Comprehensive analysis of vitamin D_3 impurities in oily drug products using SFC-MS

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Abstract

Vitamin D_3, an essential micronutrient, often requires supplementation via medicines or food supplements, which necessitate quality control (QC). This study presents the development of a method for detecting and quantifying seven impurities of vitamin D_3 in oily drug products using supercritical fluid chromatography hyphenated to mass spectrometry (SFC-MS). Targeted impurities include two esters of vitamin D_3 and five non-esters including four that are isobaric to vitamin D_3. Firstly, a screening study highlighted the Torus 1-AA column and acetonitrile modifier as adequate for the separation, followed by optimization of the SFC conditions. Secondly, make-up solvent composition and MS settings were optimized to reach high sensitivity. For both the separation and MS response, screening design of experiments proved useful. Lastly, a fast saponification and liquid-liquid extraction method was developed, enabling efficient sample cleanup and impurities recovery from the complex oily matrix. The SFC-MS method suitability was assessed in two validation studies. The first study employed ICH Q2 guideline for impurity limit test to demonstrate method specificity and establish limit of detection (LOD) and limit of quantification (LOQ) at 0.2% and 0.5%, respectively, for ester impurities. The second study conducted a comprehensive quantitative assessment for three non-ester impurities using a total error approach, determining method validity through accuracy profiles. The validated method exhibited reliable performance across impurity concentrations from 0.1% to 2.0%, with estimated LODs ranging from 2 to 7 ng/mL. This study further promotes SFC-MS as a valuable, versatile, and green tool for routine pharmaceutical QC.

Keywords

SFC-MS; Vitamin D_3; Oily drug products; Impurities; Quality control; Validation;

1. Introduction

Vitamin D_3 (cholecalciferol) is a vital micronutrient, crucial for bone homeostasis and described to be involved in several processes such as cellular growth, cardiovascular health, glucose metabolism, immune response, etc.[1–3] While it is primarily synthesized in the human skin via a non-enzymatic process triggered by sunlight, dietary intake also plays an important role. However, due to factors such as decreased sun exposure or dietary limitations, a global deficiency of vitamin D_3 is prevalent among both children and adults. This deficiency can lead to (severe) health issues and has prompted increased interest and routine prescription of supplements and medicines for its adequate intake[4,5].

This study assesses oily vitamin D_3 formulations in the context of pharmaceutical quality control. It builds on previous research that established a generic method for quantifying vitamin D_3 in medicines and supplements using supercritical fluid chromatography hyphenated to mass spectrometry (SFC-MS)[6]. The objective of the present study is to develop an SFC-MS method to detect and quantify vitamin D_3 impurities in such products. Certainly, impurities can affect the safety and efficacy of medicines, making their identification and quantification crucial in ensuring the quality of drug products. Robust and validated analytical methods are required to ensure the control of impurities to be compliant with regulatory requirements[7–9].

Seven impurities of vitamin D_3 are evaluated in this study, which can be formed during production and/or storage of vitamin D_3 drug substances and drug products. Four of these impurities are isomers of vitamin D_3 (isobaric impurities), namely 5,6-trans-cholecalciferol, lumisterol$_3$, isotachysterol$_3$, and tachysterol$_3$. 5,6-trans-cholecalciferol is formed from the photoisomerization or iodine-catalyzed isomerization of vitamin D_3. The same mechanisms lead to the formation of tachysterol$_3$ and lumisterol$_3$ from pre-vitamin D_3, a vitamin D_3 isomer. Isotachysterol$_3$ has been demonstrated to arise under acidic conditions[10,11]. Additionally, two esters of vitamin D_3, specifically vitamin D octanoate and vitamin D decanoate, are investigated. These esters form via transesterification of vitamin D_3 with triglycerides in the oily matrix[12]. Lastly, we consider dihydrotachysterol. Note that this last compound has an antirachitic activity and is used in some medicines as an analogue to vitamin D. The chemical structures and physicochemical properties of these compounds are given in Figure S1 (supplementary material).

Very few papers described the analysis of these compounds. The isobaric impurities have been mainly studied qualitatively in the context of degradation studies or to ensure method selectivity in the determination of vitamin D_3[11,13–18]. Because it is an analogue to vitamin D, some studies

have described the determination of dihydrotachysterol in biological matrices to determine the antirachitic activity, its metabolization and/or bioavailability[19,20]. For octanoate and decanoate ester impurities, Ballard et al.[12] described their detection in a degradation study of an experimental tablet formulation. Regarding quantitative analysis, Andri et al.[21] developed and validated a robust method for the determination of 5,6-transcholecalciferol (along with vitamin D_3 and pro-vitamin D_3) in vitamin D_3 raw material using SFC-UV. Mahajan et al.[22] developed a RP-PLC method to quantify trans-cholecalciferol and vitamin D_3 in cholecalciferol tablets. Thus, from the literature survey, the determination of vitamin D_3 impurities is a topic that still requires investigation. This is especially relevant for finished drug products and this study is the first to present the determination of a comprehensive set of vitamin D_3 impurities in oily formulations.

Several analytical challenges are encountered, intrinsically linked to characteristics of the impurities, the composition of the sample matrix, and the required analytical performance characteristics. The primary concern is the necessity of resolving the isobaric compounds while having adequate retention of the ester impurities. Regarding the isobaric impurities, it is mandatory to separate them chromatographically because the MS detector will not be able to differentiate them based on their identical mass-to-charge ratio (m/z). Their structural similarity also means that they can behave similarly during the chromatographic process, making their chromatographic separation difficult. Additionally, these specific impurities are isobaric to vitamin D_3 which is the active pharmaceutical ingredient (API) and thus will be at a higher concentration compared to them in the sample. The peak of vitamin D_3 is expected to be broader, which can lead to co-elution. Thus, chromatographic conditions optimisation is required to achieve adequate separation among the isobaric impurities and the API. Along with the challenges of the isobaric compounds, the method must present suitable retention of the ester impurities. Indeed, they are the more apolar impurities, which could lead to a completely different behaviour compared to the other analytes, with concerns of limited retention or very early elution in SFC conditions. Second, the oily (lipophilic) sample matrix is very complex and can cause chromatographic interferences and MS ionization matrix effects. This requires the development of a sample preparation technique that can reduce or eliminate these effects while permitting the quantitative recovery of the analytes. Finally, the sensitivity of the method must be adequate to permit the determination of very low concentrations of the impurities. To answer these challenges, SFC-MS development and validation strategies were implemented.

2. Material and methods

2.1. Chemicals, reagents, and columns

Vitamin D_3 (D_3, 100 %), dihydrotachysterol (99.6 %) and ergocalciferol (candidate internal standard) were sourced from EDQM (Strasbourg, France). Vitamin D_3 octanoate (octanoate, >80 %), vitamin D_3 decanoate (decanoate, > 80 %), lumisterol3 (lumisterol, > 90 %) and isotachysterol3 (isotachysterol, 90 %) were produced by TRC Inc. (Toronto, Canada) and supplied by LGC Standards (Molsheim, France). 5,6-trans-cholecalciferol (trans-cholecalciferol, 98.6 %) was procured from Dalton Pharma Services (Toronto, Canada). Tachysterol3 (tachysterol, 98.6 %) was obtained from Pharmaffiliates (Vilnius, Lithuania). Internal standard vitamin D_3-23,24,25,26,27-$^{13}C_5$ (≥ 97 %) was obtained from Sigma-Aldrich (Overijse, Belgium). The sample matrix was produced in-house from excipients including refined olive oil, all-rac-α-tocopheryl acetate, sweet orange extract, and polyglyceryl oleate, all of which were generously provided by an industrial partner.

Carbon dioxide (CO_2, ≥ 99.995 %) was obtained from Air Liquide (Liege, Belgium). LC-MS grades of methanol (MeOH), acetonitrile (ACN), water (H_2O), 2-propanol (2-PrOH), and n-heptane (LC grade) were sourced from Biosolve BV (Valkenswaard, Netherlands). Analysis grades of potassium hydroxide (KOH) and sodium chloride (NaCl), absolute grade of ethanol (EtOH), and LC-MS grades of formic acid (FA, ≥ 99 %), ammonium formate (AmFm), and ammonium acetate (AmAc) were supplied by VWR Chemicals (Leuven, Belgium).

Four analytical columns were purchased from Waters (Milford, MA, USA), including Viridis BEH, Viridis HSS C18 SB, Torus 1-Aminoanthracene (1-AA), and Viridis CSH Fluoro-Phenyl (FP). All columns had the dimension 100 x 3.0 mm, with a particle size of 1.7 µm, except for the HSS C18, which had a particle size of 1.8 µm.

2.2. SFC-MS instrumentation and analytical conditions

SFC analysis was carried out using a Waters Acquity UPC^2 (Waters, Milford, MA, USA), which was equipped with a binary solvent delivery system, a 10 µL loop autosampler, an active preheater column oven, a PDA detector, and a two-stage backpressure regulator (BPR). For detection via mass spectrometry, the SFC system was connected to a Waters SQ Detector 2

(Waters, Milford, MA, USA) with APCI and ESI sources. Hyphenation to mass spectrometry was performed with a SFC-MS splitter interface (Waters, Milford, MA, USA) and a make-up solvent delivery system (Isocratic Solvent Manager, Waters, Milford, MA, USA).

