

OPTIMIZATION AND VALIDATION OF A FAST SUPERCRITICAL FLUID CHROMATOGRAPHY METHOD FOR THE QUANTITATIVE DETERMINATION OF VITAMIN D3 AND ITS RELATED IMPURITIES

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KEYWORDS

SFC ; Green analytical chemistry ; Vitamin D3 ; Method validation ; Accuracy profile ; Design space

ABSTRACT

In the uprising context of green analytical chemistry, Supercritical Fluid Chromatography (SFC) is often suggested as an alternative to Normal Phase Liquid Chromatography. Indeed, SFC provides fast, efficient and green separations. In this report, the quantitative performances of SFC were challenged on a real-life case study: the Quality Control (QC) of vitamin D3. A rapid and green SFC method was optimized thanks to the Design of Experiments–Design Space (DoE–DS) methodology. It provided robust and high quality separation of the compounds within a 2 min timeframe, using a gradient of ethanol as co-solvent of the carbon dioxide. The analytical method was fully validated according to the total error approach, demonstrating the compliance of the method to the specifications of U.S. Pharmacopeia (USP: 97.0–103.0%) and European Pharmacopeia (EP: 97.0–102.0%) for an interval of [50–150%] of the target concentration. In order to allow quantification of impurities using vitamin D3 as an external standard in SFC-UV, correction factors were determined and verified during method validation. Thus, accurate quantification of impurities was demonstrated at the specified levels (0.1 and 1.0% of the main compound) for a 70.0–130.0% dosing range. This work demonstrates the validity of an SFC method for the QC of vitamin D3 raw material and its application to real samples. Therefore, it supports the switch to a greener and faster separative technique as an alternative to NPLC in the pharmaceutical industry.

1. Introduction

Nowadays, the cholecalciferol or vitamin D3 arouses lot of interests among the scientific community [1], [2], [3], [4]. Indeed, it is involved in several health benefits [5], [6], [7] such as supporting the bone health [8], helping the brain [9] and immune system [10] or lowering the risk of chronic diseases and cancers [11], [12]. Despite of its numerous benefits, a worldwide global deficiency is currently described for children and adults [13]. Indeed, its endogenous synthesis is not sufficient to provide satisfying levels of vitamin D3 for the large extent of the population [14], [15]. Consequently, dietary supplements or medicines containing vitamin D are prescribed to patients upon regular basis [16]. To ensure the quality of these various products, the quality control of the active ingredient is obviously the first mandatory step. For that purpose, manufacturers may refer to different monographs from pharmacopeia (e.g. United States Pharmacopeia (USP) [16], European Pharmacopeia (EP) [17]). Among the panel of tests proposed, EP still recommends a Normal Phase Liquid Chromatography (NPLC) to assess the cholecalciferol's purity. Due to its long analysis runtime (40 min) and the subsequent large consumption of toxic solvents, a faster and greener method would be helpful to perform this control on a routine basis.

Since few years, Supercritical Fluid Chromatography (SFC) is making a remarked comeback on the front scene of analytical sciences. Indeed, the analysts devote great interest to the modern version of SFC, which provides repeatable results and involves robust instruments. Moreover, the manufacturers have minimized void volumes, which therefore allows the use of columns packed with sub-2 μm particles with a minimal chromatographic band broadening [18]. Typically, performances achieved by these SFC instruments are higher and belong to the Ultra High Performance range (i.e. UHPSFC) [19]. Among the many novel applications of (UHP)SFC, most of them are issued from the pharmaceutical field and were reviewed by Desfontaine et al. [20]. Given its low viscosity and high diffusivity, the carbon dioxide based mobile phase allows high flow rates on sub-2 μm stationary phases. Combination of these two properties obviously provides SFC users with a fast and efficient technique, which is strongly suggested as a serious alternative to NPLC [21]. Indeed, it is known that supercritical carbon dioxide has a polarity close to n-hexane [22]. Consequently, the separation obtained using polar stationary phase (e.g. a bare silica) could be achieved by using supercritical mobile phase instead of n-hexane [23], [24]. Nevertheless, most of SFC publications were up to now mainly focused on fundamental aspects [25] and only a limited number of works reports quantitative analysis in SFC [26] demonstrating its ability to advantageously replace a NPLC method.

Since 2002, SFC instruments have a dedicated monograph in the European Pharmacopeia (4th edition – to now) but to the best of our knowledge, it seems that the technique is not yet applied. Also, regarding the applicability of the technique for fat soluble vitamins (vitamin A, D & E), recent publications reported their separation and quantification by SFC-MS in biological fluids [27], [28], [29] or SFC-UV in pharmaceuticals [30]. For vitamin D3 and its related impurities, some application notes already issued [31], [32] but none of them were validated and they didn't use the sub-2 μm packed columns available. In this context, our goal was to develop a new robust SFC method for the

quantitative determination of vitamin D3 and its related impurities. Such method would be a fast and eco-friendly alternative to the existing NPLC methods presented by pharmacopoeias. The present study was then completed by the demonstration of the reliability of the proposed method for its intended purpose: the reliable determination of vitamin D3 in SFC.

To reach this objective, the SFC method was firstly optimized in terms of separation by combining design of experiments (DoE) methodology [33], [34], [35] and Design Space (DS) concept [36], [37], [38]. Such approach permitted robust optimization of the analytical method in compliance with the current concept of Quality by Design (QbD) [39], [40] which is strongly supported by the Food and Drug Administration (FDA) [41], [42].

As the quantitative performances of the SFC method have to be better or at least similar to those of the monographs, the proposed method was challenged by validation. A total error approach based on β -expectation tolerance intervals [43], [44], [45], [46] was employed, which allowed to evaluate and control the level of risk associated to further use during routine analysis.

2. Materials and methods

2.1. CHEMICALS AND REAGENTS

Cholecalciferol (D3; 99.0%) and 7-dehydrocholesterol (PRO; 98.8%) were purchased from Sigma Aldrich (St Louis, MO, USA). 5,6-*trans*-cholecalciferol (TRANS; 99.1%) was provided by Dalton Chemical Laboratories Inc. (Toronto, Canada). Pre-cholecalciferol (PRE; >80%) was sourced from Toronto Research Chemicals Inc. (Toronto, Canada).

Ethanol (HPLC gradient grade) was supplied by Merck Millipore (Darmstadt, Germany). Methanol and *n*-heptane (HPLC grades) was purchased from J.T. Baker (Deventer, Netherlands). 2-propanol (MS grade) was sourced from Biosolve BV (Valkenswaard, Netherlands). Carbon dioxide grade 4.5 (99.995%) was purchased from Westfalen BVBA (Aalst, Belgium).

