor A (Table 83).

| Slope | | | Intercept | | |
|---|---|---|---|---|---|
| **$E_a$/R** | 5051,4 | K° | **lnA** | 13,724 | [$min^{-1}$] |
| **R** | 0,008314 | KJ/mol*K° | | | |
| **$E_a$** | 41,998 | KJ/mol | **A** | 9,13E+05 | [$min^{-1}$] |

Table 83: Activation energy $E_a$ and frequency factor A for the oxidation reaction of OLEOVIT™

The high activation energy (41,998 KJ/mol) deriving from the kinetic analysis of OLEOVIT™ showed that the previous formulation was less sensitive to oxidation than the new spray formulated (6,994 KJ/mol) (Table 84).
The addiction of emulsifier and flavouring agent in the formulation can be the reason of the considerable difference of stability. In fact, PECEOL™ as well as the aromatic groups of flavouring agent, provide double bonds, which react with the oxygen, determining the initiation of oxidative cascade.

| Sample | Activation energy $E_a$ [KJ/mol] |
|---|---|
| Vitamin $D_3$ API | 9,514 |
| PECEOL™ | 10,889 |
| MCT oil | 16,532 |
| Vitamin $D_3$ spray | 6,994 |
| OLEOVIT™ | 41,998 |

Table 84: Activation energy of API, excipients, old and new formulation

Using the activation energy and frequency factor found for vitamin $D_3$ spray and OLEOVIT™, the stability of formulation was extrapolated for 25°C and 50°C by means of relative linear regressions (Table 85).

| **Parameters** | **Vitamin $D_3$ Spray** | **OLEOVIT™** |
|---|---|---|
| | At 25°C and under air flow | |
| k[$min^{-1}$] | 4,95E-01 | 4,00E-02 |
| OIT[min] | 2 | 25 |
| | At 50°C and under air flow | |
| k[$min^{-1}$] | 0,61560 | 1,48E-01 |
| OIT[min] | 1,6 | 6,7 |

Table 85: Comparison of stability of old and new formulation at 25°C and 50°C under air flow

From data reported in Table 85, it can be seen as under the described conditions, the vitamin $D_3$ spray begins to oxidize, already after two minutes from the exposition at air flow at 25°C, while OLEOVIT™ requires 25 minutes.

At 50°C, which is the maximal temperature described for the processing in the SOP provided by the company, the reduction of the OIT is still more significant for vitamin $D_3$ spray.

Therefore, the new formulated spray needs more attention to nitrogen flush and protection during the manufacturing process as well as during the storage, because the energetic barrier which prevents the oxidation reaction is low and external factors can easily contribute to providing the energy necessary to overcome it.

## 3.8 Surface tension analysis

The surface tension analysis was performed for the new formulated vitamin $D_3$ spray, in order to estimate the ability to be spread onto the buccal mucosa.
In fact, as already discussed in Paragraph 1.8 of the introduction, the buccal absorption is strictly related to the surface tension, which is generated in the mouth, between the oily formulation and the aqueous components (e.g. saliva, mucosa). The surface tension should be as lower as possible to ensure the functionality of the dosage form. To this purpose, the role of surfactant is very important.


The instrument used for the analysis was the *Bubble pressure tensiometer-BP50* of the German company Krüss (Figure 90).
It allows the determination of the dynamic surface tension (SFT), whose unit is mN/M. The instrument is composed of a capillary, which is submerged into the liquid solution to be measured. When it is turned on, gas bubbles are produced in the liquid at the tip of the capillary [102]. Initially, the radius of curvature of the bubble is large, but then decreases. Once the radius of the bubble is equal to the radius of the capillary, the pressure maximum (Pmax) can be calculated [102].

Figure 90: Bubble pressure tensiometer-BP50 Krüss

According to the Young-Laplace equation, by knowing the radius of the capillary, the instrument is able to determine the surface tension using the following formula [102]:

$$P = \frac{2\sigma}{r} \quad \text{(Young-Laplace)} \qquad \text{Equation 8}$$

$$\sigma = \frac{(P_{max} - P_0) * r}{2}$$ Equation 9

Where $P_0$ is the hydrostatic pressure deriving from the immersion depth of the capillary and the density of liquid, while r is the radius of the capillary.

The value of surface tension, measured by the instrument, is dependent on the surface age, which is the time from the beginning of bubble formation to the registration of maximum pressure [102].

Figure 91 shows the results obtained from the analysis conducted for the vitamin $D_3$ spray, containing PECEOL™ as surfactant, MCT oil, as lipophilic excipient and water as control medium.

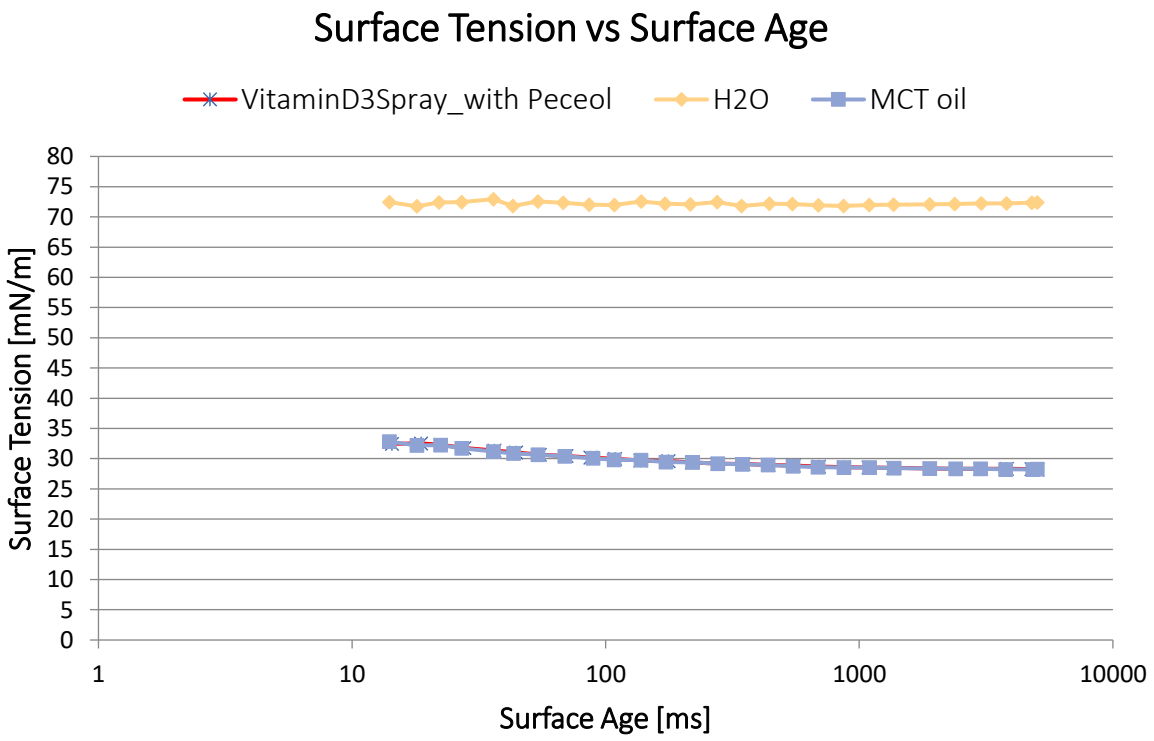



Figure 91: Analysis of surface tension of vitamin $D_3$ Spray, MCT oil and water at temperature of 22°C

The detected surface tension of the spray was rather low (~ 30 mN/m) and very close to that of MCT oil alone. Therefore, both MCT oil as well as the surfactant will contribute to facilitate spreading on the buccal mucosa.

## 3.9 Size distribution of sprayed droplets

The aim of a spray formulations is to facilitate buccal administration. However, it has to be guaranteed that the delivery of the sprayed drops occurs in the mouth, therefore, a possible distribution into the bronchial tube or lungs has to be avoided or reduced at minimum.

The particle size analysis of sprayed droplets was conducted for the new formulated vitamin $D_3$ spray with the purpose to estimate the percentage of droplets which can be inhaled during administration, compromising the intake of the therapeutic dosage.

A particular set up of the *Mastersizer 2000 Scirocco* (0.02-2000 µm) of Malvern company was used. In fact, as shown in Figure 92, the instrument was transformed to reproduce a 'real' mouth, by means of a funnel, through which the sprayed sample directly reached the cell.

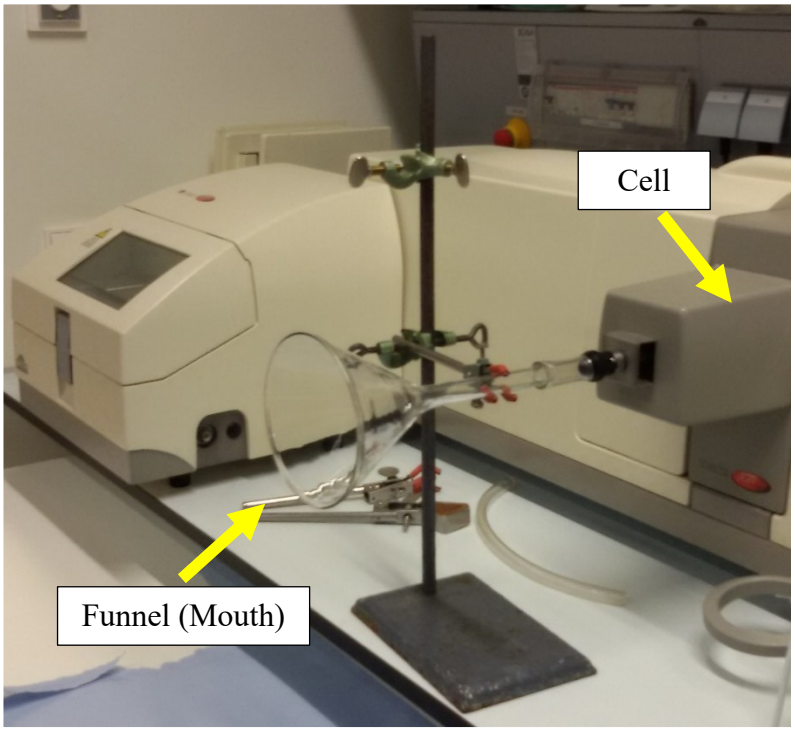



Figure 92: Set-up of Mastersizer 2000 Scirocco used for the size distribution analysis of sprayed droplets

Within the cell, the sample is analysed based on the principles of static light scattering.

From the scattering angles, on the base of the Mie theory, the Mastersizer software analyses the particle size distribution of sprayed droplets. The settings used for the analysis are listed in Table 86.

| | |
|---|---|
| **Refractive index (Oleic acid)** | 1.4585 |
| **Measurement time** | 15 seconds |
| **Measurement snaps** | 15000 |
| **Background time** | 15 seconds |
| **Background snaps** | 15000 |

Table 86: SOP for the performed size distribution analysis

The frequency distribution and the cumulative undersize distribution curve were calculated (Figure 93), in order to define the percentage of drops which were smaller than 10 µm and therefore able to reach the respiratory tract.