Optimal chromatographic conditions were achieved using the Torus 1-AA column with a mobile phase consisting of ACN as modifier. The following gradient was applied: the initial composition of the mobile phase was 3 % of modifier which was increased to 10 % in 7.5 min followed by an isocratic hold of 1.0 min. Finally, a return to the initial conditions was established in 0.5 min and maintained for 1 min (re-equilibration) for a total analysis time of 10.0 min. Mobile phase flow rate was equal to 1.5 mL/min. The column temperature was fixed at 45 °C and the backpressure at 120 bar. The injection volume was set to 3 µL and the autosampler temperature to 6°C to ensure sample stability. Methanol and 2-propanol were used as strong and weak needle wash, respectively.

Optimized MS conditions were set as follows: ESI source, capillary voltage +3.5 kV, desolvation temperature 400 °C, source temperature 150 °C, cone gas flow rate 75 L/h, desolvation gas flow rate 750 L/h, cone voltage 30 V for all analytes. MeOH/H_2O 90/10 (v/v) + 0.5 % FA was used as make-up solvent at a flow rate of 0.15 mL/min. Mass spectrometer was operated in positive mode and single ion recording (SIR). Targeted m/z values for the analytes were 385.40 for D_3 and the isobaric compounds, 381.50 for dihydrotachysterol, 511.64 for vitamin D octanoate and 539.60 for vitamin D decanoate. Both vitamin D octanoate and decanoate were also detected at m/z 367.40.

Empower 3.0 (Waters, Milford, MA, USA) was used for instrument control, data acquisition and processing. Data analysis was performed with Microsoft Excel 2019. Data analysis of the validation results for non-ester impurities was performed with E-Noval 4.1 (Pharmalex, Mont-Saint-Guibert, Belgium).

2.3. Experimental design

Definitive screening designs (DSDs) were used to investigate the effects of several factors on SFC separation and MS response, respectively. The DSD for SFC separation consisted of a set of 13 randomized experimental runs with 5 factors varied at 3 levels. The factors investigated

for SFC separation were the temperature, the pressure, the initial proportion of modifier, the final proportion of modifier and the gradient time. The DSD for ESI-MS response consisted in a set of 17 randomized experimental runs with 6 factors varied at 3 levels. The factors investigated for the MS response were the capillary voltage, the cone voltage, the desolvation temperature, the desolvation gas flow rate, the cone gas flow rate, and the make-up flow rate. Summary about the factors and their levels are presented in Tables S1-S2 (Supplementary Material). All the designs were generated and analysed using JMP® Pro 15 (SAS institute Inc., Cary, NC, USA).

2.4. Sample preparation

2.4.1. Standard and development solutions

Individual stock solutions of D_3 and impurities were accurately prepared by weighing and dissolving an adequate quantity of each analyte to reach a concentration of 1 mg/mL. For the impurities, an adequate volume of each individual solution was pipetted to prepare a mix of impurities at 10 µg/mL. Exploratory solutions of D_3 and impurities were prepared in a concentration range of 25-2500 ng/mL. Additionally, test solutions were prepared by diluting the stock D_3 solution and mixture of impurities to reach a concentration of 31.25 µg/mL for D_3 and 156.25 ng/mL for the impurities, corresponding to 0.5 % impurities concentration relative to D_3. All the solutions were prepared in pure n-heptane or in diluted matrix solution (5% v/v in n-heptane) and stored protected from light at -20 °C. The exploratory and test solutions were used for chromatographic development and MS response optimization.

2.4.2. Sample saponification protocol

0.25 mL of the oily sample solution was accurately pipetted in a 50 mL centrifuge tube, and the analytes and internal standard were added. Then, 2.5 mL of a 3M KOH solution prepared in EtOH/H_2O 80/20 (v/v) was added to the sample. The mixture was vortexed for 30 seconds and the saponification reaction was allowed to proceed for 15 minutes under ambient conditions. Following the reaction, 1.0 mL of a 10 g/L NaCl solution was added to the mixture and 2.5 mL (10x sample dilution factor) or 5.0 mL (20x sample dilution factor) of heptane was introduced to perform the extraction. The sample was vigorously shaken and vortexed for 30 seconds. Finally, the mixture was centrifuged for 5 minutes at 10000 rpm and an aliquot of the

supernatant was taken for injection. For this protocol, all standard solutions used for spiking were prepared in ethanol.

2.4.3. Validation protocol for ester impurities

Method validation for ester impurities was performed considering a limit test approach according to ICH Q2 guideline[23]. One validation series was performed. Mix impurities solutions containing octanoate and decanoate at a concentration of 15.625 µg/mL and 3.125 µg/mL were prepared by mixing and diluting stock standard solution of each ester impurity in ethanol. First, 0.25 mL of matrix sample was pipetted in volumetric flasks of 5.0 mL (20x sample dilution factor), then vitamin D_3 was added at a concentration of 31.25 µg/mL. An adequate volume of the mix impurities solutions was added to reach a concentration of 31.25 ng/mL, 62.5 ng/mL, 156.25 ng/mL and 312.5 ng/mL. These values correspond to impurities concentrations of 0.1 %, 0.2 %, 0.5 % and 1.0 % respectively, relative to the concentration of vitamin D_3. Finally, n-heptane was added as the diluent to fill the flasks.

2.4.4. Validation protocol for non-ester impurities

Full quantitative method validation for non-ester impurities was performed using a total error approach[24–27]. The validation was performed for three non-ester impurities for which high-quality analytical standards could be obtained: trans-cholecalciferol, tachysterol and dihydrotachysterol. The two other non-ester impurities were not considered due to a lack of analytical standards of sufficient quality as explained in section 3.2.2. Four validation series were performed. For each series, mix impurities solutions containing the three impurities at a concentration of 15.625 µg/mL and 3.125 µg/mL were prepared by mixing and diluting stock ethanolic solutions of each individual impurity in ethanol.

The targeted concentration of impurities was 312.5 ng/mL, which corresponds to 0.5 % impurity concentration relative to the concentration of vitamin D_3 at 62.5 µg/mL. These calculations consider a 10x sample dilution factor of a tested drug product that contains 625 µg/mL of vitamin D_3. The range was set from 0.1 % to 2.0 % relative impurity concentration.

For calibration standards (four concentration levels), one solution was prepared for each concentration level for each series by adding vitamin D_3 at a concentration of 62.5 µg/mL, and

diluting the mix impurities solutions to reach concentrations of 62.5 ng/mL, 125 ng/mL, 312.5 ng/mL and 1250 ng/mL. These values correspond to impurities concentrations of 0.1 %, 0.2 %, 0.5 % and 2.0% respectively, relative to the concentration of vitamin D_3. In each solution, the internal standard was added at a concentration equivalent to the 0.5 % impurity concentration level. The calibration solutions were reconstituted in post-extraction matrix to account for matrix effects. For this, an adequate volume of sample matrix was saponified according to the protocol described in section 2.4.2 without spiking analytes and considering a 10x sample dilution factor. This post-extraction matrix solution was used as the diluent for the calibration standards and was prepared once for each validation series.

For validation standards (five concentration levels), three independent replicates per concentration level were prepared for each series. First, 0.25 mL of sample matrix was pipetted in a centrifuge tube and vitamin D_3 was added at a concentration of 62.5 µg/mL. Then, adequate volumes of the mix impurities solutions were added to reach a concentration in the extracted solution of 62.5 ng/mL, 125 ng/mL, 312.5 ng/mL, 625 ng/mL and 1250 ng/mL. These values correspond to impurities concentrations of 0.1 %, 0.2 %, 0.5 %, 1.0 % and 2.0% respectively, relative to the concentration of vitamin D_3. The internal standard was added at a concentration equivalent to the 0.5% impurity concentration level. The saponification protocol described in section 2.4.2. was then applied with an extraction volume of n-heptane equal to 2.5 mL considering a 10x sample dilution factor.

3. Results and discussion

3.1. Chromatographic development

3.1.1. Column screening

During the method development, careful consideration was given to pre-vitamin D_3 (pre-cholecalciferol), which is a biologically active isomer of vitamin D_3 that is formed in solution from vitamin D_3 depending on temperature and time. Its formation in drug products under normal storage conditions is highly likely and was confirmed in our previous QC study encompassing a diverse array of medicines and food supplements[6]. Pre-vitamin D_3 is part of the isobaric group which also includes vitamin D_3, trans-cholecalciferol, lumisterol, isotachysterol and tachysterol and must therefore be resolved during the chromatographic process. As fresh standard solutions of vitamin D_3 standards do not always exhibit this compound, aged standard solutions were used when needed to ensure its identity and resolution from other isobaric compounds (degradants). From a broader perspective, the structural similarity of these compounds was a challenge in method development as they exhibited a similar chromatographic behaviour, which could lead to partial or full co-elution.