2.2. SFC INSTRUMENT

A Waters Acquity UPC²™ system (Waters Corp., USA) was used to carry out the whole study. This system is composed of a binary solvent manager with the standard mixing chamber (250 μ L), a Sample Manager equipped with a 10 μ L loop, working in partial loop operating in the needle overfill mode and using methanol and 2-propanol as strong and weak needle wash respectively, a back pressure regulator (BPR), a column manager with active preheaters and a PDA detector (262 nm detection) equipped with the high pressure stainless steel 8 μ L (10 mm path) flow cell. The analytical column employed in this study was an Acquity UPC² BEH (3.0 $\times$ 100 mm; 1.7 μ m) purchased from Waters.

2.3. SAMPLE PREPARATION

2.3.1. SCREENING SAMPLES

A solution of the four compounds (PRE, PRO, TRANS & D3– $\approx 200 \mu\text{g mL}^{-1}$ each) was realized in a 99:1 (v/v) mix of *n*-heptane and 2-propanol. This solution was used to assess different organic modifiers and backpressure settings.

2.3.2. OPTIMIZATION DESIGN SAMPLES

For the Design of Experiments, the samples were prepared each days, (i.e. three times), to obtain three independent solutions containing the four compounds ($200 \mu\text{g mL}^{-1}$ each- in pure *n*-heptane). For each series of experiments, fresh working solutions were prepared, meaning that 9 independent samples (divided in 3 series) were used for the complete DoE.

2.3.3. UV CORRECTION FACTOR SAMPLES

For the study of the UV spectra in order to set up a correction factor, samples were prepared identically and as following: 2.5 mg of compound (PRO/TRANS/D3) accurately weighted in 5.0 mL volumetric flasks and dissolved with pure *n*-heptane. Individual working sample solutions were obtained by dilution of the stock solutions to 62.5, 125 and $187.5 \mu\text{g mL}^{-1}$.

2.3.4. VALIDATION PROTOCOL

All the calibration and validation standards were dissolved and/or diluted with pure *n*-heptane. To ensure proper and precise handling of the volatile organic solutions, positive displacement automatic pipettes were purposely used. All the solutions were prepared protected from light to avoid degradation of the photosensitive compounds.

Four series of the different compounds (PRO/TRANS/D3) were performed. The validation of pre-cholecalciferol determination was not performed because it is i) highly instable and easily transformed to vitamin D3 [40] with potential bias issues, ii) expensive and difficult to source.

2.3.4.1. Calibration standards

For the Calibration Standard (CS) of vitamin D3, one stock solution was freshly prepared for each validation series. The stock solution was made with 20.0 mg of vitamin D3 accurately weighted and dissolved in 10.0 mL volumetric flask. The stock solutions were diluted to get 50, 100 and $150 \mu\text{g mL}^{-1}$ in 10.0 mL volumetric flasks.

For TRANS and PRO, a fresh stock solution was also prepared for each validation series: 400 μg of vitamin D3–accurately weighted by means of a microbalance- was dissolved in a 20.0 mL volumetric flask. This stock solution was diluted upon request to obtain concentrations of 2, 6 and $20 \mu\text{g mL}^{-1}$.

2.3.4.2. Validation standards

For the Validation Standards (VS) of vitamin D3, three independent stock solutions were freshly prepared for each validation series by means of independent weightings. These stock solutions were

made in the same way as the CS, which provided samples diluted to get 50, 100 and 150 $\mu\text{g mL}^{-1}$ for each of the independent solutions.

For the VS of TRANS & PRO: three fresh independent stock solutions were prepared for each validation series. These stock solutions were made by dissolving 260 μg of TRANS and 2600 μg of PRO –accurately weighted with a microbalance- in 10.0 mL volumetric flasks. Both compounds were dissolved in pure *n*-heptane and sonicated for 2 min to ensure proper dissolution of the pro-vitamin D3. The stock solutions were diluted to get 1.4, 2.0 and 2.6 $\mu\text{g mL}^{-1}$ and 14, 20 and 26 $\mu\text{g mL}^{-1}$, respectively for trans- and pro-vitamin D3.

2.4. ANALYTICAL METHOD

2.4.1. EXPERIMENTAL PARAMETERS SCREENING

Different organic modifiers, namely methanol and ethanol were employed in isocratic mode. The mobile phase was composed of a 98:2 (v/v) mixture of carbon dioxide and organic modifier. The flow rate was set constant at 2 mL min^{-1} . Column and autosampler temperatures were fixed to 40 °C and 6 °C, respectively. The injection volume was 0.5 μL .

2.4.2. METHOD OPTIMIZATION DESIGN

A three factors Central Composite Design (CCD) with three randomized orthogonal blocks and triplicate central points was used to optimize the analytical method. The selected design involved 35 experiments and allowed to take account of the potential interactions. The experiments were distributed in three series, which took place during non-consecutive days thanks to method's short runtime. Thus, variance of the instrument (potential intra and extra-days effects) and method were taken into account at the onset of the development.

Development was focused on the separation of the critical pair of peaks formed by pre- and *trans*-cholecalciferol. This required optimization of the gradient profile while limiting density and eluotropic strength of the mobile phase. In this context, the temperature (*T*), the gradient final composition at the end of gradient time (P_{EtOH}) and gradient time (t_{G}) were selected as factors of the DoE (Table 1). The separation of the critical pair of peaks was monitored under the different experimental conditions. Other parameters were kept constant (BPR (110 bar), modifier (ethanol), wash solvents (methanol & 2-propanol) and injection volume (0.5 μL)). At the end of the gradient, the proportion of ethanol was held for one minute (i.e. a one minute isocratic plateau to wash the column). Then, the column was re-conditioned to initial conditions for 30 s prior to the next injection.

Table 1. Experimental parameters and levels of the parameters used for the DoE.

DoE Parameter:	Temperature (°C)	Gradient time (min.)	Ethanol proportion (% (v/v))
Level:			
Minimum	35.0	1.0	10.0
Low intermediate	39.0	1.4	11.0
Central	45.0	2.0	12.5
High intermediate	51.0	2.6	14.0
Maximum	55.0	3.0	15.0

2.5. MODELLING AND OPTIMIZATION

2.5.1. RETENTION TIME MODELLING

As described previously by Lebrun et al. [48] and recently applied to SFC by Dispas et al. [49], [50], retention times at the beginning, apex and end (t_b , t_r and t_e , respectively) of the four peaks corresponding to the compounds of interest were recorded in the different experimental conditions of the DoE. The studied response were the normalized retention times termed k and calculated according to $k = (t_x - t_0)/t_0$ where t_x is a given retention time (e.g. t_b) and t_0 is the column dead time. Logarithmic transformations of the different responses (i.e. $\log t_b$) were modelled with a multivariate polynomial equation (see Section 3.1.2.1) and stepwise regression was used to maximize the adjusted determination coefficient of the model, which allowed high quality predictions of the retention times.