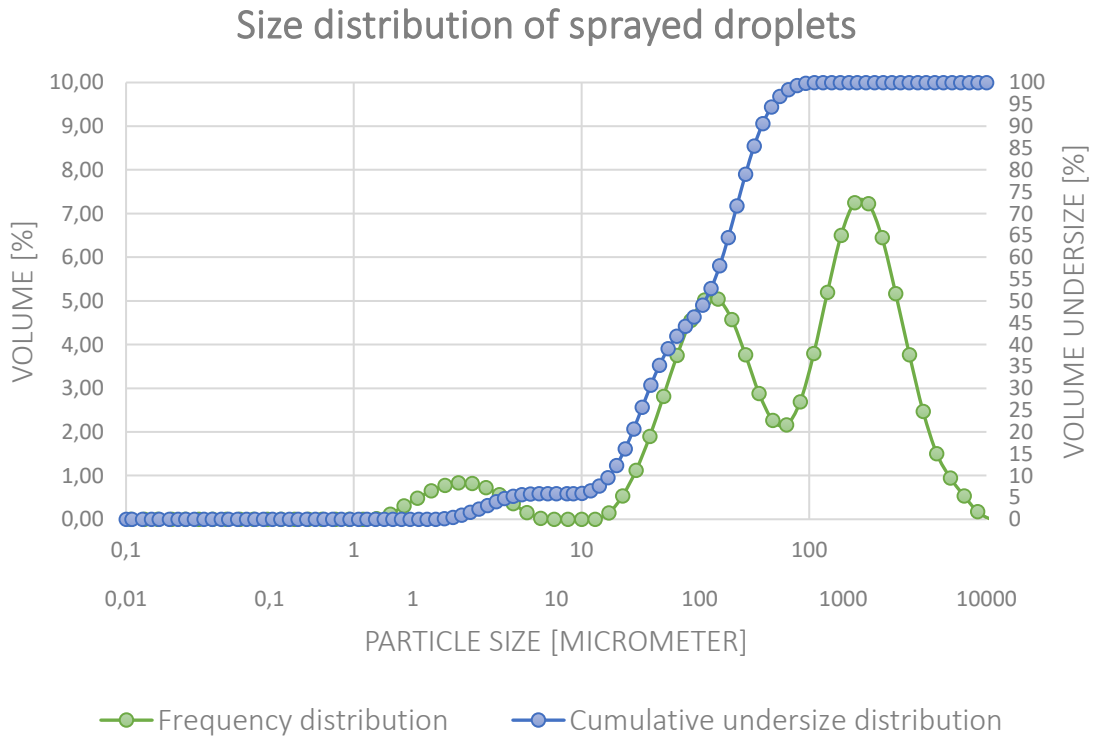



Figure 93: Size distribution of sprayed droplets

As shown in Figure 93, it is possible to see that less than five percent (volume based %) of the formulation was smaller than 10 µm, which means that the probability for the formulation to be inhaled is negligible.

For this reason, the vitamin $D_3$ spray is considered to be safe and effective, because the risk of a pulmonary application is low and the correct buccal administration of the daily dosage is assured.

# 4 Design of experiment by MODDE (MKS Umetrics)

The description of design of experiment (DoE) can be found in the ICH guideline Q8/Q(8)Ra, regarding the good practices for pharmaceutical product development. It is defined as a tool of quality by design (QbD). According to QbD, the quality has to be built in the product, meaning that a detailed knowledge of critical quality attributes (CQAs), concerning raw materials and process parameters, is necessary to determine the factors, which can influence the quality of the product [103]. After performing the risk assessment analysis, the DoE helps to increase the process understanding and to determine the design space, which is 'the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality' ("International Conference on Harmonisation (ICH); Draft Guidance: Q8 (R2) Pharmaceutical Development Revision 1", ICH Q8). The major advantage of using design of experiment (DOE) is that it considers all input variables, allowing the evaluation of their effects on responses [104].

## 4.1 Screening design of process factors

DoE analysis was performed for the development of the vitamin $D_3$ spray. The first step was to select the important factors, whose variation could be responsible for changes in the response. The factors were chosen based on prior knowledge of the process and of formulation components, without resorting to screening models.
In the table below, there is a list of factors, considered in the screening phase, used to execute the design of experiment, in order to define process parameters and optimise the manufacturing phase.

| Name (Abbreviation) | | Units | Type | Settings | Transform | Precision |
|---|---|---|---|---|---|---|
| **Temperature** | Temp | Centigrade | Quantitative | 25 to 60 | None | 0,875 |
| **Time** | Time | Min | Quantitative | 15 to 60 | None | 1,13 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Oxygen** | Oxy | Bar | Quantitative | 0,2123 to 3 | None | 0,0697 |
| **Sunlight** | Sun | $W/m^2$ | Quantitative | 500 | None | |

Table 87: Factors influencing the production process of vitamin $D_3$ spray

As already explained in previous chapters, vitamin $D_3$ is extremely sensitive to heat, oxygen and light when exposed to these for a certain period of time. Therefore, tests were performed by varying the settings shown in the table above, between a low (minimum) and high limit (maximum) value.

The sunlight value was kept constant at 500 $W/m^2$, which was, according to the average daily solar radiation, the estimated value for the time between 10 and 11 AM in the month of July, when the test was executed [105].

No transformation of the unit was set up and the precision value, which defines the limits of the 95% confidence interval and of the acceptable deviations from the given low-high range, was automatically established by the program.

Once the factors were defined, the response, which is the result of an experiment, was specified (Table 88).

| **Name (Abbreviation)** | | **Units** | **Transform** | **Type** | **Min** | **Target** | **Max** |
|---|---|---|---|---|---|---|---|
| **Vitamin $D_3$** | Vit $D_3$ | mg | None | Regular | 8,2 | 9,5 | 9,6 |

Table 88: Responses considered for the production process of vitamin $D_3$ spray

It was indicated the minimum value for the vitamin $D_3$ (8,2 mg), registered during the stress test (Paragraph 3.3.5), then the target value, represented in this case by the amount necessary for the 15% over dosage (9,5 mg) and the maximum, the amount weighed for the preparation (9,6 mg).

As well as for the factors, also for the response no specific transformation was done to adjust the estimated function, but the standard setting was used and not the derived one.

## 4.2 Response surface modelling (RSM)

After the screening phase, the response (change of vitamin $D_3$ content) was approximated by a quadratic polynomial model, in order to understand how the factors had influenced the response, to predict, optimise or find a region where to operate [106].

The most appropriate response surface modelling (RSM) design was the central composite face design (CCF) [106]. It was important to change the characteristics of factors and the way of interaction, thus defining the number of runs for the experiment.

As shown in Figure 94, a Box-Wilson Central Composite Design, usually defined as 'a central composite design,' was used [107]. It is based on a full or fractional factorial design, with factorial points, replicated center points and star points, (on the faces of the sides), that allow the estimation of curvature in the design space [107].

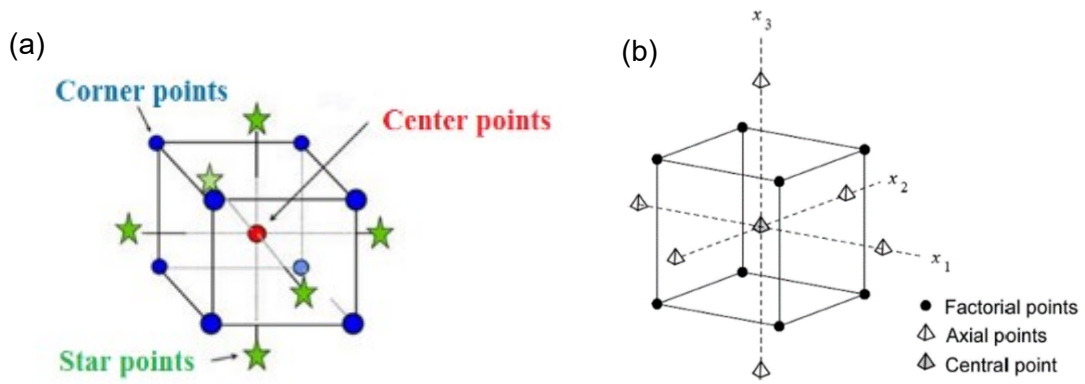



Figure 94 : Graphic representation of a central composite face design (CCF) for three factors [108, 109]

In the figure above, the fractional factorial points represent the assessment of linear and 2-way interaction terms [108]. The center points estimated the pure error and the stability of the process, while the star points represented the assessment of quadratic terms in the following mathematic model (Figure 94,a) [108]. The axes $x_1$, $x_2$, $x_3$ stand for the factors considered (Figure 94, b).

In the specific case of the thesis work, four factors (temperature, time, oxygen, sunlight) were considered and according to the factorial design, this yielded k=4 with each factor having two levels, a lower (-1) and a higher (+1). The resulting design had $2^k=2^4$ factorial points and 2*k axial points or star points [107, 109].

The number of center points runs, which are experimental runs where the factor value is usually set halfway between the low and high settings, should be between 3 and 5, according to the rule of thumb [110]. Threfore, it was set up at 3.
The number of total runs provided by MODDE was 17, where 14 were the design runs (including factorial design and star points) and 3, the center points runs.

## 4.3 Fitting of the experimental data

After setting the experimental runs, the software processed the results obtained for each conducted test and the data collected were used to estimate the coefficients of the model, which correlates the response y and the factors x.
The model which gave the best fit was the partial least square (PLS) which considers several responses simultaneously and shows the effect of factors on responses [106]. The results of the fit are shown in the Figure 95.

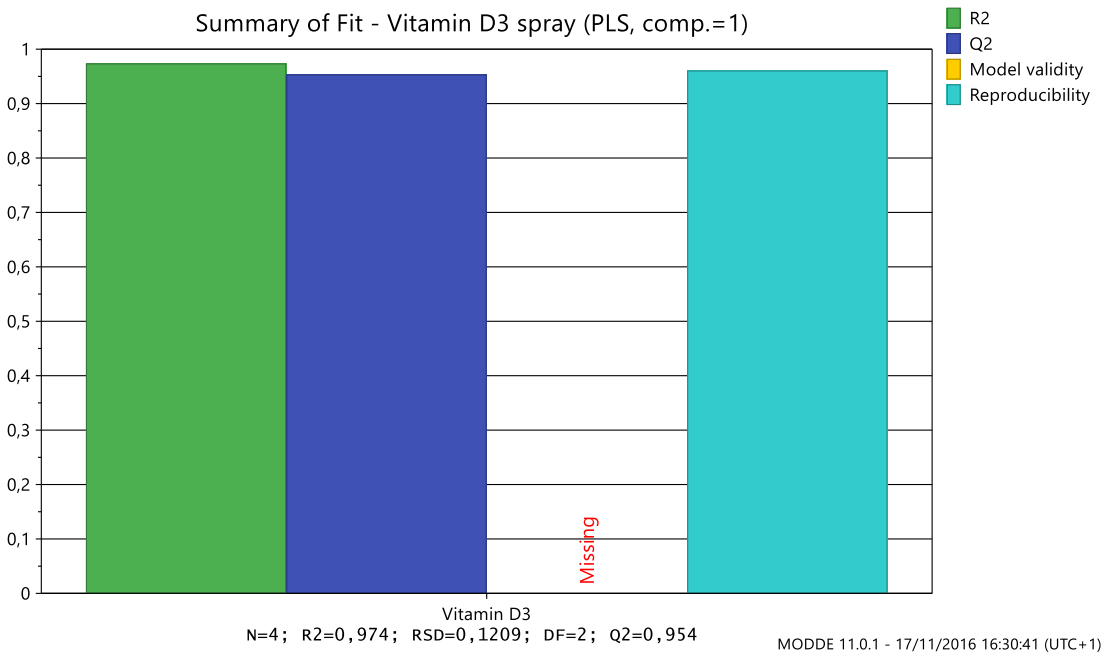



Figure 95: A summary in four parameters of the basic model statistics

The parameter $R^2$ indicates how good was the fitting of the experimental data through the PLS and it results from the following equation [106]:

$$R^2 = 1 - \frac{SS_{res}}{SS_{tot}} = 1 - \frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{\sum_{i=1}^{n}(y_i - \bar{y})^2} \qquad \text{Equation 10}$$

Where $SS_{res}$ is the sum of squares (squared deviations) of residuals , i.e. the sum of squared deviations between the observed and fitted values of each response, corrected for the mean [106].

Since a regression considers how much of the variance in the dependent variable (factor, X) can be predicted by variation in the independent or response variable (Y), the $SS_{res}$ actually represents the variation in the dependent variable X that is not predicted by the model. Therefore, it could also be defined as error sum of squares.

$SS_{tot}$ is the total sum of squares of response corrected for the mean and $\bar{y}$ is the mean response equal to Equation 11 [106]:

$$\bar{y} = \frac{1}{n}\sum_{i=1}^{n} y_i \qquad \text{Equation 11}$$

In order to have a better understanding of what $SS_{res}$ and $SS_{tot}$ indicate, the figure below shows the geometrical meaning of these terms.