Initial evaluation of the tested columns was carried out using a range of modifiers, including methanol, ethanol, 2-propanol, acetonitrile, and methanol/acetonitrile mixtures. Generic gradients with modifier proportions ranging from 2 to 15-30 % and gradient times between 2.5 and 10 minutes were employed. The flow rate was set at 1.5 mL/min, temperature at 40°C, and BPR at 130 bar. The BEH and CSH-FP columns exhibited low retention, co-elution of isobaric analytes, and poor peak shapes, while the C18 and 1-AA columns demonstrated better performances, warranting further testing. These columns have demonstrated to be particularly suited for the analysis of fat-soluble vitamins and their related substances[28]. Regarding interactions type, these results indicate that hydrophobic and/or π-π interactions are necessary to successfully retain and separate these compounds. The bare silica of the BEH column mainly provides polar interactions such as dipole-dipole and hydrogen bonding[29]. The CSH-FP column (pentafluorophenyl ligand) also exhibits some polar interactions but has a strong capability for π-π interactions which could theoretically be adequate for these compounds. However, this is not the case in practice since low retention is observed on this column. This observation aligns with the findings of Petruziello and al. in the analysis of fat-soluble vitamins[28], suggesting that other factors may diminish π-π interactions on this stationary phase or that π-π interactions

alone are insufficient for these analytes. On the other hand, the 1-AA column (1-amino-anthrancene ligand) also provides strong π- π interactions for these compounds[30]. Additionally, the large anthracene ring of this stationary phase can also provide hydrophobic interactions[31]. Thus, a combination of these interactions may explain the good results obtained on this column. Finally, the C18 column with an octadecyl ligand provides hydrophobic interactions for these analytes.

Acetonitrile was identified as the most suitable modifier for both the C18 and 1-AA columns as it permitted to separate isobaric compound. Compared to the alcohol modifiers, acetonitrile has a lower elution strength which proved to be an important parameter for the separation of these analytes in SFC conditions. This has also been observed in the literature for lipid separation[32]. However, using pure acetonitrile resulted in a lower MS intensity, most likely due to its non-protic nature. The addition of low proportion of methanol in acetonitrile was tested but was inconclusive as the resolution of the isobaric compounds was significantly affected, even accounting for adjusted gradient profiles. For both columns, ester impurities were eluted at the beginning of the run, similar to what was reported by Socas-Rodriguez et al.[33] in the determination of vitamin D and its metabolites including esterified metabolites by UHPSFC-MS/MS. This is attributed to the fact that at the beginning of the run there is a higher proportion of apolar supercritical CO_2 which preferentially elutes the less polar ester analytes. For the C18 column, dual peaks could be observed for the ester analytes as seen in Figure S2 (Supplementary Material). No discernible differences in the mass spectra were observed for the peaks and it was difficult to assess the presence of isomers such as esters of pre-vitamin D_3 for example. As only these impurities were affected, it could also simply be a case of severe peak splitting in the defined analytical conditions. This column also required a high proportion of modifier up to 30 % to elute the compounds, resulting in higher backpressure and this limits the possibility of increasing the flow rate to reduce the run time. Additionally, we observed that fatty components from the matrix were strongly retained on this stationary phase. They could not be eluted within the time frame and were detected in subsequent injections, necessitating an additional strong washing step at the end of the gradient and an adequate re-equilibration time. This also contributed to an increase of the run time. For these reasons, the 1-AA column was ultimately selected. It had adequate resolving power of isobaric compounds, adequate retention of esters and permitted to elute all analytes and matrix components in a short time.

3.1.2. SFC factors screening

DSD are a class of experimental designs developed for efficient factor screening by estimation of main, two-factor interaction and even quadratic effects in the presence of potentially large numbers of factors. For continuous factors, the possibility to estimate quadratic effects is enabled by its three-level design, unlike more traditional screening designs which usually evaluate two levels for the factors. The interested reader is directed to more specialized literature for a complete description of these screening designs[34,35].

For each experiment, a standard mix containing vitamin D_3 and impurities at 0.5 % concentration level were injected. Individual solutions of the isobaric compounds trans-cholecalciferol, lumisterol and isotachysterol were also injected to ensure peak traceability throughout the experiments. The separation criterion S was selected as response for the DSD. For two analyte peaks eluting one after the other, it is calculated as the difference between the retention time at the beginning of the second peak and the retention time at end of the first peak. It represents therefore the time in the space between both peaks. Baseline separation is achieved when $S \geq 0$ min and negative values represent peak co-elution. Considering this criterion, data analysis was preferentially performed for the individual injections to ensure proper determination of retention times at the beginning and end of the analyte peaks.

No modification of elution order was observed throughout the experimental domain. Two critical pairs were determined: lumisterol–D_3 and D_3–isotachysterol. At the target concentration of vitamin D_3, the peak is intense and large with some tendency of slight tailing (symmetry factor of 1.3), which can have an impact on neighbouring peaks. This highlights the importance of the separation between impurities before and after the peak of vitamin D_3 in the isobaric group. S-criterion was calculated for the critical pairs and the DSD was analysed. Summary of the important factors that were highlighted is given in Table 1. The effects of these factors were also estimated (Table 1) and the resulting models were used to build a profile for the responses in the experimental domain as illustrated in Figure 1. Note that the model for S-criterion for lumisterol-D_3 is high (R^2 adjusted of 0.91). However, the model for S-criterion for D_3-isotachysterol is lower (R^2 adjusted of 0.50), likely due to the tendency of tailing of the large vitamin D_3 peak which adds variability in the determination of the retention time at the end of the peak for some experiments. The resulting model therefore has lower accuracy to capture the observed data.

Three factors were highlighted: the temperature, the final proportion of modifier, and the gradient time. The main effects were the most statistically significant and have the largest impact. For both S-criterion, values are maximized at low final proportion of modifier and long gradient times (10 % and 7.5 minutes respectively, in the experimental domain). Taken together, it suggests that the proportion of modifier per minute (gradient slope) needs to be low to increase the separation between D_3 and neighbouring peaks in this experimental setup. This is an expected chromatographic behaviour as a slow gradient profile is usually beneficial for complex separations of structurally related substances. The range of modifier in the gradient is mainly driven by the final modifier proportion in this setup because a higher experimental range was tested for the final proportion compared to the initial proportion. This could explain why this factor was found to be important while the initial proportion was not. While not evaluated in this study, it could also then be theoretically possible to adjust other parameters such as slightly increasing the flow rate to further decrease the gradient steepness and possibly improve the separation. This is particularly interesting in SFC as an increase of flow rate is generally feasible considering the increase in pressure is moderate and high efficiency is achieved even at high flow rates. Note that the separation in SFC in also governed by the pressure, which could have unintended effects. In this study however this factor was not highlighted as having an impact on the separation of the critical pairs for a backpressure range between 120 and 150 bar. Thus the effect of increasing the flow rate is analysis dependent and must be evaluated on a case-by-case basis. An increase of temperature was also found beneficial for the separation between lumisterol and D_3. This factor also had a statistically significant quadratic effect which suggests a local maximum as illustrated in Figure 1. For the same S-criterion, other second order effects were highlighted, but to lesser degree of statistical significance. Of note an interaction between the final proportion and the gradient time is suggested, which is most likely linked to the discussion above for the distinct main effects.

No further optimization of the three highlighted factors was performed as an adequate separation was achieved at this stage as illustrated in Figure 2. They were set at the levels tested in the experimental domain that provided the highest S-criterion. The other parameters were set at user-chosen levels as reported in section 2.2.

3.2. ESI-MS response optimization

3.2.1. ESI-MS make-up screening

Different make-up solvent compositions were first tested for the optimization of ESI+ MS response, including pure methanol, and methanol with the addition of 5%, 10%, and 20% of water v/v. A key finding from these experiments revealed that most analytes were not detected when using pure methanol as a make-up solvent as seen in Figure 3. However, the addition of water to the make-up solvent, even only 5 % (v/v), resulted in the successful detection of the analytes. Moreover, as the proportion of water in the make-up solvent was increased to 10 % or 20 % (v/v), signal intensity for the analytes also increased sharply, more than three times for several analytes. This effect however seems analyte dependant and not uniquely related to the proportion of water in the make-up. Indeed, some analytes such as trans-cholecalciferol or dihydrotachysterol had a better MS response at 10 % (v/v) water proportion compared to 20 % (v/v). Other analytes such as decanoate or lumisterol did not exhibit much difference in MS response at 10 % or 20 % (v/v) water proportion. It is also most likely that a decrease of desolvation efficiency at higher proportion of water plays a role and limits the possible MS response improvement from only adding water in the make-up.

The effect of adding water in the make-up to increase ionization of some analytes in ESI+ has been reported in the literature[36,37]. The current understanding is that one of the drivers for the electrospray ionization in SFC is via the formation of alkoxycarbonic acid when CO_2 reacts with an alcohol such as methanol (formation of methoxylcarbonic acid) or water (formation of carbonic acid). This decreases the apparent pH of the mobile phase, facilitating proton transfer in positive mode [36,38,39].