2.5.2. DS COMPUTATION

The model of the retention was used to predict the retention of the beginning, apex and end of the different peaks. A quality criterion, also known as Critical Quality Attribute (CQA) was employed to estimate the quality of the chromatographic results. Separation (S) is defined as the difference between the beginning of a peak and the end of the previous one (e.g. $S = t_{b,Trans-} - t_{e,Pre-}$). In this case, critical separation (minimal separation between the four peaks: $S > 0$) was used as CQA. If S is positive ($S > 0$), it means that all peaks are baseline-resolved. PRE and TRANS are the two peaks less resolved on the majority of the chromatograms, therefore forming the critical pair in most of the case.

Prediction error obtained for the model were propagated to the CQA and then, as described by Lebrun & Debrus et al. [48], [51], MonteCarlo simulations were applied to obtain, for any experimental condition, the predictive distribution of S according to $t_{e,Pre-}$ and $t_{b,Trans-}$. On this basis, DS was computed taking into account the posterior probability of having S superior to a certain quality level, which in mathematical terms corresponds to:

$$DS = \{x_0 \in \chi | E_{\theta}[P(S \geq \lambda) | \theta, \text{data}] \geq \pi\} \quad (1)$$

Where x_0 is a given point of the experimental domain (χ), λ is the acceptance limit for the criterion S (e.g. here $\lambda = 0$), θ is the set of estimated parameter for the model established and π the quality level threshold, while P and E account respectively for the probability and the mathematical expectations. Thus, given the observed data, the Design Space defines a zone of the experimental domain where the assurance of quality is provided. Indeed, in this sub-space of the experimental domain, it exists a probability to reach the desired achievement of the chromatographic method: the separation of all peaks and especially the critical ones (pre- & *trans*-cholecalciferol) at a pre-defined level of quality.

2.6. SOFTWARE

UPC² instrument and data handling was operated with Empower 2 (build 2154) from Waters. JMP 10.0 (SAS Institute, Cary, NC, USA) for Apple Mac OS X and R (v 3.1.3) (The R project for Statistical Computing, available as a free-ware at <https://www.r-project.org>) were used to carry out the statistical analysis. Analytical method validation and accuracy profile were computed using Enoval 3.0 (Arlenda sa, Liège, Belgium).

3. Results and discussions

3.1. ANALYTICAL METHOD DEVELOPMENT AND OPTIMIZATION

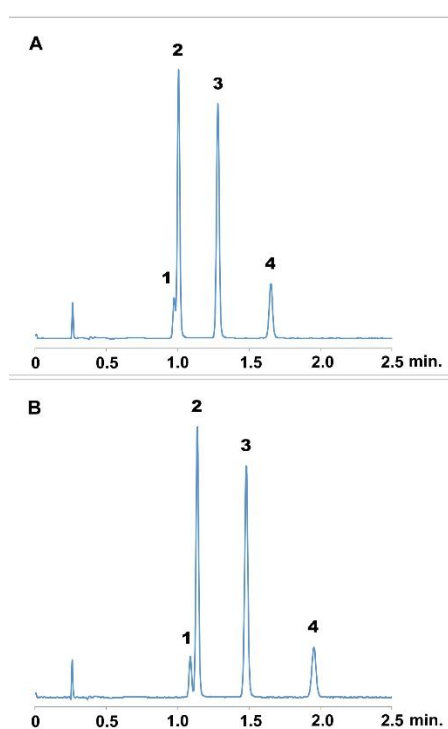
3.1.1. EXPERIMENTAL PARAMETERS SCREENING RESULTS

At first, total co-elution of the critical pair of peaks (PRE & TRANS) was observed in presence of methanol when the backpressure value was set to 150 bar. When BPR set up was reduced to lower value (110 bar), the separation was improved (Figure 1a) but still required improvement through some optimization (e.g. assessment of a weaker modifier). This observation suggests to control and limit mobile phase's elutropic strength and density for the separation of those lipophilic compounds in these experimental conditions. So, ethanol was assessed on the Acquity UPC² BEH column, which improved the resolution of the critical pair (Figure 1b).

Given its overall superiority (overall greenness, good peaks shape, fast runtime), pure ethanol was selected as organic modifier. Due to elutropic strength and density dependence of the separation, a solvent gradient elution was required to separate the critical pair and provide fast analysis. DoE-DS methodology was used to determine the optimal parameters: proportion of ethanol, gradient time and column temperature. In the DoE, the effect of the pressure in itself was not investigated because i) the separation of some peaks was decreased while the BPR set up was increased, ii) in these conditions where the fraction of organic modifier is reduced and the flow rate is fixed to 2 mL min⁻¹, the pressure is slightly impacted by the temperature (i.e. pressure decreases when temperature increases). So, the variation of the temperature allows some kind of fine-tuning of the

pressure in the same way. For the DoE, a maximal temperature of 55 °C was used to improve the lifetime of the analytical column. A low temperature also limits the compressibility of the mobile phase, which would also improve the robustness of the future method.

Figure 1. Chromatograms obtained in isocratic mode (2% (v/v) of organic modifier in mobile phase); flow rate = 2 mL min⁻¹ and BPR setting = 110 bar. Chromatogram (A) is obtained with pure methanol as co-solvent; for chromatogram (B) pure ethanol was used as co-solvent. The elution order is 1: PRE; 2: TRANS; 3: D3 and 4: PRO on both chromatograms (A & B). Although still not optimal, it demonstrates the benefits of the use of a lesser polar modifier to enhance the separation of PRE and TRANS.



3.1.2. METHOD OPTIMIZATION DOE

3.1.2.1. Response modelling

The basis of the empirical models of retention is the polynomial Eq. (2), which takes into account significant effects of the DoE's factors and their interactions:

$$\text{Log}k_{\text{tr}} = \beta_0 + \beta_1 \cdot T + \beta_2 \cdot P_{\text{EtOH}} + \beta_3 \cdot tG + \beta_4 \cdot T^2 + \beta_5 \cdot P_{\text{EtOH}}^2 + \beta_6 \cdot tG^2 + \beta_7 \cdot T \cdot P_{\text{EtOH}} + \beta_8 \cdot T \cdot tG + \beta_9 \cdot P_{\text{EtOH}} \cdot tG + \varepsilon \quad (2)$$

where $\beta_0, \beta_1, \dots, \beta_9$ accounts for the estimated parameters of the model and ε is the estimated error of the model. Stepwise regression allowed maximization of the R^2_{adj} for each of the twelve (3 retention times $\times$ 4 compounds) models of retention time. Thus allowing building high quality model of the retention times. On Figure 2 (top), good agreement between the predicted and observed

retention times resulting from the developed model were found. Also, the residuals of the models are comprised within a narrow interval (Figure 2-bottom). As residuals account for the variability of experiments and models, it is thus reasonable to assume that retention time prediction error is really low over the whole experimental domain.