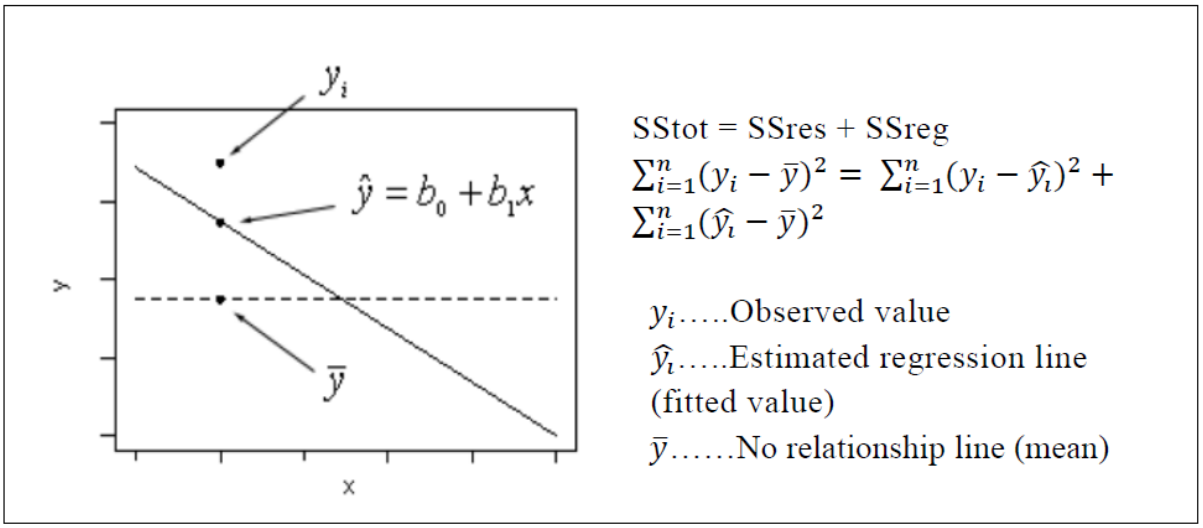



Figure 96: Graphical representation of $SS_{res}$, $SS_{reg}$ and $SS_{tot}$

The $R^2$ value is always between 0 and 1. Usually, a model with $R^2$ of 0.5 is a meaningful model [106], but it is not this the case because the $R^2$ had a value of 0.974 (Figure 95), very close to 1 (100%) indicating the high reliability of the model.

The $Q^2$ is important to estimate the precision of future prediction [106]. It is expressed in the same units as $R^2$ and is defined by the Equation 12:

$$Q^2 = 1 - \frac{PRESS}{SS_{tot}} = 1 - \frac{PRESS}{\sum_{i=1}^{n}(y_i - \bar{y})^2} \quad \text{Equation 12}$$

where PRESS is the prediction residual sum of squares and depends on the mathematical model used for fitting, while $SS_{tot}$ is the same as described for $R^2$ [106].

In case of PLS, the PRESS is computed in a *cross-validation procedure*, where a part of the data (e.g., 1/Kth of the samples) is kept out of the model, which is then based only on the kept samples. The prediction for the excluded samples is conducted afterwards with this model (i.e., the rest of the (K - 1)/K samples) [106]. In this case the PRESS is computed through the Equation 13 [106]:

$$PRESS_{PLS} = \sum_{i=1}^{n}(y_i - \hat{y}_i^{cv})^2 \quad \text{Equation 13}$$

Where $\hat{y}_i^{cv}$ is the predicted response value from the cross-validation procedure [106]. The $Q^2$ can be less than or equal to 1. $Q^2$ higher than 0.1 indicates a meaningful model and a value higher than 0.5 is obtained for a good one. For this model, it assumes the value of 0.954 which is an indicator for a very good statistical accuracy.

The other parameter related to the PLS is the *model validity* (Figure 95), which allows understanding if the model had problems, such as the presence of outliers, transformation problem or innaccuracy [106].

As shown in the summary of fit, no value was calculated for the model validity and the term *Missing* is related to the fact that the pure error was very small and replicates were recognised as identical by MODDE [106].

The fourth bar in the summary of fit plot (Figure 95) is the *reproducibility* and refers to the variation of the replicates respect to the whole variability [106]. A desired value would be higher than 0.5 and it was close to 1 in this model.

## 4.4 Assessment of the model adequacy

MODDE elaborated the data deriving from the experiments (Table 89) and their fitting through PLS. On the base of the collected results, different plots were originated, in order to determine the significance of the model terms.

| Experiment | | Run | Included factors | Temperature | Time | Oxygen | Sunlight | Vitamin $D_3$ |
|---|---|---|---|---|---|---|---|---|
| Number | Name | Order | | | | | | |
| 1 | N1 | 3 | Incl | 25 | 15 | 0,21232 | 500 | 9,36 |
| 5 | N5 | 9 | Incl | 25 | 15 | 0,21232 | 500 | 9,39 |
| 6 | N6 | 12 | Incl | 60 | 60 | 3 | 500 | 8,21 |
| 8 | N8 | 13 | Incl | 60 | 60 | 3 | 500 | 8,45 |

Table 89: Worksheet of the design of experiment

The following *coefficients plot* (Figure 97) shows that temperature, time and oxygen were all significant terms, considering a confidence level of 95%. In fact, the green bar is far away negatively from y=0 and the uncertainty level does not extend across y=0[106].

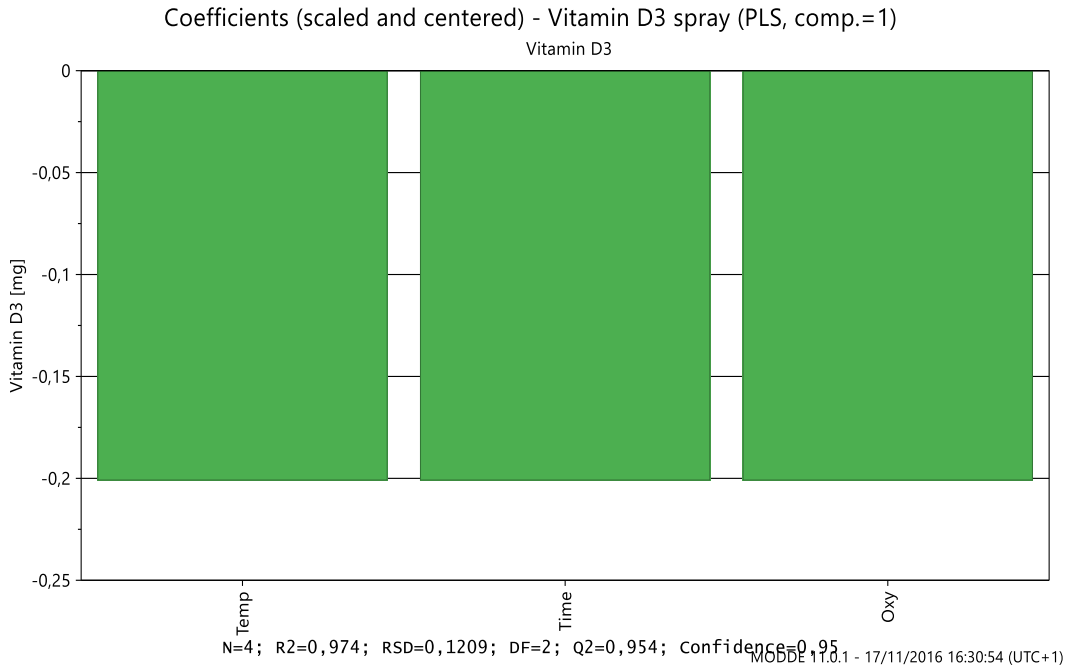



Figure 97: Coefficients plot for the significance of model terms

The *residual normal probability plot* shown below (Figure 98) is a report of the residuals on a cumulative normal probability scale [106]. The standardised residuals ($estd,_i$, Equation 15) come from the difference between observed ($y_i$) and predicted ($\hat{y}_i$) values ($e_i$, Equation 14), divided by the residual standard deviation (S) [106]:

$$e_i = y_i - \hat{y}_i \quad \text{Raw residuals} \qquad \text{Equation 14}$$

$$estd,_i = e_i / s \quad \text{Standardised residuals} \qquad \text{Equation 15}$$

It has to be considered for a better understanding of the program that in the case of a PLS regression, the standardised residuals on x-axis are presented by default [106].

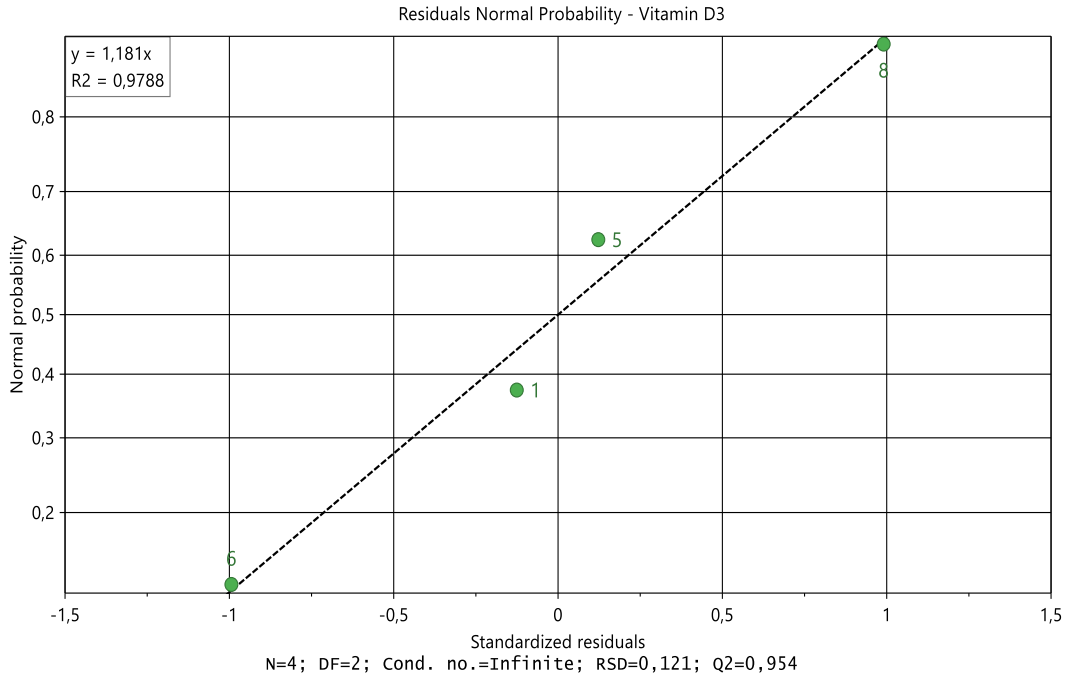



Figure 98: Residual normal probability plot

The plot allows detecting the distribution of residuals. If the residuals are normally distributed, the points on the plot follow almost a straight line, meaning that the difference between the predicted and the observed value is not so significant and hence the model provides a good approximation of results [106].

In the case of plot resulting from the model for the vitamin $D_3$ spray, the $R^2$ for the regression was equal to 0.9788. Therefore, despite the two outliers, slightly deviated respect to the normal probability line, the model gave a good fit for the experimental data. This was also confirmed by the plot *observed vs predicted* (Figure 99), where the observed values represented by the points were distributed very close to the straight line, constructed on the predicted values.