Published studies generally use methanol as modifier and thus it is expected that methoxylcarbonic acid is already produced during the chromatographic process as demonstrated by Fujito et al[39]. This phenomenom does not occur in this study since only acetonitrile is used as modifier, which does not react with CO_2 to form an acidic species. It is only started with the addition of the make-up. It is possible that, for this set of compounds and this MS interface configuration, an equilibrium is not achieved fast enough to promote proton capture by the analytes with methoxycarbonic acid when pure methanol is used in the make-up. Thus, the addition of water and the formation of carbonic acid could permit to reach this equilibrium, especially since carbonic acid is a stronger acid than methoxycarbonic acid [38]. Devaux et al.[40] performed the RPLC x SFC analysis of biofuels and developed a 1D-SFC method using MeOH/ACN 50/50 (v/v) as a modifier with a similar MS interface. They also observed a significant improvement of ESI+ response when water was added in the methanolic make-up solution. Thus, the addition of water in the make-up solution could prove to be an important strategy for ESI+ optimization when a high proportion of acetonitrile is used in the

modifier. The condition at 10 % water (v/v) was selected since pressure issues were encountered at 20 % water proportion.

Further evaluation of the MS response was performed by addition of additives in the make-up (Figure S3). Ammonium formate strongly decreased the signal up to - 90 % of all analytes. This result could be explained by (i) ion suppression and/or (ii) disruption of the formation and/or effects of the alkoxycarbonic acids as reported by Fujito et al.[39] Formic acid however improved the sensitivity for the isobaric compounds and dihydrotachysterol. This was not the case for ester impurities for which a decrease in sensitivity was observed, suggesting other factors such as ion suppression could still play a role. Since the sensitivity for the ester impurities was still adequate, the final conditions used 0.5 % formic acid to ensure high sensitivity for the non-ester impurities.

3.2.2. ESI-MS factors screening

DSD was used to identify critical instrumental MS parameters to ensure high sensitivity for the analytes. All experiments were performed in real conditions with the developed method and optimized make-up solvent. European pharmacopoeia signal-to-noise ratio (S/N) was used as response and was automatically calculated by the instrument software on raw data (unsmoothed signal). For each experiment, a blank injection of n-heptane, the sample diluent, was first performed to permit the calculation of noise. A mixture of vitamin D_3 and impurities at 250 ng/mL was then injected. The highlighted factors and their estimates after data analysis are given in Table 2. Data for trans-cholecalciferol is not presented because it could not be detected, this observation will be discussed at the end of this section.

As seen in Table 2, capillary voltage (main effect) was found to be significant for all analytes. The highest S/N values were obtained when it was set at the lowest setting of 3.5 kV. Additionally, the range for the capillary voltage was chosen after preliminary univariate testing which indicated a decrease in signal for values below 3 kV. Taken together, these observations suggest an optimal of capillary voltage for all analytes between 3 and 3.5 kV. Note that this parameter had already been identified as important in the preliminary testing, the DoE analysis is a confirmation.

Models from parameters estimates have adjusted R^2 ranging between 0.3 and 0.9. The highest adjusted R^2 are calculated for the ester impurities (0.74 and 0.90). For these compounds, more statistically significant effects were highlighted. Notably, an increase of desolvation temperature had a negative impact on S/N values for octanoate. The desolvation gas flow rate

had a main and a quadratic effect for decanoate, indicating a local maximum that is found close to the mid-level that was tested. A negative effect when increasing the make-up flow rate was also highlighted for decanoate and is most likely related to a decrease of desolvation efficiency at the higher flow rates. This is corroborated by the second order effect of interaction between the make-up flow rate and desolvation temperature for which there is a positive effect on S/N when both factors are increased. This means that the higher temperature counterbalances the decrease of desolvation efficiency at higher make-up flow rates.

The non-ester compounds have the least accurate models with adjusted R^2 between 0.33 and 0.53. Looking at boxplots for the distribution of S/N values from the experimental domain in Figure S4, we observe a grouping at low S/N values and significantly less variation compared to the esters. On one hand, it could mean that the non-ester compounds are less influenced by the studied MS parameters in the ranges evaluated in the screening design. However, MS ionization process variability could also play a role and, arguably, it can have a greater impact at low signal intensity. Thus, if there is a high measurement uncertainty at low S/N values, it lowers the likelihood of finding statistically significant effects and decreases the model accuracy. The only other significant effect that could be found for the non-ester compounds is the cone voltage as main effect for isotachysterol and quadratic effect for dihydrotachysterol. Nevertheless, as a screening step, the DSD permitted to highlight several critical parameters and provided valuable insights on the ESI+ MS response.

The low S/N values that were observed for the non-ester analytes was investigated, especially since trans-cholecalciferol could not be detected. It appeared that the response highly varied considering the standard used to prepare the solutions, and some standard vials produced almost no response at all. This could be highlighted only for the isobaric impurities for which multiple standard vials were available. Other vendors of these compounds were thus evaluated prior to method validation and reported in section “material and methods”. High-quality analytical standards are of paramount importance for pharmaceutical quality control. Such standards used in method development and validation enhance the accuracy and reliability of the analytical data, promoting trust in the obtained results in support of the safety and efficacy of the drug products. The use of poor-quality standards can cause to underestimate or overestimate the proportion of impurities, leading to inaccurate decisions in the release of a batch of tested products. There is thus a risk for both the consumer and the manufacturer. It is therefore important to select reliable providers that adhere to rigorous quality control that permit to ensure authenticity, purity, and traceability of the analytical standards.

3.3. Sample preparation

3.3.1. Saponification and extraction

One of the issues of analysing impurities is that a concentrated sample must be injected to ensure proper detection of the analytes. This in turn can lead to co-injection of a large quantity of matrix, depending on the sample type and the sample preparation step. For this case study, a low dilution factor of the original sample is used, between 10x and 20x, meaning that between 5 % and 10 % (v/v) oily matrix is injected considering a simple dilute-and-shoot sample preparation. Several chromatographic interferences coming from the matrix as well as ionization matrix-effects could be observed for some analytes in the operating conditions. For oily matrices, selective extraction of analytes requires a sample clean-up to limit the presence of triacylglycerols (TAG), which are the main components. The most common procedure is based on a saponification approach[41]. The principle is to hydrolyse the TAGs, which are fatty acid esters, with a strong base. Then, the analytes of interest are recovered by extraction and purification of the resulting mixture.

Common saponification protocols are performed at high temperatures (60-100 °C) for a duration usually under 1 hour, or at room temperature for a long time, usually overnight for 16-18 hours[41]. The high temperature of the saponification could prove to be detrimental for the impurities. The saponification protocol developed in this study is performed at room temperature for a short period of time in 15 minutes. The analytes are extracted by liquid-liquid extraction (LLE) with n-heptane, which can be directly injected in SFC, with or without an additional purification step. Note that the ester impurities determined in this study will also be hydrolysed and thus this technique cannot be used for their determination.

Based on this protocol, the saponification reaction could be considered as a soft saponification process but sufficient to clean-up the samples. As seen in Figure 4, the chromatographic interferences from the matrix are significantly removed after application of the preparation protocol. For dihydrotachysterol (Figure 4A), a return to baseline is achieved and the matrix signal suppression at the end and after the peak is corrected. The signal for ergocalciferol (candidate internal standard) in Figure 4B is not drown by the matrix and can be integrated for analysis. Finally, low volumes of solvents and reagents were used, which permitted to perform the reaction and extraction in a simple centrifuge tube and enabled the possibility to easily process several samples in parallel.

3.4. Ester impurities method validation

Method validation for the ester impurities was performed as a limit test according to ICH Q2 guideline[23] in this study. The validation was performed as a limit test instead of a full quantitative approach for two main reasons. First, there are no safety concerns or maximal content specification reported for these analytes. Vitamin D_3 esters are expected to be hydrolysed during absorption, realising vitamin D_3[42]. This has been studied in human neonates[12]. Moreover, formulations containing esters of vitamin D_3 were proposed and have been the subjects of patents[12,43,44]. Second, the degree of trans-esterification reactivity (drug-excipient) that leads to their formation has been described to be minor. They could be considered as markers of API stability during long-term storage of the drug product.

For a limit test, the criteria to assess are selectivity/specificity and limit of detection (LOD). The determination of the limit of quantification (LOQ) was also included in this validation. The validation results are presented in Table 3. Specificity in sample matrix was achieved for both analytes. LOD and LOQ were determined according to the signal-to-noise ratio with $s/n \geq 3$ for LOD and $s/n \geq 10$ for LOQ at the same m/z for which method selectivity was demonstrated. For both analytes, the LOD and LOQ were estimated at 0.2 % and 0.5 % impurity concentration, respectively. Figure 5 showcases the chromatograms for both analytes at the LOD.

3.5. Non-ester impurities method validation

Method validation for the non-ester impurities followed a total-error approach, employing accuracy profiles based on β-expectation tolerance intervals. This approach is also referred to as a "combined approach for accuracy and precision" in ICH Q2(R2) guideline and represents a modern and enhanced approach for method validation. Data were computed using the internal standard, specifically, peak area ratios of analyte over the internal standard. The calibration model used was the weighted (1/X) quadratic regression model. The accuracy profiles obtained are presented in Figure 6. The dashed blue lines represent the β-expectation tolerance limits. These limits define an interval within which future analytical results are expected to fall with a predetermined probability, which was set at 95% for this study. The method is considered as valid within the range for which the accuracy profiles are in the acceptance limits, indicated by the dashed black lines. The maximum risk level chosen (risk of having measurements falling

outside of the acceptance limits) was set to 5%. The validation results are presented in Tables 4 – 6.