Thanks to these models, it is possible to follow the behaviour of the different retention times for each of the parameters investigated with the DoE (percentage of modifier at the end of gradient time, temperature and gradient time). So, regarding the final percentage of ethanol (Figure 3a), it appears that modifiers proportions higher than $\approx 13.5\%$ would provide low S values for the critical pair (e.g. $t_{e,Pre} \approx t_{b,Trans}$). Also, in terms of retention times, the pro-vitamin D3 varies the most with the percentage of modifier. Indeed, pro-vitamin D3 is the more polar compound and thus, the last eluted from the polar column (BEH: bare hybrid silica).

Higher column temperature decreases the speed of the experiments by increasing the retention of all peaks. This is related to the lowered elutropic strength of the mobile phase at higher temperature (lower density of the mobile phase). Figure 3b also shows that in spite of having significant effect on retention time, the column temperature doesn't really impact the selectivity of this method. Thus, the different retention times have similar and parallel evolution when the temperature is modified.

For the case of gradient time (Figure 3c), deleterious effect of short gradient time on critical pair separation was observed. Indeed, critical pair is prone to co-elute when gradient slope is too steep (< 1.5 min). However, according to the models, longer gradient time favours enlargement and tailing of peaks.

Figure 2. Results of the retention modeling with predicted vs. observed retention time (top) and residuals of the model (bottom).

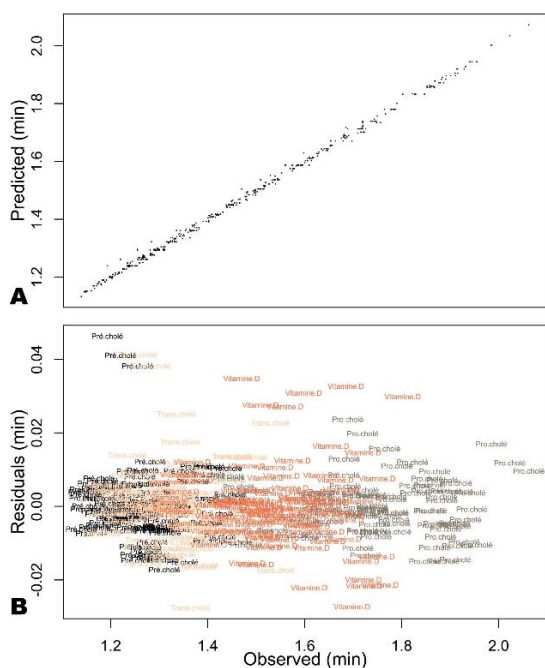
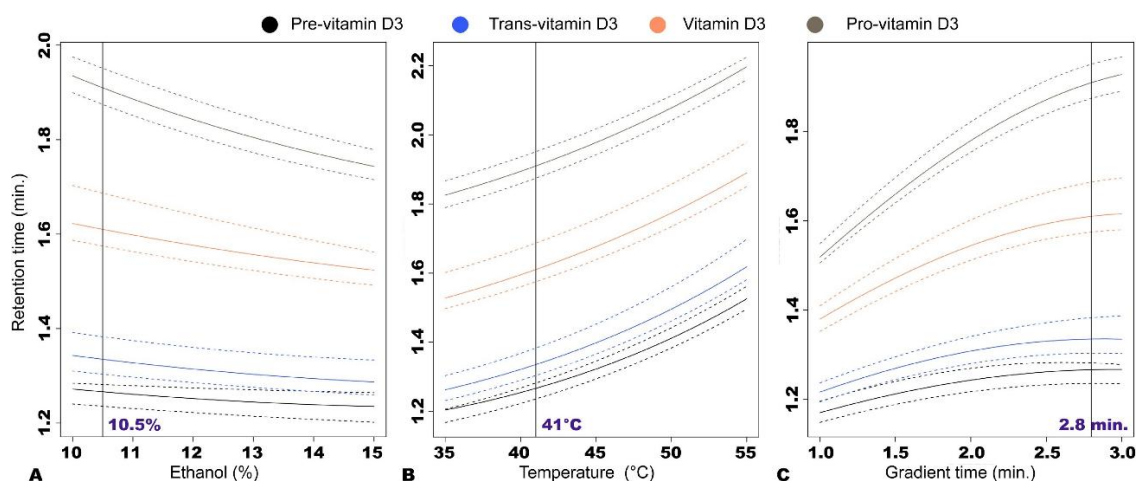


Figure 3. Models of the retention of the compound according the three parameters assessed during the DoE. The different figures and curves express the evolutions of retention time according to one of the experimental parameter monitored. To each slice of these figures corresponds a predicted chromatogram in the experimental conditions defined. The vertical black line refers to the optimal values defined (dark blue) for the experimental parameters studied. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



3.1.2.2. Optimal experimental parameters

On the basis of the different model established, DS was computed with the CQA $S > 0$ for the different peaks (Figure 4). It allowed the identification of an optimal separation of the four compounds of interest when $P_{\text{EtOH}} = 10.5\%$, $T = 41^\circ\text{C}$ and $t_G = 2.8$ min. In these conditions, the minimal quality level, π , was found to be equal to 0.895, which indicates a probability close to 90% to obtain the baseline separation under the defined experimental conditions. This therefore guarantees high quality and robustness for further use of the analytical method. Indeed, ICH affirms that “*working within the design space is not considered as a change to the procedure*” [52]. So, it means that small variation of the experimental parameters inside the white zone defined in Figure 4 will provide results of similar quality. For instance, it is possible to use the column at a lower (e.g. 38°C) or higher (e.g. 42°C) temperature and obtain similar quality of separation. Also, under these optimal conditions, a good agreement was found between the predicted and actual chromatogram (Figure 5). Retention time of the last eluted peak (pro-D3) is a little bit longer than predicted due to the higher error/variability of prediction.

Figure 4. Probability surfaces $P(S > 0 \text{ min})$ for the critical pair separation: a) ethanol percentage vs. gradient time (min), b) ethanol percentage vs. temperature ($^{\circ}\text{C}$) and c) gradient time (min.) vs. temperature. The DS ($\pi = 89.5\%$) is the white zone encircled on the different probability maps. (For the interpretation of the colours, the reader is kindly referred to the online version of this article).