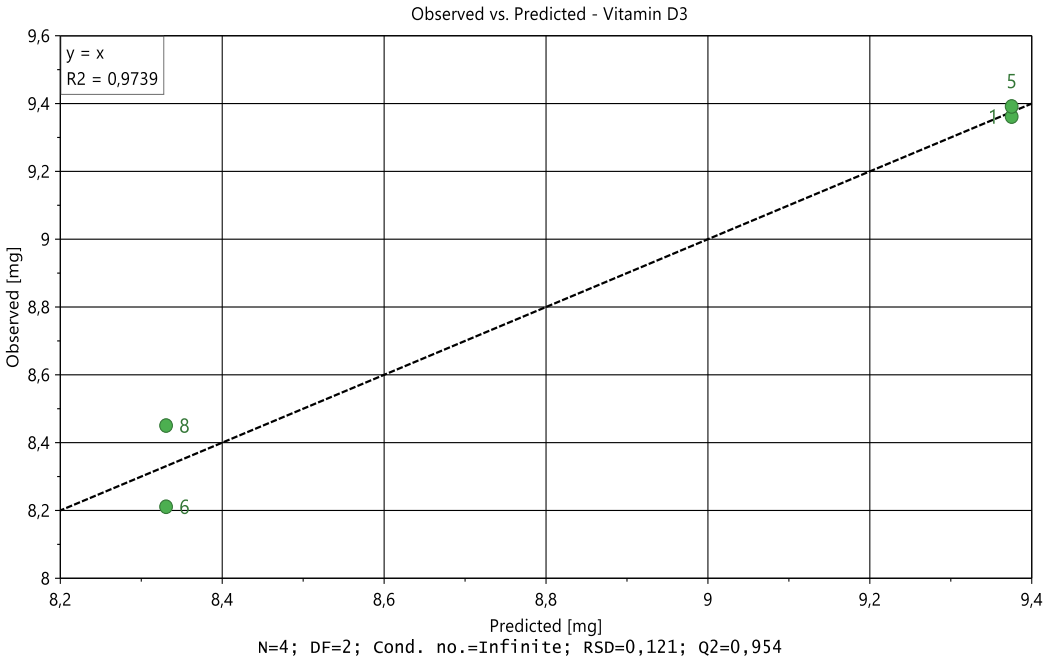



Figure 99: Observed vs predicted plot

## 4.5 Evaluation of the model according to ANOVA

The validity of the model was supported not only by the graphical representation shown before, but also from the analysis of variance (ANOVA).
It decomposes the total sum of squares of a selected response Y (sum of squares corrected for the mean) into a part due to the regression model and a part due to the residuals, as expressed by Equation 16 [106]:

$$SS_{tot} = SS_{reg} + SS_{res} \qquad \text{Equation 16}$$

Where $SS_{tot}$ and $SS_{res}$ have been already defined before, while $SS_{reg}$ is the sum of squares of the regression (Equation 17), corrected for the mean [106]:

$$SS_{reg} = \sum_{i=c1}^{n}(\hat{y}_i - \bar{y})^2 \qquad \text{Equation 17}$$

The equation above determines how much variation in the data can be explained by the variables used in the model. The corresponding decomposition of the degrees of freedom (Equation 18) is considered as well [106]:

$$DF_{tot} = DF_{reg} + DF_{res} \qquad \text{Equation 18}$$

Where $DF_{tot}$=n-1(n, number of factors) is the degree of freedom of the population variance of the dependent variable x, $DF_{res}$=n-k (k, number of model) is the degrees of freedom of the estimate of the population error variance and $DF_{reg}$=k-1 (in case of linear regression is 1), the degree of freedom of the fitted values respect to the overall mean [106].

| **Vitamin $D_3$** | **DF** | **SS** | **MS variance** | **F** | **p** | **SD** |
|---|---|---|---|---|---|---|
| Total | 4 | 314,588 | 78,6471 | | | |
| Constant | 1 | 313,467 | 313,467 | | | |
| Total corrected | 3 | 1,12131 | 0,373769 | | | 0,611367 |
| Regression | 1 | 1,09206 | 1,09206 | 74,6708 | **0,013** | 1,04502 |
| Residual | 2 | 0,02924 | 0,014625 | | | 0,120934 |
| Lack of Fit | 0 | -- | -- | -- | **--** | -- |
| (Model error) | | | | | | |
| Pure error | 2 | 0,02925 | 0,014625 | | | 0,120934 |
| (Replicate error) | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| N | = 4 | $Q^2$ | **0,954** | Condition. number. | Infinite | |
| DF | = 2 | $R^2$ | **0,974** | RSD | **0,1209** | |
| Component | = 1 | $R^2$ adj. | **0,961** | | | |

Table 90: ANOVA table resulting from the PLS model applied for the considered formulation

The ANOVA table (Table 90) contain the parameters with different meaning, which contribute to defining the statistical goodness of the model. Some of the parameters displayed in the table above, such as $R^2$ and $Q^2$ have been previously defined, while the *$R^2$ adjusted* is a new term.

It is the variance of the response described by the model, but adjusted according to the degrees of freedom of the model (Equation 19) [106]:

$$R^2_{adj} = 1 - \frac{SS_{res}/DF_{res}}{SS_{tot}/DF_{tot}} \qquad (R^2_{adj} \leq R^2 \leq 1) \qquad \text{Equation 19}$$

The value of and the model used for the vitamin $D_3$ spray gave $R^2_{adj}$ equal to 0.961, which was lower than $R^2$ (0.974) and hence exactly in the range.

It indicates that the model could detect all possible variance of the response, when influencing factors were changed.

Another important parameter which allows defining the goodness of fit is the *F value*.

It tests the significance of the regression model by means of the Equation 20 [106]:

$$F = \frac{\frac{SS_{reg}}{DF_{reg}}}{\frac{SS_{res}}{DF_{res}}} = \frac{SS_{reg} * DF_{res}}{SS_{res} * DF_{reg}} = \frac{Regression\ mean\ square\ (MSReg)}{Residual\ mean\ square\ (MSRes)} \qquad \text{Equation 20}$$

As shown in Table 90, a high value of F (74,6708) associated with a low p value (0.013) with corresponding DF=2, indicates that the variation of data ($MS_{Reg}$), which could be predicted by the model, was higher than the variation associated with random error (*residual mean square*), which could not be therefore predicted [106].
For this reason, the model was statistically meaningful, because it allowed to evaluate the 'significant effects' that a new or modified factor could have on the considered response (e.g. vitamin $D_3$ content).
Closely correlated to the F value is the *p value*, which was equal to 0.013 for the specific model. It indicates that the probability for the regression was significant at 95% and hence the model was statistically good.
In the specific case, there were replicated experiments (observations, runs), therefore the residual sum of squares derived from the contribute of the *pure error* ($SS_{pe}$) and the *lack-of-fit* ($SS_{lof}$) of the model (Equation 21) [106]:

$$SS_{res} = SS_{pe} + SS_{lof} \qquad \text{Equation 21}$$

Where the degrees of freedom is decomposed as shown in Equation 22:

$$DF_{res} = DF_{pe} + DF_{lof} = \sum_k (n_k - 1) + (n - p - \sum_k (n_k - 1)) \qquad \text{Equation 22}$$

where $n_k$ is the number of replicates in the $k$th set of replicates [106].
For the considered model, no lack of fit was found, which attested once again its statistical goodness.
In the ANOVA plot in Figure 100, the resulting bars derived from a comparison of regression components with residual components.

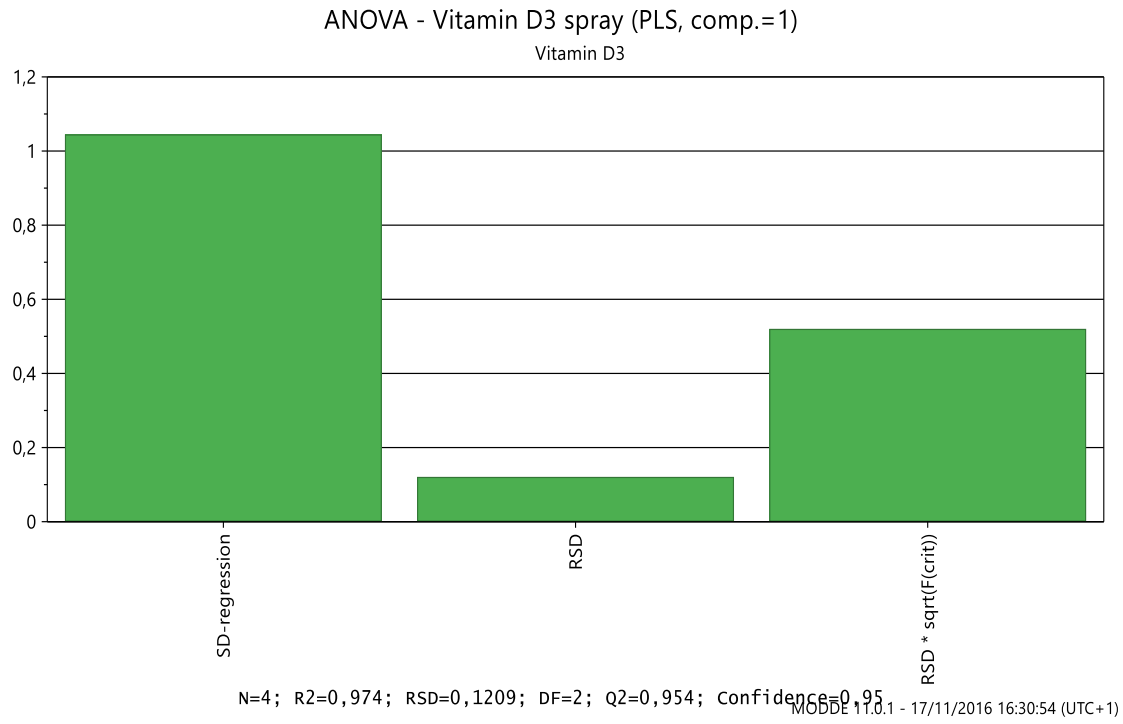



Figure 100: ANOVA table resulting from the PLS model applied for the considered formulation

The *SD Regression* indicates the variation of the response described by the model, taking into account degrees of freedom [106]. It results from the square root of MS (mean square) regression. For the model of vitamin $D_3$ spray, the SD regression bar is around 1.

As concerns the *RSD* (residual standard deviation), about the variation of the response not described by the model, the value resulting from this specific model is very low (0,1209). This means that the model is able to respond to any possible change of input parameters.

Another important value represented in Figure 100 is the *RSD*sqrt(F(crit))*, where the critical F is the value of the F-distribution which assures that SD regression is significant at the 95% confidence level [106].

For the specific model, the *RSD*sqrt(F(crit))* bar was smaller than the first of the *SD Regression*, meaning that the probability of observing a value F outside the 95% confidence level was less than 0.05.

## 4.6 Prediction on the base of the fitted model

The prediction phase is important to evaluate how the factors (temperature, oxygen, sunlight) can influence the response (vitamin $D_3$ content) during the manufacturing process.

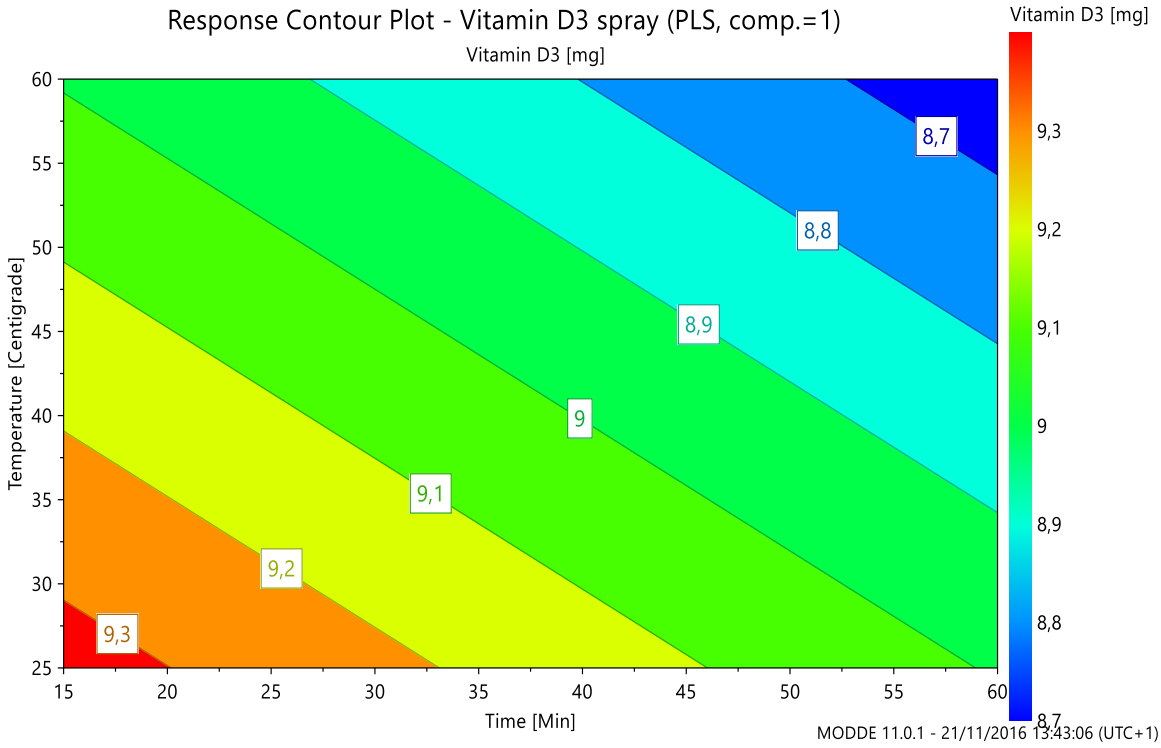



Figure 101: 2D Response contour plot with time and temperature as affecting parameters

In the *response contour plot* (Figure 101), it is shown how the response, which was the content of vitamin $D_3$, will be affected by the temperature, as well as by the time for which the formulation is kept at that temperature.