3.5.1. Selectivity

The selectivity of the method was demonstrated by analysing the extracted matrix solution. No interfering compounds could be detected at the same retention time with the analyte of interest.

3.5.2. Method trueness

Trueness, expressed as the mean relative bias (%), provides an indicator of systematic error during the measurement process. It characterizes the degree of concordance between the average measured values and the true values of spiked samples. The evaluation of trueness was performed for each concentration level by comparing the mean introduced concentrations and the mean back-calculated concentrations (based on the calibration model and data) of the validation standards. The calculated relatives bias (%) are presented in Tables 4 – 6. For trans-cholecalciferol and dihydrotachysterol, the measured relative bias is less than |5 %|. A small negative bias at low concentration levels is observed for trans-cholecalciferol and a positive bias on the other levels. For dihydrotachysterol it is a mostly positive bias that is observed. For tachysterol, a negative bias is observed on the whole dosing range between |8 and 10 %| at the lower concentration levels and around |5 %| at the higher concentration levels. On average, a +2% bias is calculated for trans-cholecalciferol and dihydrotachysterol, while it is an average of -5% bias for tachysterol. This negative bias for tachysterol could be attributed to an incomplete recovery of this analyte during the sample preparation step. However, considering that the three analytes have very similar chemical structures, it is unlikely that the extraction coefficient of tachysterol (during the sample preparation) would be sensibly different compared to the two other analytes, leading to a negative bias. This suggests that other factors are affecting this specific analyte such as the internal standard correction. To verify this aspect, data analysis was performed without accounting for the internal standard (peak areas instead of ratios). In this case, a similar bias is observed for all the analytes, with an average of -38% ± 2%. Thus, while the internal standard, which is the same for all analytes, helps in correcting the bias; it is not performed in the same way for tachysterol compared to the two others. The use of deuterated internal standards could be better suited however they are not commercially available for the impurities tested. Nevertheless, the results obtained during the validation highlight that the

internal standard used in this study is a good compromise and the calculated bias values are fully acceptable and demonstrate the good trueness of the method.

3.5.3. Method precision

Precision provides information on random errors. It is expressed in terms of relative standard deviation (RSD (%)) and was evaluated at two different levels: repeatability (intra-series) and intermediate precision (intra and inter-series). The RSD values are presented in Tables 4 – 6. A similar trend is observed for all the analytes. As could be expected, the highest RSD are calculated for the lowest concentration level. Repeatability between 2 and 4 % and intermediate precision between 8 and 12 % are obtained. The inter-series variability is the most prominent at this concentration level. When calculated on absolute values by omitting the internal standard, repeatability is similar, but the intermediate precision is diminished in the range 5 to 6 %. Thus, the internal standard has an impact on method precision, especially at the lowest concentration level. For the other concentration levels, the variability is less pronounced with repeatability between 2 and 4% and intermediate precision between 3 and 6 %. No major difference in RSD values was observed for these concentration levels when calculated on absolute values. Overall, method precision is considered very good for the scope of the analysis, considering the low concentration of impurities and that it was evaluated across a total of 60 completely independent sample preparations.

3.5.4. Method accuracy, LOQ, LOD

Accuracy refers to the degree of agreement between test results and a recognized reference value. It results from the combination of trueness and precision (i.e., the total error). The computation of accuracy profiles is the core principle of the total-error approach. The acceptance limits were set *a priori* to 30 % considering the scope (impurities determination in a complex matrix) and some potential error sources that could be highlighted in method development such as (i) the use of a MS detector (ionization process variability and matrix effects, (ii) the low concentrations targeted, (iii) the sample preparation technique (potential for analytes loss and variability between preparations) and (iv) the same internal standard for all the analytes (potential source of variability in the signal correction). As seen in Figure 6, the method is considered accurate for the determination of dihydrotachysterol on the whole dosing range. The accuracy of the method is impacted at the lowest concentration level for the other

two analytes as could be expected from trueness and precision results. Accuracy profiles of both analytes are fully in the acceptance limits for the rest of the dosing range. From these results, lower and upper limits of quantification (LLOQ and ULOQ) can be derived by calculating the smallest and highest concentrations beyond which the accuracy profiles are outside the acceptance limits (Tables 4 – 6). They represent the smallest and highest quantity that can be assayed with well-defined accuracy, respectively. The LLOQ, expressed in relative to the API, is equal to 0.1 % for dihydrotachysterol and trans-cholecalciferol, and 0.2 % for tachysterol. The ULOQ for the three analytes corresponds to 2.0 % relative impurity concentration.

The limit of detection (LOD), which represents the smallest quantity that can be detected by the method but not accurately quantified, was estimated following the Miller & Miller methodology[45]. LODs in the range 2 – 7 ng/mL concentrations were calculated for the three analytes, equivalent to 0.003 % – 0.01 % relative impurity concentrations.

3.5.5. Linearity

The linearity is the ability of an analytical method to obtain results proportional to the concentration of the analyte in the sample. It was assessed by fitting a linear regression model of the back-calculated concentrations as a function of the introduced concentrations. A good linearity of the results is calculated for all the analytes. The slope is close to 1 with R^2 values superior to 0.99.

4. **Conclusion**

The determination of impurities is one of the more challenging tasks in pharmaceutical quality control but is crucial in ensuring the safety and efficacy of medicines. Moreover, the difficulty can be compounded in finished drug products, especially if the matrix is complex.

The present work demonstrates a comprehensive approach for the determination of vitamin D_3 impurities in oily drug products using SFC-MS. On the separation side, method development permitted to achieve adequate resolution of isobaric impurities along with the API while maintaining adequate retention for ester impurities for a total analysis time of 10 min. On the MS detection side, the optimization of the composition of the make-up solvent, by the addition

of water, proved to be crucial in reaching high sensitivity for the analytes. For both separation and MS optimization, screening designs were useful in understanding the processes and finding suitable method parameters. A fast and easy saponification-LLE protocol was developed for sample preparation step and permitted to eliminate matrix interferences while achieving recovery of the analytes. Finally, the fitness-of-purpose of the SFC-MS method were demonstrated during two validation studies. In the first part of this study, a limit test approach from ICH Q2 guideline was used for the ester impurities and permitted to demonstrate the specificity, LOD and LOQ of the method. In the second part, the full quantitative determination of three non-ester impurities was validated using a total error approach. The computed accuracy profiles permitted to state the validity of the method while controlling the risks associated with the future use of the method in routine analysis. The validated dosing range was between 0.1 and 2.0 % impurities concentration and LODs were estimated between 2 – 7 ng/mL. These validation results are highly promising. Indeed, the SFC-MS method is able to reach the required quantitative performance for reliable QC of impurities in a complex matrix. Moreover, the proposed methodology using the SFC-MS conditions and the sample preparation protocol is relatively easy and reduces solvent consumption compared to traditional approaches. It could also be applied for the analysis of impurities in oily food supplements provided adequate determination of matrix effects.

This work adds further proof that SFC-MS is a viable tool in this framework. Considering the versatility and greenness of SFC, along with its role as a complementary analytical technique, its implementation in QC laboratories is a sound strategy and represents a step towards more sustainable and efficient analytical practices.

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Appendices

Figures captions

Figure 1: Response profile considering the models for A) S-criterion lumisterol-D_3 and B) S-criterion D_3-isotachysterol.

Figure 2: Example chromatograms for a solution containing impurities at 0.5% relative concentration level in n-heptane in the final conditions. 1) pre-vitamin D_3 2) trans-cholecalciferol 3) lumisterol 4) isotachysterol 5) tachysterol. Chromatographic conditions in text.

Figure 3: Relative difference of ESI+ MS response with make-up solvents of MeOH, MeOH/H_2O 90/10 (v/v) or MeOH/H_2O 80/20 (v/v). Reference is ESI+ MS response with make-up solvent MeOH/H_2O 95/5 (v/v). Responses calculated on peak area.

Figure 4: Overlay chromatograms for a sample prepared in i) reconstituted matrix solution in n-heptane with 5% (v/v) matrix (red line and orange fill) versus ii) sample after application of saponification and extraction protocol (black line and white fill). A) Dihydrotachysterol SIR B) Ergocalciferol SIR (m/z 397.40) C) Isobaric analytes group SIR.

Figure 5: Method validation for ester impurities: chromatograms of vitamin D_3 octanoate and vitamin D_3 decanoate at the LOD. Red lines: validation solution at 0.2% impurities concentration. Blue lines: matrix blank.

Figure 6: Accuracy profiles computed for trans-cholecalciferol, tachysterol and dihydrotachysterol in vitamin D_3 oily drug product. The plain red line represents the relative bias, the dashed blue lines are the 95% β-expectation tolerance limits, the black dotted curves are the acceptance limits, and the coloured circles represent the relative back-calculated concentrations and are plotted in respect to their targeted concentration. The calibration model used is the weighted (1/X) quadratic regression and the acceptance limits were fixed at ±30%.

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Tables

Table 1: DSD for SFC-MS separation: effect summary and factors estimate.