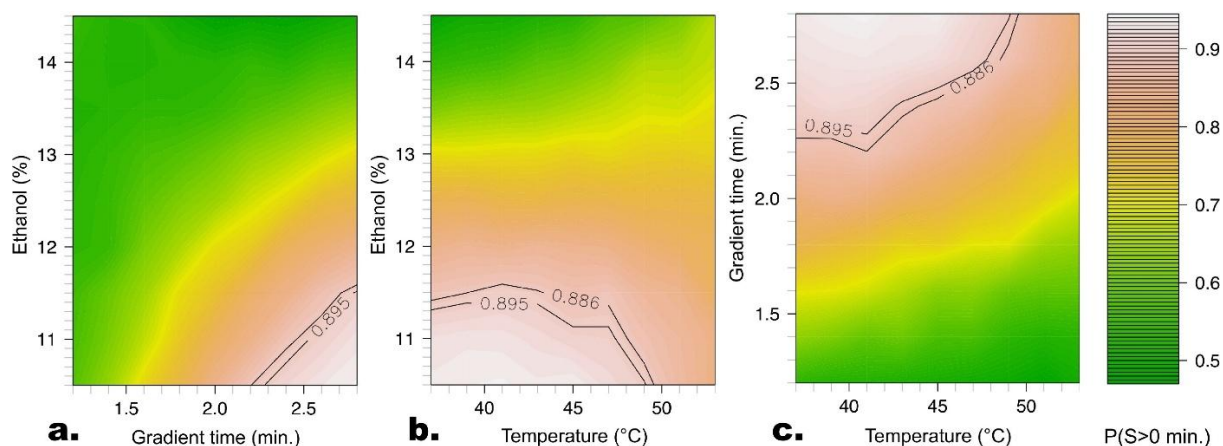
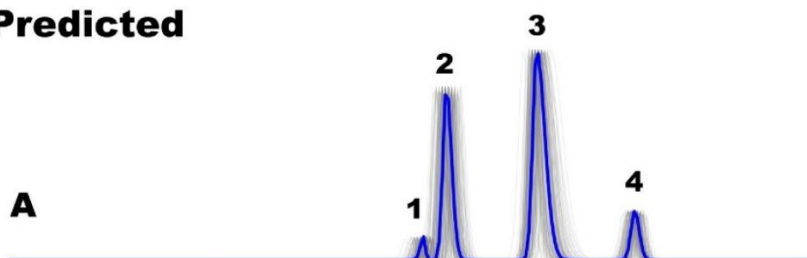
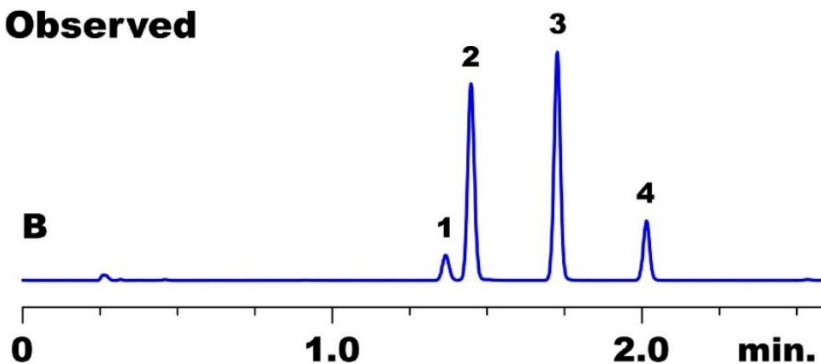


Figure 5. Predicted chromatogram (a.) with predictive uncertainties (grey shaded area) vs. actual chromatogram (b.) observed in the optimal experimental conditions defined with DoE-DS. Elution order is 1: PRE; 2: TRANS; 3: D3 and 4: PRO on both chromatograms.

Predicted



Observed



3.2. STUDY OF THE RESPONSE IN SFC-UV

3.2.1. DETERMINATION OF THE RESPONSE OF THE COMPOUNDS

Usually, in monographs, the impurities are quantified according to the analytical response of the main compound (e.g. “Impurity A: not more than the area of the principal peak in the chromatogram obtained with the reference solution (c) (0.1 per cent)” [17]).

However, it was observed with the samples used for the DoE that similar concentrations of these compounds did not provide peak with equivalent areas (Figure 5). Thus, it means that normalization on the basis of peak area is not applicable. Therefore, prior to the validation of the analytical method, UV response factor of trans- and pro-vitamin D3 were defined according to the response of the main compound.

Regarding pre-cholecalciferol, the reduced peak area may be explained by the instability of the compound, which cause the formation of vitamin D3 [47], [53]. To limit the reaction, this compound has to be stored at a temperature of -80°C , whereas the pro-/trans-vitamin D3 are stored at upper temperatures (i.e. -20°C).

Regarding pro-D3, its structural difference may explain the low UV response. Indeed, pro-D3 has a steroid backbone while the three other compounds are seco-steroids (opening of the 1–3 cyclohexadiene ring under action of light [54]). It was also demonstrated that the molar absorption coefficient of a solute in SC-CO₂ is dependent of the density of the fluid [55], [56], [57]. However in the optimal conditions, the fluid density is quite low. Thus, Pro-D3, which has a higher polarity than the three other compounds, may have a limited solvation, which may contribute to decrease the UV response of this compound.

To determine these correction factors, each of the three compounds was injected at three concentration levels. In order to ensure precision of the injection according to the performances of the injector (RSD = 0.76% for 6 injections (2.0 μL) of vitamin D3 (2 $\mu\text{g mL}^{-1}$)), the injection volume was raised to 2.0 μL without issues thanks to the robust optimization (Figure 6) and high performances of the method. To take into account potential instability of the solutions and avoid bias of the results, special attention was devoted to the injection sequence. Samples were injected by cycles of increasing concentrations (3 levels). Based on the short runtime of the method, less than 3 h was needed for the repetition of 10 cycles (30 injections). During this period, concentrations of the solutions were found to be stable (Supplementary material Figure 1) and it was not possible to evidence any carry-over (assessed against blank injection of pure n-heptane; data not shown).

A calibration curve for the vitamin D3 was determined on the basis of the response recorded (262 nm) for each samples. The slope of this curve was compared to the slope of the calibration curves of pro- and trans- vitamin D3. Thus, it was possible to express the slope of the curve of the two impurities according to the response of vitamin D3. These data are summarized in Table 2. Figure 7 presents the plot of the responses according to the UV response of the main compound.

Figure 6. Chromatogram corresponding to a 2.0 μL injection of a 2 mg mL^{-1} solution of vitamin D3. This sample also contains the maximal allowed amount of trans-vitamin D3 and pro-vitamin D3. The peaks have the same nomenclature as used in Figure 5.