The region defined by the red color, indicates that the reachable maximum, predicted by the model, was around 9.3 mg, very close to the value obtained from the stress test (Table 89). According to the prediction, resulting fom the conducted tests, by increasing the temperature and the time of stress, the content of vitamin $D_3$ will decrease and move towards the blue region. Here, the amount will be still close to the required dose (8.3 mg) but rather less than the weighed one (9.6 mg).

By increasing the oxygen flow from 0 to 3 bar, the prediction was similar (Figure 102). The oxygen determines decomposition of formulation components.

Therefore, on the base of the results of stress tests with oxygen (Table 89), the decrease of vitamin $D_3$ content was predicted by the model, in case of moving from a region at low oxygen pressure to a region at high pressure, by increasing the time of exposure.

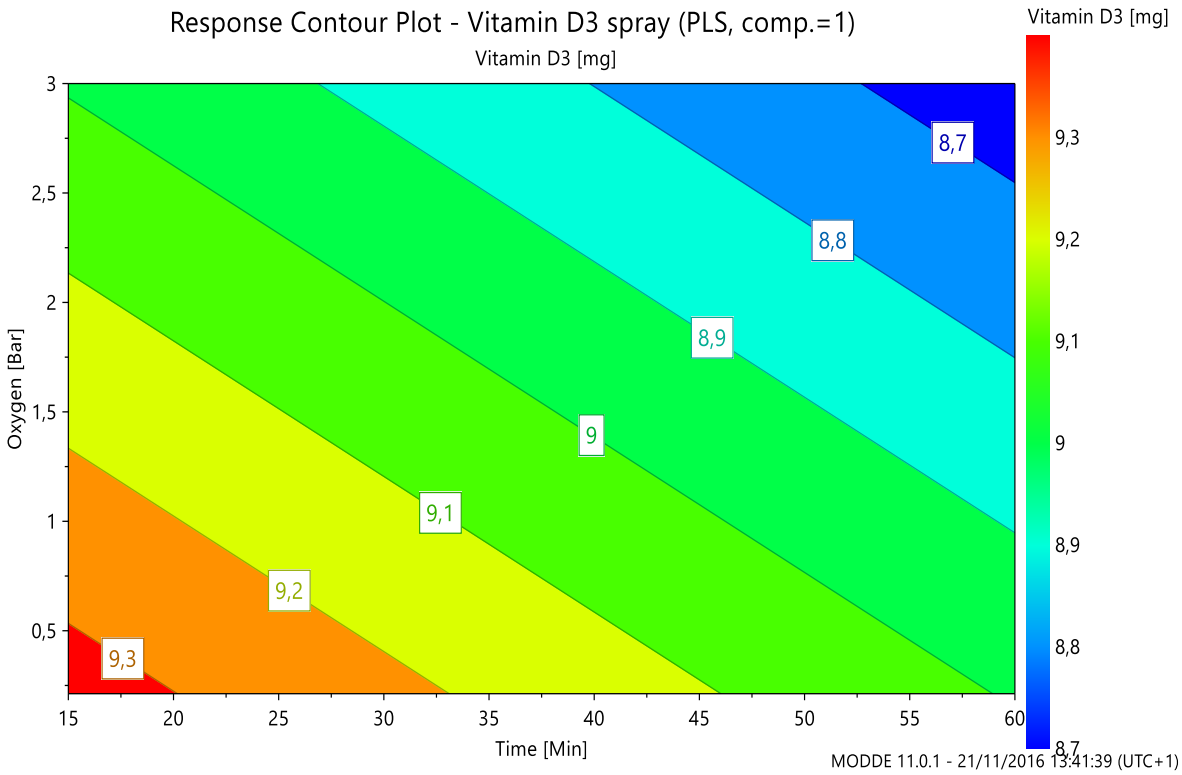



Figure 102: 2D Response contour plot with time and oxygen as affecting parameters

In both plots of Figure 101 and 102, the sunlight was excluded by default from the graphic, because constant. Moreover, in Figure 101 the oxygen is also constant at the value of 0.4540 bar, while in Figure 102 the temperature was constant at 28.5°C.

In order to have a complete overview of the relation between temperature, time, oxygen and response, the 4D plot (Figure 103) was derived.

The simultaneous consideration of the most important parameters can help to optimise the manufacturing process by reducing the waste of finished products, which do not respect quality criteria.

As shown in the figure below, the effect of oxygen on vitamin $D_3$ will become more significant when a high temperature is kept for many minutes.

In fact, in the three diagrams of Figure 103, the predictive model indicates that vitamin $D_3$ will undergo a progressive reduction from 9.6 mg (initial amount) to 9 mg when stressed at oxygen pressure of 0.21232 bar and temperature of 50°C, for 30 minutes (Figure 103, (a)). The decrease will be accentuated (8.8 mg), by increasing the oxygen pressure at 3 bar and keeping it for 30 minutes, at the temperature of 50°C (Figure 103, (c)).

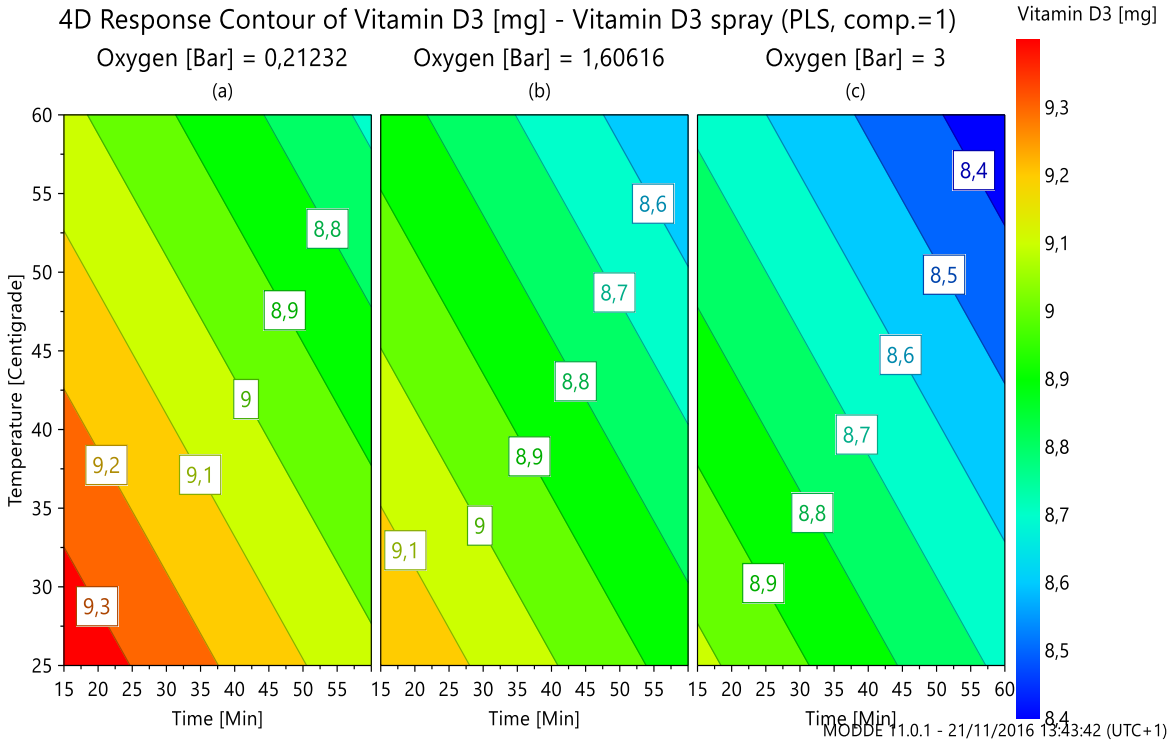



Figure 103: 4D Response contour plot-simultaneous correlation among temperature, time, oxygen and vitamin $D_3$ amount

Although the stressed conditions, the predicted vitamin $D_3$ content remains in the range defined in Table 88 and this will allow a dosage form production to provide the appropriate API content.

The *design space analysis* in Figure 104 is useful to determine the probabilty of failure, which means in this specific case, the probability to have a content of vitamin $D_3$ in the formulation lower than that weighed and far from the required content.

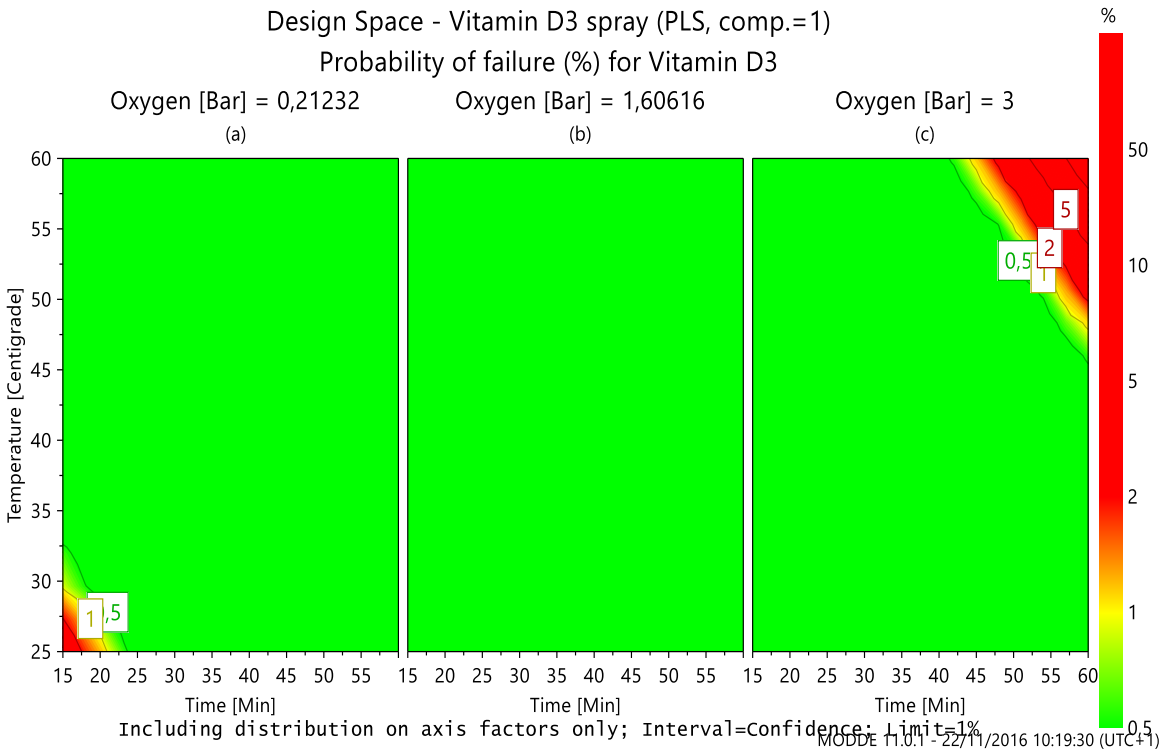



Figure 104: 4D Design space plot

In the Figure 104, (a), the red area, representing the probability of failure, is very narrow around time 15-20 minutes, between 25-30°C at environmental pressure of oxygen (0.21232 bar). This can happen during the phase of packaging, when the formulation is distributed into the amber bottles.