Source	S-criterion Lumisterol-D_3		S-criterion D_3-Isotachysterol	
	Estimate	*p*-value	Estimate	*p*-value
Intercept	-0.01321	0.2618	0.0415	0.0056*
Temperature (35,50)	0.022	0.0025*	-	-
Final proportion (10,20)	-0.036	0.0002*	-	-
Gradient time (3.5,7.5)	0.022	0.0025*	0.05	0.0040*
Final proportion*Gradient time	-0.01255	0.0657	-	-
Temperature*Temperature	-0.03519	0.0153*	-	-
Gradient time*Gradient time	0.01736	0.1108	-	-
Model adjusted R^2	0.91		0.50	

* Statistically significant effect on response (*p*-value < 0.05)

Table 2: DSD for ESI+ SFC-MS response: effect summary and factors estimate.

Source	Lumisterol S/N		Isotachysterol S/N		Tachysterol S/N		Dihydrotachysterol S/N		Octanoate S/N		Decanoate S/N	
	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value
Intercept	21.58	< 0.0001*	47.71	< 0.0001*	20.23	< 0.0001*	66.02	< 0.0001*	249.33	< 0.0001*	131.23	0.0001*
Capillary voltage (X_1)	-9.83	0.0091*	-9.52	0.0479*	-9.77	0.0013*	-17.60	0.0195*	-136.89	< 0.0001*	-73.31	< 0.0001*
Cone voltage (X_2)	-	-	-13.75	0.0482*	-	-	-0.20	0.9830	-	-	-	-
Desolvation temperature (X_3)	-	-	-	-	-	-	-	-	-39.60	0.0041*	-6.15	0.7675
Desolvation gas flow rate (X_4)	-	-	-2.62	0.2432	-	-	-	-	-	-	-15.36	0.0066*
Cone flow rate (X_5)	-	-	-	-	-	-	2.79	0.4050	-	-	-	-
Make-up flow rate (X_6)	-	-	-	-	-	-	-	-	-	-	-66.12	0.0045*
X_2^2	-	-	-10.72	0.0634	-	-	-22.93	0.0179*	-	-	-	-
X_4^2	-	-	-11.11	0.0718	-	-	-	-	-	-	-53.35	0.0016*
X_1*X_2	-	-	7.39	0.1640	-	-	-5.97	0.4416	-	-	-	-
X_1*X_3	-	-	-	-	-	-	-	-	-	-	29.92	0.0173*
X_3*X_4	-	-	-	-	-	-	-	-	-	-	11.39	0.0424*
X_3*X_6	-	-	-	-	-	-	-	-	-	-	60.10	0.0169*
Model adjusted R^2	0.33		0.53		0.47		0.50		0.74		0.90	

* Statistically significant effect on response (*p*-value < 0.05)

Table 3: Method validation results for ester impurities in drug product.

	Octanoate	Decanoate
Specificity	✓ (m/z 367.40)	✓ (m/z 539.60 / 367.40)
LOD (s/n ≥ 3) (% impurity)	0.2 %	0.2 %
LOQ (s/n ≥ 10) (% impurity)	0.5 %	0.5 %

Table 4: Method validation results for the quantification of transcholecalciferol in drug product.

	Response function (p = 4; m = 4; n = 1)	
Calibration model	Weighted (1/X) quadratic regression	
Calibration levels	62.5 – 125 – 312.5 – 1250 ng/mL	
	Trueness (p = 4; m = 5; n = 3)	
Concentration (ng/mL)	Relative bias (%)	
62.5	-2.35	
125	-1.97	
312.5	0.02	
625	3.78	
1250	0.98	
	Precision (p = 4; m = 5; n = 3)	
Concentration (ng/mL)	Repeatability (RSD (%))	Intermediate precision (RSD (%))
62.5	3.45	9.00
125	2.61	6.13
312.5	2.92	3.58
625	2.50	5.14
1250	2.29	4.51
	Accuracy (p = 4; m = 5; n = 3)	
Concentration (ng/mL)	Relative β-expectation tolerance limits (%)	
62.5	[-31.02 , 26.32]	
125	[-21.04 , 17.11]	
312.5	[-8.81 , 8.85]	
625	[-11.66 , 19.23]	
1250	[-12.39 , 14.35]	
	Linearity (p = 4; m = 5; n = 3)	
Range (ng/mL)	[62.5 – 1250]	
Slope	1.015 (95% confidence interval [0.9988 – 1.032])	
Intercept	-0.9113	
R^2	0.9961	
LOD (ng/mL)	LLOQ (ng/mL)	ULOQ (ng/mL)
7.26	68.86	1249

p: number of series of experiments, m: number of concentration levels, n: number of replicates per concentration levels and per series

Table 5: Method validation results for the quantification of tachysterol in drug product.

	Response function (p = 4; m = 4; n = 1)	
Calibration model	Weighted (1/X) quadratic regression	
Calibration levels	62.5 – 125 – 312.5 – 1250 ng/mL	
	Trueness (p = 4; m = 5; n = 3)	
Concentration (ng/mL)	Relative bias (%)	
62.5	-9.63	
125	-8.47	
312.5	-7.28	
625	-5.17	
1250	-5.30	
	Precision (p = 4; m = 5; n = 3)	
Concentration (ng/mL)	Repeatability (RSD (%))	Intermediate precision (RSD (%))
62.5	3.54	12.43
125	2.82	4.59
312.5	2.53	3.88
625	2.28	3.68
1250	2.24	3.21
	Accuracy (p = 4; m = 5; n = 3)	
Concentration (ng/mL)	Relative β-expectation tolerance limits (%)	
62.5	[-51.15 , 31.89]	
125	[-21.21 , 4.27]	
312.5	[-17.83 , 3.28]	
625	[-15.35 , 5.02]	
1250	[-13.75 , 3.16]	
	Linearity (p = 4; m = 5; n = 3)	
Range (ng/mL)	[62.5 – 1250]	
Slope	0.9509 (95% confidence interval [0.9390 – 0.9630])	
Intercept	-4.195	
R^2	0.9977	
LOD (ng/mL)	LLOQ (ng/mL)	ULOQ (ng/mL)
5.37	105.4	1236

p: number of series of experiments, m: number of concentration levels, n: number of replicates per concentration levels and per series

Table 6: Method validation results for the quantification of dihydrotachysterol in drug product.

	Response function (p = 4; m = 4; n = 1)	
Calibration model	Weighted (1/X) quadratic regression	
Calibration levels	62.5 – 125 – 312.5 – 1250 ng/mL	
	Trueness (p = 4; m = 5; n = 3)	
Concentration (ng/mL)	Relative bias (%)	
62.5	0.7831	
125	4.973	
312.5	-0.4985	
625	2.311	
1250		
	Precision (p = 4; m = 5; n = 3)	
Concentration (ng/mL)	Repeatability (RSD (%))	Intermediate precision (RSD (%))
62.5	2.08	7.76
125	4.40	5.73
312.5	3.33	5.81
625	2.48	2.48
1250	1.73	3.76
	Accuracy (p = 4; m = 5; n = 3)	
Concentration (ng/mL)	Relative β-expectation tolerance limits (%)	
62.5	[-25.32 , 26.88]	
125	[-9.56 , 19.50]	
312.5	[-17.05 , 16.05]	
625	[-3.39 , 8.01]	
1250	[-9.71 , 13.22]	
	Linearity (p = 4; m = 5; n = 3)	
Range (ng/mL)	[62.5 – 1250]	
Slope	1.018 (95% confidence interval [1.005 – 1.031])	
Intercept	-0.2899	
R^2	0.9976	
LOD (ng/mL)	LLOQ (ng/mL)	ULOQ (ng/mL)
2.096	62.80	1256

p: number of series of experiments, m: number of concentration levels, n: number of replicates per concentration levels and per series