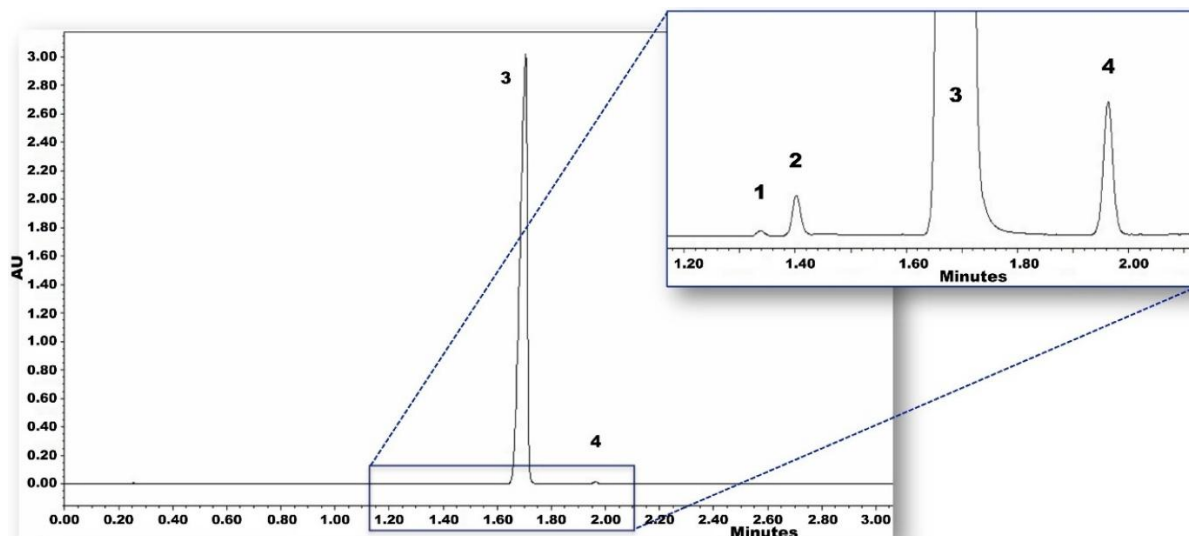
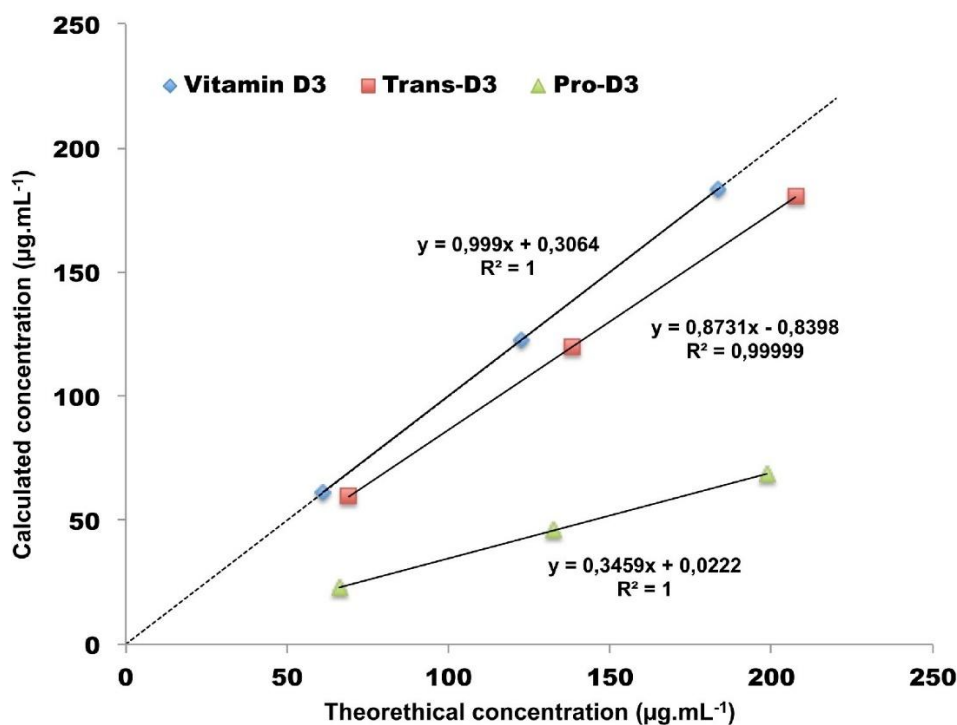


Table 2. Summary of the SFC-UV responses and correction factors for Trans- & Pro-vitamin D3.

Compound	Vitamin D3	Trans-vitamin D3	Pro-vitamin D3
Slope	0.999	0.8731	0.3459
Intercept	0.3064	-0.8398	0.0222
R ²	1.0000	0.9999	1.0000
RSD (%)	<i>n</i> = 10	<i>n</i> = 10	<i>n</i> = 10
Level 62.5 $\mu\text{g mL}^{-1}$	0.5004	0.3207	0.7339
Level 125 $\mu\text{g mL}^{-1}$	0.3437	0.3646	0.3900
Level 187.5 $\mu\text{g mL}^{-1}$	0.3091	0.1674	0.3510
Correction factor:	/	1.1453	2.8910

n = number of replicates per concentration level.

Figure 7. Representation of the theoretical vs. observed concentrations for Trans- & Pro-vitamin D3 according to the UV response of Vitamin D3. The different slopes observed on this plot are explained by the UV response of each compound in SFC-UV. Dashed line serves as a reference and has a slope equal to 1.00. Equations of the curves are summarized in Table 2.



3.2.2. DETERMINATION OF THE CORRECTION FACTOR FOR PRO-AND TRANS-VITAMIN D3

Due to the significant differences of UV responses, it was necessary to determine a correction factor to quantify the impurities according to vitamin D3, as it is mentioned in monographs. On the basis of the pre-established model of the UV response, the correction factor was calculated as the inverse of the slope of the linearity curve. Such approach has already been described in the AFNOR guidelines [58] and is used to e.g. correct a constant uncompleted recovery. The corresponding correction factors are indicated in Table 2.

3.3. ANALYTICAL METHOD VALIDATION

In this study, quantitative performances of the optimized analytical method were assessed for Vitamin D3, Pro- and Trans-vitamin D3 by means of a total error approach validation using accuracy profile based on β -expectation tolerance intervals [59]. The computation of these intervals takes into account the bias and the estimated variability of the results to define an interval where a pre-defined proportion β of future results are expected to fall inside (e.g. 95%). For each level of concentration, these intervals of tolerance are calculated and allow the control and evaluation of the risk associated to the acceptance of unsuitable results (e.g. 5%). Practically, if the β -expectation is comprised inside the acceptance limits, it ensures that future results will be comprised within the

acceptance criteria with at least a pre-defined probability. So as, the analytical method is suitable for its intended purpose and is considered as valid. When methods are validated using the total error approach, the validation criteria (precision, accuracy, linearity...) required by ICH Q2 R1 guidelines are fulfilled [60].

According to USP 38 [16] or EP 8.0 [17], the acceptance criterion of cholecalciferol as a drug substance is (97.0–103.0%) or (97.0–102.0%) respectively of the nominal content. Therefore, accuracy profiles were computed with acceptance limit set to $\pm 3.0\%$ of the targeted concentration of the analytes (i.e. $\pm 3.0\%$ of relative total error of the method is tolerated). In this study, the target concentration of $100 \mu\text{g mL}^{-1}$ was purposely selected (20 folds dilution of a 2 mg mL^{-1} of vitamin D3). The dosing range investigated is equal to $\pm 50.0\%$ of the target concentration [$50\text{--}150 \mu\text{g mL}^{-1}$].