The surrounding green area can refer to the phase of manufacturing, when the formulation components are mixed and stirred at a certain temperature to guarantee the homogeneity of blend. According to the design space plot, even increasing the oxygen flow (Figure 104,(b, c)), the probability of failure will be still very low and will increase only when the formulation will be stressed at oxygen pressure of 3 bar, by heating up to 60°C for about 60 minutes (red area, Figure 104,c).

The overview provided by the design space allows, at industrial level, choosing with accuracy how to adjust and control process parameters, which can influence the quality of the formulation. In this way, the quality is not tested on the finished product, but can be controlled and preserved already during the manufacturing process.

The mathematic model, derived by using the lab experiments conducted on the new formulated vitamin $D_3$ spray, can be applied to define the industrial process and contribute to improving it.

# 5 Conclusions

The development of the new formulation for a vitamin $D_3$ spray was focused on the design of a dosage form, which provides smooth administration and high patient compliance.

The starting point was the selection of principal excipients (Chapter 2). It was based not only on the chemical and physical properties, but also on the suitability to improve the taste. Therefore, besides the use of MCT oil as lipophilic medium, where vitamin $D_3$ could be dissolved, the introduction of PECEOL$^{TM}$ as emulsifier and Tetrarome(R) Orange as flavouring agent, containing vitamin E with antioxidant function, was chosen to get an innovative formulation.

The stability tests, performed by RP-HPLC (Paragraph 3.3) on samples stored at 25°C and 60% RH, demonstrated that the formulation preserved a certain stability within the period of six months, with a mean of recovery equal to 100 % (Figure 36, Paragraph 3.3.4.1). Moreover, the comparison analysis conducted among samples of two identical batches yielded that for the first four months, the observed variability of vitamin $D_3$ amount was similar, with a slight difference in the standard deviation (Figure 39, Paragraph 3.3.4.1). This was considered to be an evidence of analytical accuracy and relative stability of the formulation.

Further positive results were obtained by analysis of chromatograms (Paragraph 3.3.4.2), which were characterized by recognisable peaks, not only for vitamin $D_3$ but also for the excipients. The absence of significant changes, during the tests carried out for about six months, confirmed stable drug content.

The stability tests were additionally executed on samples stressed (Paragraph 3.3.5) by oxygen (3 bar), sunlight and high temperature (60°C), in order to investigate the decomposition processes and thus to define the potential changes in the peak detection, which do not usually occur at standard conditions. Although the decrease of vitamin $D_3$ amount was observed, the values registered were not critically in terms of drug content. However, even though it has to be considered that the influencing factors, used for stability tests, can be adjusted and controlled in the manufacturing process. In fact, given the low variability of vitamin $D_3$ content in stress conditions, it can be ensured that the resistance to change is preserved during the ordinary conditions of production and storage.

The estimation of the principal physical properties such as viscosity (Paragraph 3.4), density (Paragraph 3.5) and refractive index (Paragraph 3.6) showed how the influence of MCT oil, prevailed over the other excipients of formulation, hence causing the high similarity of the mentioned properties between MCT oil and vitamin $D_3$ spray. The same similarity was found out, by carrying out the thermal behaviour analysis by means of DSC (Paragraph 3.7). Indeed, repeated steps of crystallisation and melting (Paragraph 3.7.1) testified that the formulation had a profile, comparable with that of MCT oil, but most of all, that it did not undergo irreversible changes during thermal transitions.

In order to investigate the oxidation kinetics of the formulation, the DSC was also used to conduct isothermal oxidation tests (Paragraph 3.7.2), which resulted the OIT to understand how to adjust process parameters (e.g. temperature, air), ensuring the quality of the product, already during manufacturing.

The obtained results for the new formulated spray, were compared to that of OLEOVIT$^{TM}$ and a significant difference in the activation energy characteristic for the oxidation reactions was observed. In fact, the vitamin $D_3$ spray showed a higher sensitivity to oxidation, due to the lower value of $E_a$, necessary to activate the reaction. This can be related to the presence of other excipients, besides the MCT oil, in the new formulation, with respect to the trade product.

According to this results, it is understandable that the exposition to oxygen at processing temperature (e.g. 50°C) has to be avoided, by creating inert, operation conditions (e.g. by fumigation with nitrogen).

The spray formulation was designed to administer the drug in the mouth onto the buccal mucosa. Therefore, it is not admitted that the drug distributes in other parts of the respiratory tract, such as lungs or bronchial tube. At this purpose, the surface tension analysis (Paragraph 3.8) and the size distribution of sprayed droplets (Paragraph 3.9) were determined.

The low surface tension, calculated for the new formulation, guarantees the reduction of interfacial tension between oily and aqueous (e.g. saliva) components in the mouth. In this way, the adsorption of surfactant on the buccal mucosa is promoted and thus the absorption of the lipophilic API through the cell membranes occurs much more quickly.

The drop size distribution proved that less than five percent of drops were smaller than 10 µm, which is the safety size to be respected, in order to avoid the inhalation of the drug into the lungs.

Finally, the data resulting from the stability and stress tests were collected for the development of a mathematical model (Chapter 4) constructed by means of MODDE and based on the fitting of experimental data, obtained by changing the influencing factors (temperature, time, oxygen, sunlight).

The implemented model gave the possibility to predict, through the response contour plot (Paragraph 4.5), the variability of vitamin $D_3$ content, based on the modification of factors and through the design space (Paragraph 4.5), the definition of the optimal region for operating procedures. Therefore, the manufacturing process included the QbD principles.

The developed new formulation of vitamin $D_3$ spray represents the prototype of a dosage form, designed in every detail, in terms of stability and compatibility, but also including the quality of the process itself.

# Abbreviations

| | |
|---|---|
| RH | Relative Humidity |
| RP-HPLC | Reversed phase high performance liquid chromatography |
| DSC | Differential scanning calorimetry |
| ICH | International Council for Harmonisation |
| OIT | Oxidation induction time |
| IOT | Isothermal oxidation test |
| RSM | Response surface modelling |
| QbD | Quality by design |
| DoE | Design of experiment |
| 25(OH)D | 25-hydroxyvitamin D = calcidiol |
| 1,25(OH)2D | 1,25-dihydroxyvitamin D =calcitriol |
| Vitamin D | calciferol |
| Vitamin $D_2$ | ergocalciferol |
| Vitamin $D_3$ | cholecalciferol |

# Materials

| | |
|---|---|
| Reference Vitamin $D_3$ | CH.B.:10186907 |
| Tetrahydrofuran (THF) | HiPerSolv CHROMANORM, VWR Prolabo Chemicals (Product code: 28559.320) |
| 2-Propanol (2-PrOH) | HiPerSolv CHROMANORM, VWR Prolabo Chemicals (Product code: 20880.320) |
| Acetic acid (glacial) 100% (HAc) | Merck |
| Acetonitrile (ACN) | HiPerSolv CHROMANORM, VWR Prolabo Chemicals ((Product code: 83639.320) |
| Water | Milli-Q® |
| Uvasol$^R$ (n-Pentane) | Merck |
| Tetrarome Orange | Firmenich (Product code:987431) |
| Peceol™ | Gattefossè (Product code: 3088BA2) |
| MCT oil | Fresenius Kabi Austria GmbH (Product code:09000061) |

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# Appendix

IPW-Karl-Franzens Universität

**H E R S T E L L U N G S V O R S C H R I F T F Ü R**
***( P R O D U C T I O N I N S T R U C T I O N F O R )***

**Vitamin D3 Spray *(Vitamin D3 Spray)***

Geltungsbereich *(area of validity)*: **Finalisierung** *(Finalization)* Dok.Nr.: **SOP_NewFormulation.docx**

Exemplar **1 - 2 / 2** Seite *(page)* **1** von **16**

**0.** Ausgabe vom **14.12.2016** *(Ideal edition of December 14th 2016)* Gültig ab *(valid from)*: **14.12.2016**

**Genehmigt und in Kraft gesetzt** *(approved and put into force)*:

Herstellungsleiter Finalisierung:
*Head of Production*: .....

**Genehmigt** *(approved)*:

Qualitätssicherung:
*Quality Assurance:*

.....

Qualified Person:

.....

Leiter Finalisierung:
*Head of Department:*

.....

**Ersteller** *(creator)*:

GMP-/Process-Support:

.....

**Verteiler** *(mailing list)*:

Exemplar **1** von **2**: **GMP-/Process-Support**
Exemplar **2** von **2**: **Leiter Zulassung** ***(Head of Registration)***

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**A C H T U N G !**
**JEDE KOPIE IST AUF DER VORLAGE ZU VERMERKEN.**
**DER INHABER DER VORLAGE (KOPIEGEBER) IST VERANTWORTLICH FÜR DIE AKTUALISIERUNG DER VON SEINEM DOKUMENT ERSTELLTEN KOPIEN.**
*A T T E N T I O N !*
*EACH COPY TAKEN MUST BE NOTED ON THE ORIGINAL.*
*THE OWNER OF THE DOCUMENT (COPY GIVER) IS RESPONSIBLE FOR UPDATE THE COPIES WHICH WERE MADE FROM HIS DOCUMENT.*

**INHALTSVERZEICHNIS** *(LIST OF CONTENTS)*:

IPW-Karl-Franzens Universität

**HERSTELLUNGSVORSCHRIFT FÜR**
***(PRODUCTION INSTRUCTION FOR)***

## Vitamin D3 Spray *(Vitamin D3 Spray)*

Geltungsbereich *(area of validity)*: **Finalisierung** *(Finalization)* Dok.Nr.: **SOP_NewFormulation.docx**

Exemplar **1 - 2 / 2** Seite *(page)* **3** von **16**

**0.** Ausgabe vom **14.12.2016** *(Ideal edition of December 14th 2016)* Gültig ab *(valid from)*: **14.12.2016**

ZUSAMMENSETZUNG FÜR **1** ML *(COMPOSITION FOR 1 ML)*

| **ARTIKELBEZEICHNUNG** *(article name)* | **FK** | **MENGE** *(quantity)* | **ME** |
|---|---|---|---|
| Colecalciferol Ph.Eur. | (W) | 0,09583 | MG |
| 1% w/w PECEOL™, Gattefossè *(glycerol mono-oleate)* | (D) | 9,400 | MG |
| 0.5% w/w Tetrarome Orange, Firmenich | (D) | 4,700 | MG |
| MITTELKETTIGE TRIGLYCERIDE (medium-chain triglycerides) | (D) | 925,804 | MG |
| Gesamtgewicht *(total weight)*: | | 940 | MG |

**Erläuterungen** *(notes)*:

FK = Funktion *(function)*
(W) = Einfluss auf die Wirksamkeit *(influence on the effectiveness)*
(D) = andere Bestandteile *(other components)*

**Lagerhinweise** *(storage instructions)*:

Lichtschutz erforderlich *(sunscreen required)*: **ja** *(yes)*

Lagertemperatur *(storage temperature)*: **15 - 25 °C**

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**H E R S T E L L U N G S V O R S C H R I F T F Ü R**
***( P R O D U C T I O N I N S T R U C T I O N F O R )***

## Vitamin D3 Spray *(Vitamin D3 Spray)*

Geltungsbereich *(area of validity)*: **Finalisierung** *(Finalization)* Dok.Nr.: **SOP_NewFormulation.docx**

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Rezeptur (Stückliste) für **966,2 Liter**
*formulation (bill of materials):*