Figure 1

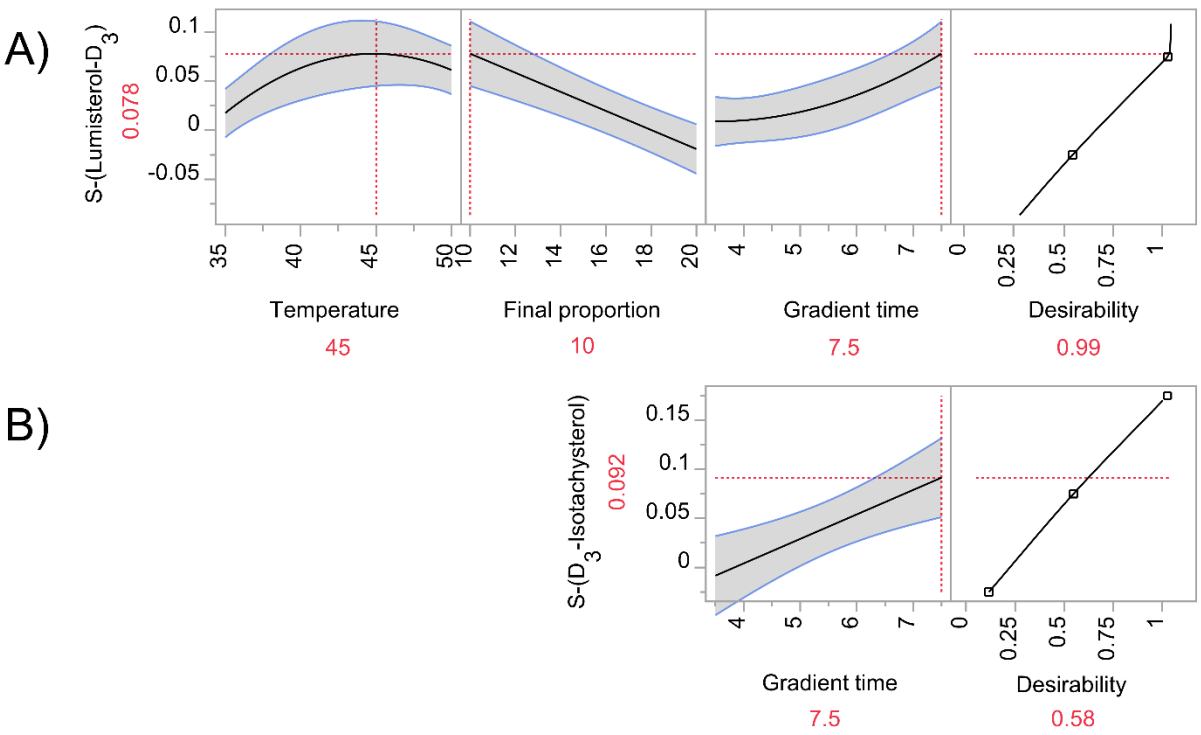

A)
S-(Lumisterol-D3)
0.078
0.1
0.05
0
-0.05
35
40
45
50
10
12
14
16
18
20
4
5
6
7
0
0.25
0.5
0.75
1
Temperature
45
Final proportion
10
Gradient time
7.5
Desirability
0.99
B)
S-(D3-Isotachysterol)
0.092
0.15
0.1
0.05
0
4
5
6
7
0
0.25
0.5
0.75
1
Gradient time
7.5
Desirability
0.58

Figure 2

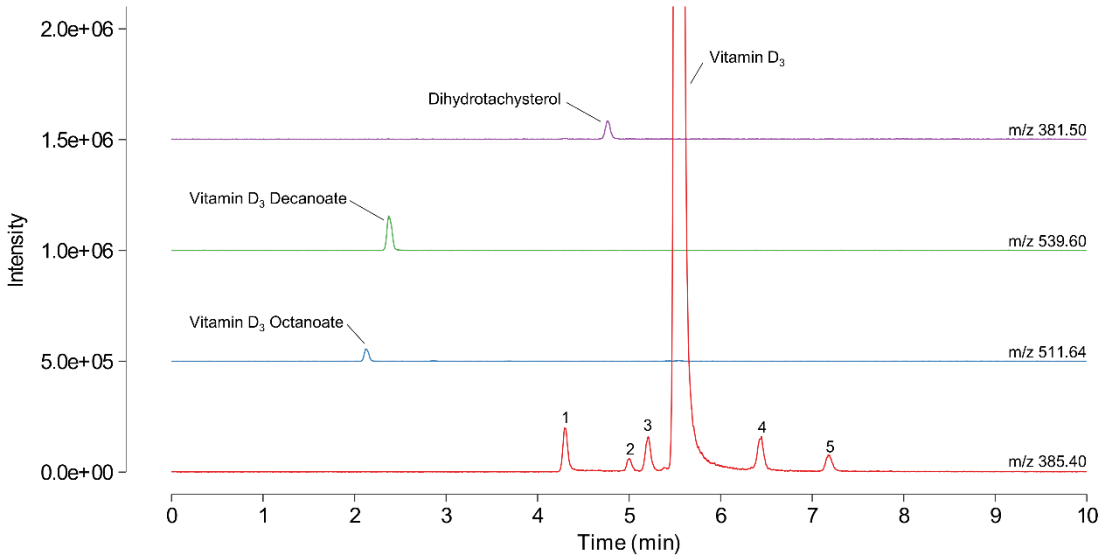

2.0e+06
1.5e+06
1.0e+06
5.0e+05
0.0e+00
Intensity
Vitamin D3
Dihydrotachysterol
m/z 381.50
Vitamin D3 Decanoate
m/z 539.60
Vitamin D3 Octanoate
m/z 511.64
1
2
3
4
5
m/z 385.40
0
1
2
3
4
5
6
7
8
9
10
Time (min)

Figure 3

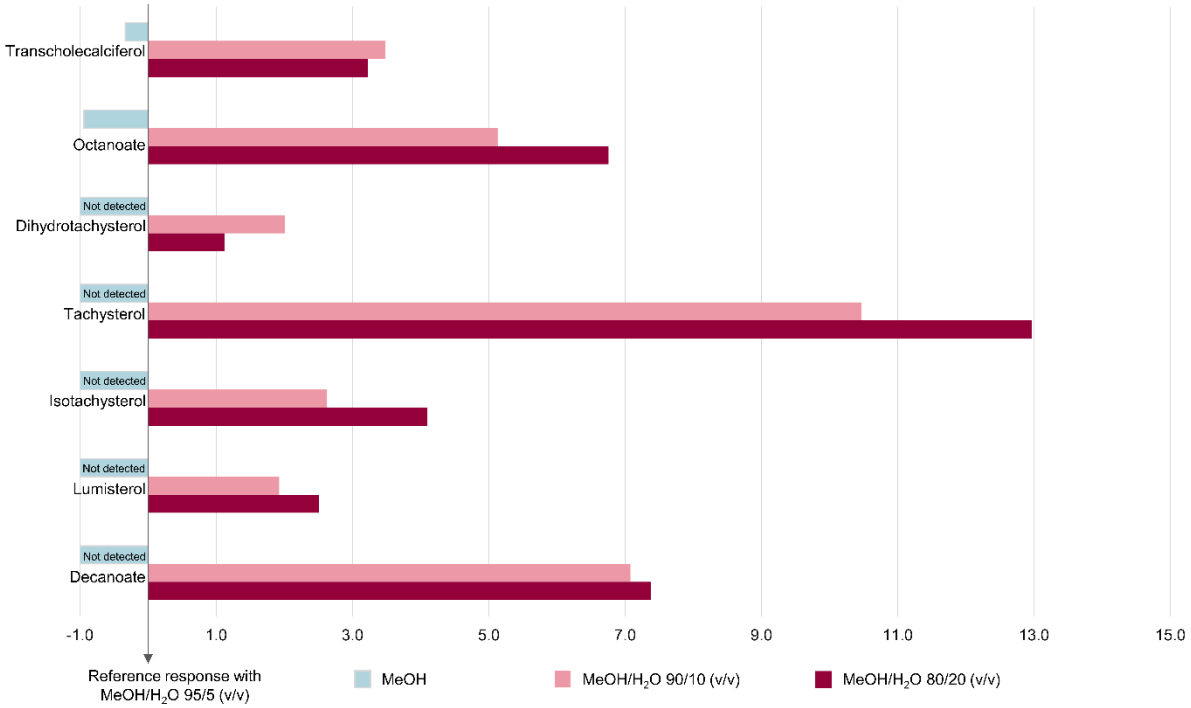

Transcholecalciferol
Octanoate
Not detected
Dihydrotachysterol
Not detected
Tachysterol
Not detected
Isotachysterol
Not detected
Lumisterol
Not detected
Decanoate
-1.0
1.0
3.0
5.0
7.0
9.0
11.0
13.0
15.0
Reference response with MeOH/H2O 95/5 (v/v)
MeOH
MeOH/H2O 90/10 (v/v)
MeOH/H2O 80/20 (v/v)

Figure 4

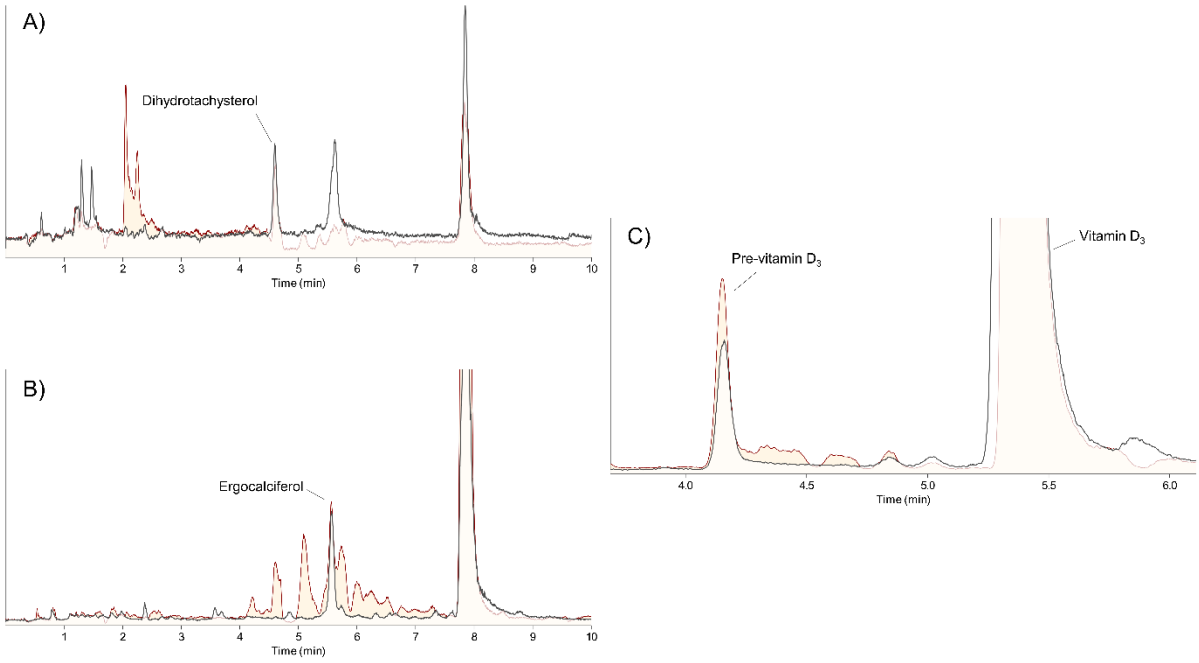

A)
Dihydrotachysterol
1
2
3
4
5
6
7
8
9
10
Time (min)
B)
Ergocalciferol
1
2
3
4
5
6
7
8
9
10
Time (min)
C)
Pre-vitamin D3
Vitamin D3
4.0
4.5
5.0
5.5
6.0
Time (min)