Regarding Pro- and trans-vitamin D3, maximal specifications are 1.0% and 0.1% of the main compound according to the EP monograph. Without dilution of an initial sample (2 mg mL^{-1}), the respective target concentrations are $20 \mu\text{g mL}^{-1}$ and $2 \mu\text{g mL}^{-1}$. For both impurities, acceptance limits were set to $\pm 10.0\%$ and the dosing range investigated is equal to $\pm 30\%$ of the target concentrations ([$14\text{--}26 \mu\text{g mL}^{-1}$] and [$1.4\text{--}2.6 \mu\text{g mL}^{-1}$]). Their calibration curves were established with vitamin D3 ($2\text{--}20 \mu\text{g mL}^{-1}$ [0.1: 1%]) as an external standard to avoid normalization approach. So, the correction factors defined during method validation were employed to compute the results. Obviously, these factors were equivalent to those previously defined (Table 2).

At these target concentrations, $2.0 \mu\text{L}$ injections allowed linear UV response across the dosing ranges. Thus, it permitted the use of linear regression through 0 fitted at the target concentration as calibration model for D3, TRANS & PRO. Such model has the advantage to only require one-point calibration curves, which really eases use of the method in routine analysis. For all compounds, β -expectations tolerance intervals were set to 95%, which means that a maximum of 5% of the results may fall outside of the acceptance limits. The different validation parameters are summarized in Table 3.

The selectivity of the method was demonstrated at the beginning of the validation process, by the injection of i) blank solution containing only *n*-heptane, ii) vitamin D3 calibration standard (solution of vitamin D in *n*-heptane) iii) Pro- & trans-vitamin D3 calibration standard (solution of vitamin D in *n*-heptane – lower concentration) iv) validation standard (solution of D3, TRANS & PRO in *n*-heptane). No chromatographic interference was observed among the peaks of D3, TRANS & PRO and the injection solvent. Also, the chromatogram obtained for CS and VS were the same (data not shown).

Table 3. Summary of the validation criterion assessed for vitamin D3, trans-vitamin D3 and pro-vitamin D3.

Vitamin D3					Trans-vitamin D3				Pro-vitamin D3			
Response Function												
(s=4; l=3; r=3; n=3)					(s=4; l=3; r=3; n=2)				(s=4; l=3; r=3; n=2)			
Model	Linear regression through 0 (fit at target level) [50 - 100 - 150µg.mL ⁻¹]				Linear regression through 0 (fit at target level) [1.4 - 2 - 2.6 µg.mL ⁻¹]				Linear regression through 0 (fit at target level) [14 - 20 - 26 µg.mL ⁻¹]			
Series	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4
Slope	2405	2445	2417	2423	2449	2461	2459	2485	2336	2406	2387	2393
Intercept	0	0	0	0	0	0	0	0	0	0	0	0
R ²	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Trueness	(s=4; r=3; n=3)				(s=4; r=3; n=2)				(s=4; r=3; n=2)			
Level	Concentration (µg.mL ⁻¹)		Relative bias (%)		Concentration (µg.mL ⁻¹)		Relative bias (%)		Concentration (µg.mL ⁻¹)		Relative bias (%)	
1	50		-0.385		1.4		-1.698		14		-0.938	
2	100		0.029		2.0		-2.136		20		-0.549	
3	150		-0.706		2.6		-2.800		26		-0.882	
Precision	(s=4; r=3; n=3)				(s=4; r=3; n=2)				(s=4; r=3; n=2)			
Level	Repeatability %RSD		Intermediate precision %RSD		Repeatability %RSD		Intermediate precision %RSD		Repeatability %RSD		Intermediate precision %RSD	
1	0.570		0.822		2.364		2.364		1.186		1.972	
2	0.582		0.746		1.782		1.782		1.014		1.493	
3	0.505		0.896		1.526		1.526		0.966		1.961	
Accuracy	(s=4; r=3; n=3)				(s=4; r=3; n=2)				(s=4; r=3; n=2)			
Level	Relative β-expectation tolerance limits (%)				Relative β-expectation tolerance limits (%)				Relative β-expectation tolerance limits (%)			
1	[-2.382; 1.613]				[-6.728; 3.331]				[-6.174; 4.297]			
2	[-1.677; 1.735]				[-5.927; 1.656]				[-4.291; 3.194]			
3	[-2.871; 1.945]				[-6.048; 0.448]				[-6.522; 4.759]			
Linearity	(s=4; l=3; r=3; n=3)		(s=1; l= 5; r=1; n=3)		(s=4; l=3; r=3; n=2)		(s=1; l= 5; r=1; n=3)		(s=4; l=3; r=3; n=2)		(s=1; l= 5; r=1; n=3)	
Range	[52.17: 152.5 µg.mL ⁻¹]		[51.3: 153.9 µg.mL ⁻¹]		[1.49: 2.767 µg.mL ⁻¹]		[1.40: 2.61 µg.mL ⁻¹]		[14.07: 26.12 µg.mL ⁻¹]		[14.84: 27.56 µg.mL ⁻¹]	
Slope	0.995		0.964		0.959		0.941		0.992		0.965	
Intercept	0.226		26.674		0.038		0.098		0.006		0.287	
R ²	1.000		1.000		0.994		0.999		0.995		1.000	
LOD	LLOQ		ULOQ		LOD	LLOQ		ULOQ	LOD	LLOQ		ULOQ
(µg.mL ⁻¹)	(µg.mL ⁻¹)		(µg.mL ⁻¹)		(µg.mL ⁻¹)	(µg.mL ⁻¹)		(µg.mL ⁻¹)	(µg.mL ⁻¹)	(µg.mL ⁻¹)		(µg.mL ⁻¹)
n.a.	52.17		152.50		0.452	1.490		2.767	4.264	14.070		26.120

3.3.1. TRUENESS

Trueness is reported as the mean bias observed between the series of measurements and the targeted concentrations. Trueness is back calculated from the calibration curve established and expressed in terms of relative bias (%). Trueness of the SFC method was assessed at three concentration levels and was found satisfactory for the three compounds. The relative bias at the target concentration (100 $\mu\text{g mL}^{-1}$ – 100%) is equal to 0.029% and thus very close to 0 (Table 3). Regarding TRANS & PRO, the relative bias at the target concentration (2 and 20 $\mu\text{g mL}^{-1}$) is equal to –2.136% and –0.5485%.

3.3.2. PRECISION

Precision expresses the ability of the method to provide close results from multiple measurements of the same samples, under the same experimental conditions. Precision is evaluated at two levels: repeatability and intermediate precision. For each of those levels, relative standard deviation (RSD (%)) is computed for any concentration levels.

As shown in Table 3, the precision of the method is satisfactory. Indeed, the RSD values are not higher than 0.582% and 0.895% for the repeatability and the intermediate precision, respectively, of the main compound. For pro-vitamin D3, RSD values were 1.186% and 1.972% for the repeatability and the intermediate precision, respectively (at the lowest concentration level).

For the case of trans-vitamin D3, RSD values for repeatability and intermediate precision were not higher than 2.364%.