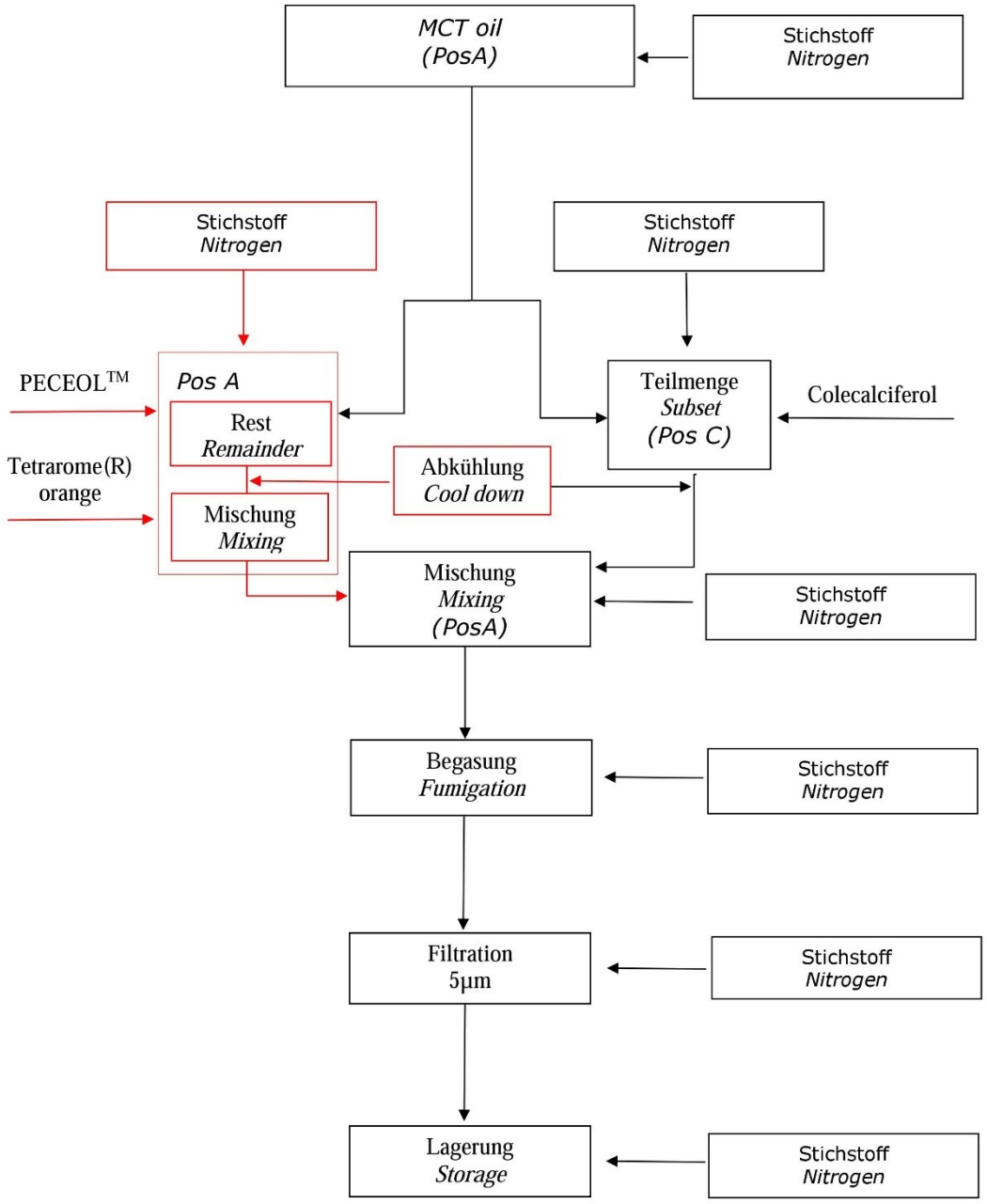
| POS | ARTNR | ARTIKELBEZEICHNUNG *(article name)* | MENGE *(quantity)* | ME |
|---|---|---|---|---|
| 1 | M011405 | MITTELKETTIGE TRIGLYCERIDE *(medium-chain triglycerides)* | 894,512 | KG |
| 2 | A0000869 | Colecalciferol Ph.Eur. | 92,594 | G |
| 3 | 3088BA2 | 1% w/w PECEOL™, Gattefossè *(glycerol mono-oleate)* | 9,08228 | KG |
| 4 | 987431 | 0.5% w/w Tetrarome Orange, Firmenich | 4,54114 | KG |
| 5 | A0080325 | Stickstoff Ph.Eur. *(Nitrogen Ph.Eur.)* | 0,001 | M3 |
| | | Gesamtgewicht *(total weight)*: | 908.228 | G |

**Geräte- und Apparateliste (Major Equipment):**
*Major equipment- and instrument list:*

| POS | **Apparatebezeichnung** *(equipment name)* |
|---|---|
| A | gewidmeter Lagertank 1000 l aus nichtrostendem Stahl *(dedicated storage tank 1000 l stainless steel)* |
| B | Produktfilter Sartofine PP 5 µm (D0002310) *(product filter Sartofine PP 5 µm D0002310)* |
| C | Fass 20 l *(barrel 20 l)* |
| D | Impellerpumpe *(impeller pump)* |
| E | gewidmeter Lagertank 1000 l aus nichtrostendem Stahl *(dedicated storage tank 1000 l stainless steel)* |
| F | Ansatzschläuche (D0000109) *(approach hoses D0000109)* |
| G | Handrührer *(hand mixer)* |

Erläuterungen zur Rezeptur (Stückliste):
*Explanation to the formulation (bill of materials):*

POS = Laufende Nummer in Rezeptur (*running number in the composition)*
ARTNR = Artikelnummer *(article no)*
MENGE = Einzusetzende Soll-Menge (*quantity ⇒ required quantity)*
ME = Mengeneinheit entsprechend Materialstamm
(*quantity unit according to the material master)*


MCT oil
(PosA)
Stichstoff
Nitrogen
Stichstoff
Nitrogen
Stichstoff
Nitrogen
PECEOL™
Pos A
Rest
Remainder
Tetrarome(R)
orange
Mischung
Mixing
Abkühlung
Cool down
Teilmenge
Subset
(Pos C)
Colecalciferol
Mischung
Mixing
(PosA)
Stichstoff
Nitrogen
Begasung
Fumigation
Stichstoff
Nitrogen
Filtration
5µm
Stichstoff
Nitrogen
Lagerung
Storage
Stichstoff
Nitrogen

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**HERSTELLUNGSVORSCHRIFT FÜR *(PRODUCTION INSTRUCTION FOR)***

**Vitamin D3 Spray *(Vitamin D3 Spray)***

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Verarbeitungsanweisung für. Stücklisten-Nr. **B0085134** *(handling instruction for bill of materials number: B0085134):*

| | **SOLL** *(required)* | **IST** *(actual)* | **U/D** *(S/D)* |
|---|---|---|---|
| **Arbeitsvorbereitung:**<br>**Saubere Arbeitsbekleidung (Reinraum-Overall)** sowie **Kopfbedeckung** und **Handschuhe** (lange Handschuhe die über die Bündchen reichen, oder zusätzlich Ärmelschoner) entsprechend Anweisung **LSOPQS0345D** werden getragen.<br>***Work preparation:***<br>*Clean working clothes (cleanroom overall), headgear and gloves (long gloves that extend over the cuffs, or in addition oversleeves) in accordance to SOP LSOPQS0345D has to be worn.* | | **Erledigt**<br>*(completed)*<br>☐ | |
| **Line Clearance:**<br>Die Produktionsräume sind frei von Resten vorangegangener Produktion.<br>***Line Clearance:***<br>*The production rooms are clear of leavings from previous production.* | | **Erledigt**<br>*(completed)*<br>☐ | |
| | **Vorcharge:**<br>*(Previous batch:)* | | |
| **Raumreinigung:**<br>Die Produktionsräume wurden nach der letzten Aktivität (Produktion oder Anlagenreinigung) gereinigt und desinfiziert.<br>(Vergleich Aktivitäten-Plan vs. Produktionslogbuch)<br>***Line Clearance:***<br>*The production rooms were cleaned and disinfected after the last activity (production or equipment cleaning). (Comparison activity plan vs. production logbook)* | **Räume sind nach letzter Aktivität gereinigt und desinfiziert**<br>*(Rooms are cleaned and disinfected after last activity)* | **Erledigt**<br>*(completed)*<br>☐ | |

IPW-Karl-Franzens Universität

**HERSTELLUNGSVORSCHRIFT FÜR *(PRODUCTION INSTRUCTION FOR)***

**Vitamin D3 Spray *(Vitamin D3 Spray)***

Geltungsbereich *(area of validity)*: **Finalisierung** *(Finalization)*
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| | **SOLL** *(required)* | **IST** *(actual)* | **U/D** *(S/D)* |
|---|---|---|---|
| **Die Produktionsräume sind trocken.**<br>*Production rooms are dry.* | **Böden der Räume sind trocken**<br>*(The room floors are dry)* | **Erledigt**<br>*(completed)*<br>☐ | |
| **Datum letzte Reinigung / Desinfektion:**<br>*Date of last cleaning / disinfection:* | **Datum:**<br>*(Date:)* | | |
| **Equipment-Reinigung:**<br>Die zur Herstellung verwendeten Apparate und Geräte sind nach **Anweisung LSOPFI2442D und LSOPFI1977D** gereinigt und die Reinheit wurde überprüft.<br>**Achtung ! Erläuterungen zur Arbeitsvorschrift sind zu beachten !**<br>***Equipment cleaning /:***<br>*The apparatus and equipment used for production are cleaned in accordance to SOP LSOPFI2442D and LSOPFI1977D and the cleanness was checked.*<br>***Attention ! Notes to the operating instructions must be observed!*** | | **Erledigt**<br>*(completed)*<br>☐ | |
| **Verwendete Einweg-Materialien *(used disposables):***<br>**Ansatzschläuche (D0000109) (Pos F)**<br>*(approach hoses D0000109) (Pos F)* | **Chargen-Nr.:**<br>*(batch no.)* | | |
| **Produktfilter Sartofine PP 5 µm (D0002310) (Pos B)**<br>*(product filter Sartofine PP 5 µm (D0002310) (Pos B)* | **Chargen-Nr.:**<br>*(batch no.)* | | |

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Geltungsbereich *(area of validity)*: **Finalisierung** *(Finalization)* — Dok.Nr.: **SOP_NewFormulation**
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| | **SOLL** *(required)* | **IST** *(actual)* | **U/D** *(S/D)* |
|---|---|---|---|
| **Schritt 1:** | | | |
| MITTELKETTIGE TRIGLYCERIDE **(M011405)** werden mittels einer Impellerpumpe **(Pos E)** in den gewidmenten 1000 l Lagertank **(Pos A)** vorgelegt. | **mind. 100 l/Minute** *(at least 100 l/minute)* | **l/Min.** | |
| Anschließend wird durch das vorgelegte MITTELKETTIGE TRIGLYCERIDE sterilfiltrierter Stickstoff Ph.Eur. **(A0080325)** geleitet. | **mind. 15 Minuten** *(at least 15 minutes)* | **Min.** | |
| ***Step 1:*** *Medium-chain triglycerides (M011405) are submitted with an impellerpump (Pos E) into the dedicated 1000 l storage tank (Pos A). Afterwards sterile-filtered nitrogen Ph.Eur. (A0080325) is passed through the submitted medium-chain triglycerides.* | **Wägestreifen von M011405 beilegen** *(enclose weighing strip of M011405)* | **Erledigt** *(completed)* ☐ | |
| **Verwendeter Lagertank:** *(used storage tank)* | **Lagertank-Nr.** *(storage tank no.)* | | |