Figure 5

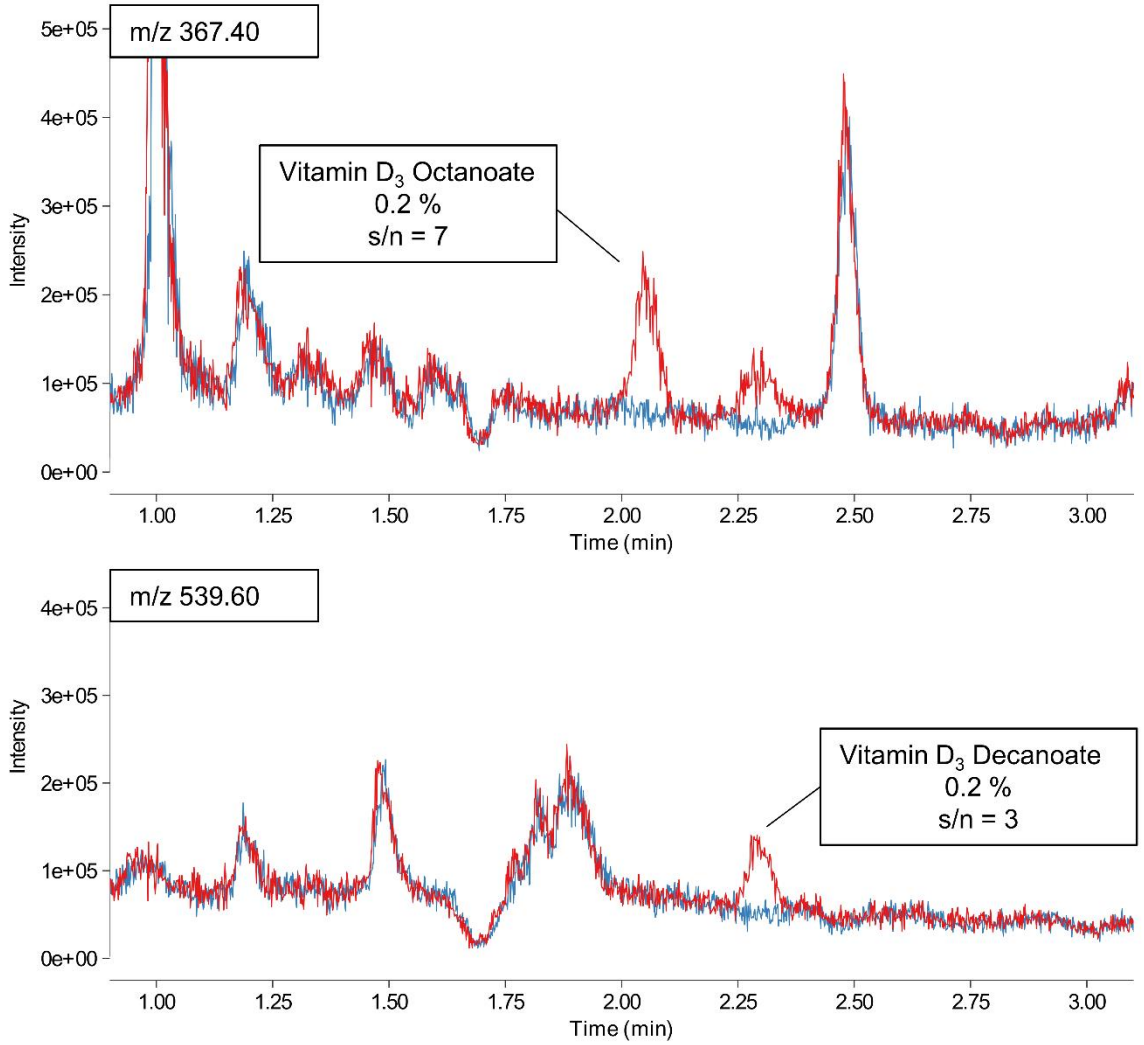
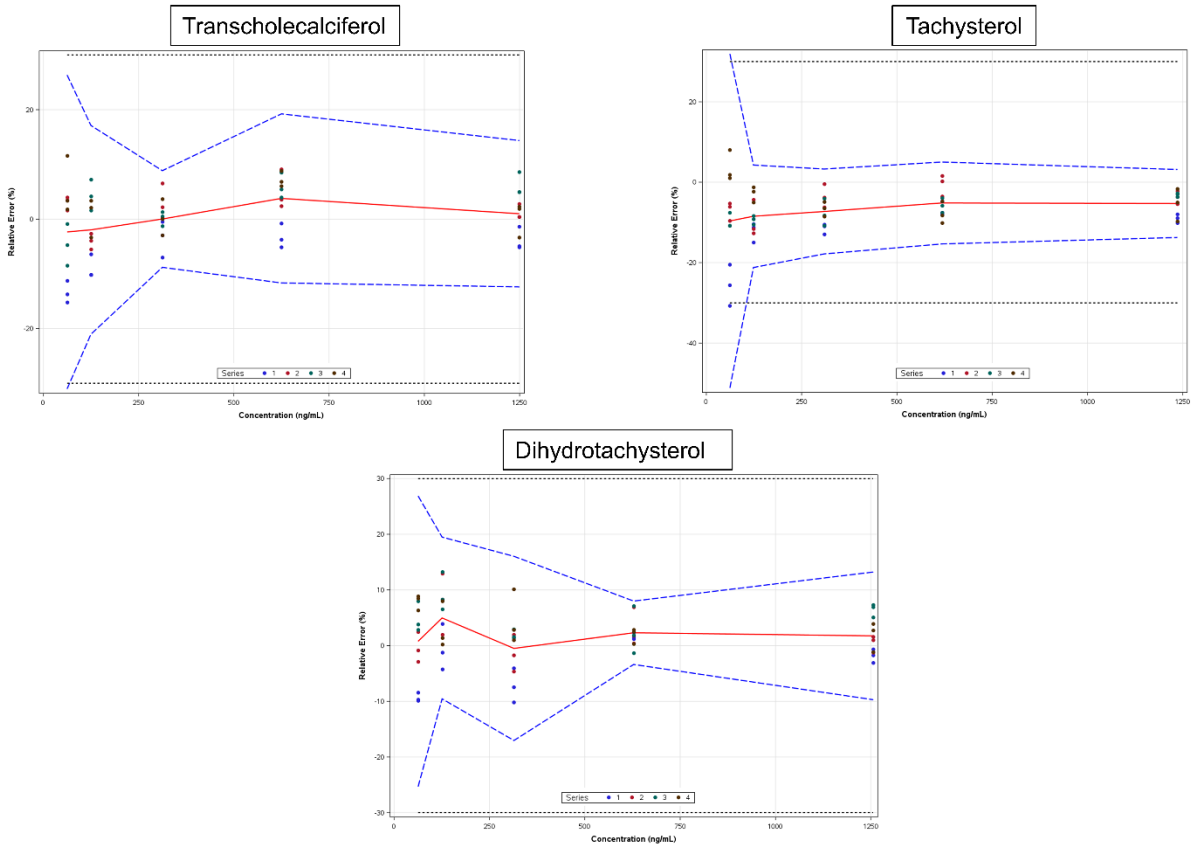

m/z 367.40
Vitamin D3 Octanoate
0.2 %
s/n = 7
Intensity
5e+05
4e+05
3e+05
2e+05
1e+05
0e+00
1.00
1.25
1.50
1.75
2.00
2.25
2.50
2.75
3.00
Time (min)
m/z 539.60
Vitamin D3 Decanoate
0.2 %
s/n = 3
Intensity
4e+05
3e+05
2e+05
1e+05
0e+00
1.00
1.25
1.50
1.75
2.00
2.25
2.50
2.75
3.00
Time (min)

Figure 6


Transcholecalciferol
Relative Error (%)
Concentration (ng/mL)
Series 1 2 3 4
Tachysterol
Relative Error (%)
Concentration (ng/mL)
Series 1 2 3 4
Dihydrotachysterol
Relative Error (%)
Concentration (ng/mL)
Series 1 2 3 4