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**HERSTELLUNGSVORSCHRIFT FÜR *(PRODUCTION INSTRUCTION FOR)***

**Vitamin D3 Spray *(Vitamin D3 Spray)***

Geltungsbereich *(area of validity)*: **Finalisierung** *(Finalization)* — Dok.Nr.: **SOP_NewFormulation**
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| | **SOLL** *(required)* | **IST** *(actual)* | **U/D** *(S/D)* |
|---|---|---|---|
| **Schritt 2:** | | | |
| Mindestens 10 Liter MITTELKETTIGE TRIGLYCERIDE aus Schritt 1 werden im Fass 20 Liter **(Pos C)** vorgelegt. | **mind. 10 l** *(at least 10 l)* | **l** | |
| | **max. 50°C** | **°C** | |
| Darin werden unter leichtem Erwärmen und Durchleiten von sterilfiltriertem Stickstoff Ph.Eur. **(A0080325)** Colecalciferol Ph.Eur. **(A0000869)** ohne Luft einzurühren, gelöst. | **mind. 15 l/Minute** *(at least 15 l/minute)* | **l/Min.** | |
| **Siehe Erläuterungen 1, 2 und 3 zur Arbeitsvorschrift !!!** | **Lösungsdauer max. 30 Minuten** *(solution time max. 30 minutes)* | **Min.** | |
| ***Step 2:*** *At least 10 liter Medium-chain triglycerides from Step 1 are submitted into a 20 liter barrel (Pos C). Therein under gentle heating and passing of steril-filtered nitrogen Ph.Eur. (A0080325) Colecalciferol Ph.Eur. (A0000869) is dissolved without stir in air. See notes 1, 2 and 3 to the operating instruction !!!* | | **Gelöst** *(solved)* ☐ | |
| | **Wägestreifen von A0000869 beilegen** *(enclose weighing strip of A0000869)* | **Erledigt** *(completed)* ☐ | |

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Geltungsbereich *(area of validity)*: **Finalisierung** *(Finalization)*
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|---|---|---|---|
| **Schritt 3:**<br>Gleichzeitig wird das PECEOL™ **(3088BA2)** dem restlichen Teil des MITTELKETTIGE TRIGLYCERIDE (Pos A) unter Rühren mit Auf- und Ab-Bewegungen, unter leichtem Erwärmen und Durchleiten von sterilfiltriertem Stickstoff Ph.Eur. **(A0080325)** zugegeben.<br>***Step 3:***<br>*Afterwards* PECEOL™ *is added to the remaining medium-chain triglycerides under stirring with up and down movements, under gentle heating and passing of sterile-filtered nitrogen Ph.Eur. (A0080325).* | **mind. 15 l/Minute** *(at least 15 l/minute)* | **l/Min.** | |
| | **max. 40°C** | **°C** | |
| | **Lösungsdauer max. 15 Minuten** *(solution time max. 15 minutes)* | **Min.** | |
| **Schritt 4:**<br>Man läßt die Mischung PECEOL™ und MITTELKETTIGE TRIGLYCERIDE auf Raumtemperatur abkühlen. Danach wird das TETRAROME(R)Orange **(987431)** der Mischung unter Rühren mit Auf- und Ab-Bewegungen und immer unter Durchleiten von sterilfiltriertem Stickstoff Ph.Eur. **(A0080325)** zugegeben.<br>***Step 4:***<br>*The mixed solution PECEOL™ und MCT is allowed to cool at room temperature. Afterwards the flavor Tetrarome orange is added to the mix under stirring with up and down movements and underpassing of sterile-filtered nitrogen Ph.Eur. (A0080325).* | **mind. 15 l/Minute** *(at least 100l/minute)* | **l/Min.** | |
| | **T~ 25°C** | **°C** | |
| | **mind. 15 Minuten** *(at least 15 minutes)* | **Min.** | |

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|---|---|---|---|
| **Schritt 5:**<br>Die Lösung von Colecalciferol Ph.Eur. **(A0000869)** und MITTELKETTIGE TRIGLYCERIDE aus Schritt 2 hat sich inzwischen auf Raumtemperatur abgekühlt. Sie wird der Mischung aus Schritt 4 unter Rühren mit Auf- und Ab-Bewegungen und immer unter Durchleiten von sterilfiltriertem Stickstoff Ph.Eur. **(A0080325)** zugegeben.<br>***Step 5:***<br>*The solution of Colecalciferol Ph.Eur. (A0000869) and MEDIUM-CHAIN TRIGLYCERIDE from step 2 has meanwhile cooled to room temperature. It is added to the mixture from step 4 with stirring up and down movements and always passing through sterile-filtered nitrogen Ph.Eur. (A0080325).* | **mind. 15 l/Minute** *(at least 15 l/minute)* | **l/Min.** | |
| | **mind. 5 Minuten** *(at least 5 minutes)* | **Min.** | |
| **Schritt 6:**<br>Danach wird der gewidmente 1000 l Lagertank **(Pos A)** verschlossen und unter Durchleiten von sterilfiltriertem Stickstoff Ph.Eur. **(A0080325)** bei **geöffnetem Entlüftungsventil** begast.<br>***Step 4:***<br>*Afterwards the dedicated 1000 l storage tank (Pos A) is sealed and under passing of sterile-filtered nitrogen Ph.Eur. (A0080325) by open vent valve fumigated.* | **mind. 100 l/Minute** *(at least 100l/minute)* | **l/Min.** | |
| | **mind. 15 Minuten** *(at least 15 minutes)* | **Min.** | |

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|---|---|---|---|
| **Schritt 7 - Filtration:**<br>**CCP 1:** Für die Filtration ist ein Filter mit einer Porenweite von **5 µm** zu verwenden. Filter-Etikette dem Herstellbericht beilegen.<br>***Step 5 - Filtration:***<br>*CCP 1: For filtration a filter with a pore size of 5 µm must be used. Label of the filter must be enclosed to the batch record* | **CCP 1: 5 µm Filter einzubauen** *(CCP 1: install 5 µm filter)* | **Erledigt** *(completed)* ☐ | |
| | **Filteretikette beilegen** | | |
| **Verwendeter Lagertank:** *(used storage tank)*<br>Bevor die Lösung aus Schritt 4 in den gewidmenten Lagertank **(Pos E)** gedrückt wird, wird dieser mit Hilfe von sterilfiltriertem Stickstoff Ph.Eur. **(A0080325)** begast.<br>*Before the solution from Step 4 is pressed into the dedicated storage tank (Pos E), it will be fumigated with help of sterile-filtered nitrogen Ph.Eur. (A0080325).* | **Lagertank-Nr.** *(storage tank no.)* | | |
| | **mind. 100 l/Minute** *(at least 100 l/minute)* | **l/Min.** | |
| | **mind. 35 Minuten** *(at least 35 minutes)* | **Min.** | |

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|---|---|---|---|
| Anschließend wird die Lösung aus Schritt 4 aus dem gewidmenten 1000 l Lagertank **(Pos A)** durch geeignete Filter mit einer Porenweite von 5 µm **(Pos B)** absolut (z.B. Nylonfilterelemente mit glasfaserverstärktem Nylonstützkörper) in den gewidmeten Lagertank **(Pos E)** aus nichtrostendem Stahl gedrückt. Die Filtrationszeit wird mit Filtrationsbeginn und –ende dokumentiert.<br>*Afterwards the solution from Step 4 is pressed from the dedicated 1000 l storage tank (Pos A) through suitable filters with a pore size of 5 µm (Pos B) absolutely (e.g. nylon filter elements with glass fiber reinforced nylon support body) into the dedicated storage tank (Pos E) of stainless steel. The filtation time is documented with start and end of filtration.* | **Filtrationszeit: (z.B. bei 1 bar Überdruck, 3 - 4 Stunden)** *(filtration time: e.g. at 1 bar over-pressure, 3-4 hours)* | **Datum/Uhrzeit BEGINN:** *(date/time–beginning)* | |
| | | **Datum/Uhrzeit ENDE:** *(date/time-ending)* | |
| | | _____ **bar** | |
| **Aufbewahrung bis zur Abfüllung:**<br>Die filtrierte Lösung wird bis zur Abfüllung in einem gewidmeten Lagertank aus nichtrostendem Stahl **(Pos E)** dicht verschlossen aufbewahrt, der entsprechend **Anweisung LSOPQS3335D** gekennzeichnet wird.<br>Bis zur Abfüllung ist der Lagertank unter einem Überdruck zu halten.<br>***Storage until filling:***<br>*The filtered solution is storaged tightly closed until filling in the dedicated storage tank made of stainless steel (Pos E), which is labeled in accordance to SOP LSOPQS3335D. Until filling the storage tank should be kept under overpressure.* | **mind. 0,2 bar $N_2$** *(at least 0.2 bar $N_2$)* | **Erledigt** *(completed)* ☐ | |

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|---|---|---|---|
| **Musterzug B0085134:**<br>Musterzug erfolgt entsprechend **Anweisung LSOPQC1454D.**<br>***Sampling B0085134:***<br>*Sampling is done in accordance to SOP LSOPQC1454D.* | | | |
| **Untersuchungsmustermenge:** *(quantity of investigation samples)* | **100 ml** | **ml** | |
| **Muster bereitgestellt:** *(samples provided)* | **100 ml** | **ml** | |
| **Ausbeutegrenzen: 98,7 – 101,1 %:** *(yield limits: 98,7 – 101,1 %)* | **901 - 923 kg** | **kg** | |
| **Die Herstellung erfolgte gemäß der gültigen Vorschrift innerhalb der vorgegebenen Grenzen?**<br>*The manufacturing was carried out in accordance to the instruction and within the limits?* | | **JA / NEIN**[*)]<br>*)nicht Zutreffendes streichen!<br>*(Yes / No*[*)]<br>*)crossing out the not applicable!)* | |

| **Unterschrift Herstellungsleiter** *(Signature Head of Production)*: | **Unterschrift Leitungsteam** *(Signature Executive team of Department)*: |
|---|---|
| **Bei Abweichung Work-Flow-Nr.** *(In case of deviation Work-Flow-No.)*: | |

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**Erläuterung zur Arbeitsvorschrift** *(Explanation to the working procedure)*:

**A C H T U N G !** *(Attention)*

**1.: Vorsicht ! Colecalciferol Ph.Eur. ist sehr giftig !**
*(Caution ! Colecalciferol Ph.Eur. is very toxic !)*

Warnhinweise im Ausgangsstoffindex sind zu beachten und **geeignete Schutzausrüstung (Handschuhe, Schutzbrille und Staubmaske)** sind zu tragen !
**Colecalciferol Ph.Eur.** wird in evakuierten bzw. schutzbegasten Behältnissen geliefert.
Beim **Öffnen** ist darauf zu achten, dass der Inhalt nicht verstaubt.

*Warnings in the starting material index should be considered and appropriate protective equipment (gloves, googles and dust mask) are to wear! Colecalciferol Ph.Eur. is supplied in evacuated respectively protect-fumigated container. When opening, make sure, that the content does not gather dust.*

**2.: Colecalciferol Ph.Eur. ist sehr empfindlich gegen Einwirkung von Luft, Licht, oxidierenden Stoffen und Säuren !**
*(Colecalciferol Ph.Eur. is very sensitive to the action of air, light, oxidizing materials and acids !)*

Ein längeres **Stehenlassen** der Lösung ohne Lichtschutz ist unbedingt zu vermeiden.

*A longer standing of the solution without sunscreen must be avoided.*

**3.: Nach dem Öffnen ist Colecalciferol Ph.Eur. sofort einzuwägen**,
in der vorgesehenen Menge MITTELKETTIGE TRIGLYCERIDE zu lösen und bis zur weiteren Verarbeitung in einem geeigneten, **dicht verschlossenem Behältnis**, dessen Kopfraum vor dem Verschließen mit sterilfiltriertem **Stickstoff begast** wurde, **lichtgeschützt** aufzubewahren.

*After opening, Colecalciferol Ph.Eur. has to be weight in immediately, solved in the provided amount of medium-chain triglycerides and stored, protected from light, until further processing in a suitable, tightly closed container, whose headspace was fumigated with sterile nitrogen before sealing.*

**4.: Achtung Schnittgefahr!**
*(Attention danger of cuts !)*

Das Öffnen der Originalitätsverschlüsse der Ölfässer darf nur mit **Schnitthandschuhen** und Kombizange erfolgen !

*Opening oft he original closures of the oil drums may only be done with cut gloves and pliers !*