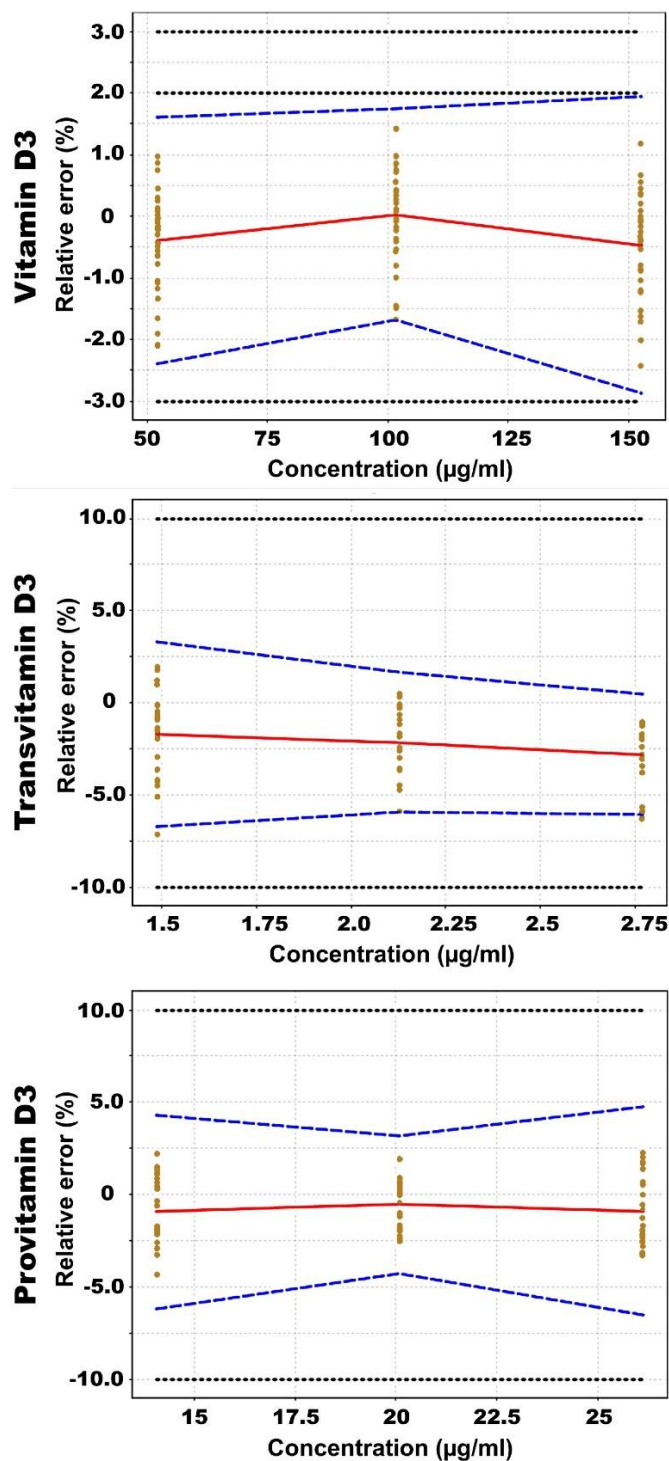
3.3.3. ACCURACY

Accuracy refers to the closeness of agreement between the test result and the accepted reference value, conventionally accepted as the true value. The accuracy takes into account the total error (i.e. random & systematic errors) associated to the analytical results.

For vitamin D3, the upper and lower bound of the β -expectations intervals were comprised within the acceptance limits defined by USP (97.0–103.0%) and European Pharmacopeia (97.0–102.0%) at all concentration levels.

The same results were obtained for both impurities studied with $\pm 10.0\%$ acceptance limits. Therefore, the method is considered accurate and future results will fall inside the acceptance limits with a minimal probability of 95%. The different accuracy profiles are presented in Figure 8 (top: vitamin D3/middle: trans-vitamin D3/bottom: pro-vitamin D3).

Figure 8. Accuracy profile obtained while considering linear regression to 0 (fitted at respective target concentration) to validate the quantitative performances of SFC method for vitamin D3 (top), trans-vitamin D3 (middle) and pro-vitamin D3 (bottom). On this figure, the black dotted lines are the acceptance limits (e.g. $\pm 3.0\%$); the blue dashed lines are the respective upper and lower bounds of the β -expectations tolerance intervals considering 5% of risk; green points are the individual results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



3.3.4. LIMIT OF DETECTION, LIMIT OF QUANTIFICATION, DOSING RANGE

The Limit of Detection (LOD) is the smallest quantity of the compound that can be detected but not accurately quantified in the sample. In the case of vitamin D3 as a raw material, LOD is not a mandatory validation parameter and was not determined. The LOD of pro- vitamin D3 and trans- vitamin D3 were calculated according to Eq. (3).

$$\text{LOD} = \frac{\text{LLOQ}}{3.3} \quad (3)$$

In these conditions, LOD of pro-vitamin D3 was equal to 4.264 $\mu\text{g mL}^{-1}$. Regarding the trans-vitamin D3, a LOD of 0.452 $\mu\text{g mL}^{-1}$ was determined.

The Limit of Quantification (LOQ) is the quantity of substance in the sample that can be analysed with a defined accuracy under the experimental conditions. Two different LOQ are described: the Lower LOQ (LLOQ) and the Upper LOQ (ULOQ). On the accuracy profile, LLOQ and ULOQ can be visually identified. Indeed, these limits are respectively the smallest and highest concentration for which β -expectations fall outside of the acceptance limits.

As all β -expectations are comprised within the acceptance limits, the LLOQ and ULOQ are respectively 52.17 and 152.5 $\mu\text{g mL}^{-1}$ for vitamin D3. The same rule applies for trans-vitamin D3 and pro-vitamin D3, which have LLOQ equals to 1.49 and 14.07 $\mu\text{g mL}^{-1}$ and ULOQ of 2.767 and 26.12 $\mu\text{g mL}^{-1}$; respectively. Thus, the dosing ranges of the main compound and its impurities are the intervals included between those respective values.

3.3.5. LINEARITY

The linearity of the results expresses the ability of the analytical method to provide results directly proportional to the concentrations.

A simple linear regression model was fitted between the estimated vs. true values results, to evaluate their linearity. For vitamin D3, the coefficient of determination (R^2) obtained was higher than 0.999, highlighting the consistence of the calibration model employed. However according to ICH, linearity has to be assessed on 5 different levels of concentration. So to be ICH compliant, further experiments -with 5 concentrations levels (i.e. two additional levels with concentrations of 75 and 125 $\mu\text{g mL}^{-1}$ were employed)- were carried out and very close results were found. The slight differences observed for the slope and the intercept of the curve may be related to the differences of sample sizes/statistical power. Indeed, for the validation four series of experiments were done with three independent samples at each concentration. In the other case, only one series with five samples (triplicate injections) was realized.

For impurities, good linearity of the results was also demonstrated on 3 levels ($R^2 > 0.994$) and 5 levels ($R^2 \geq 0.999$) (i.e. two additional levels with concentrations of 1.7 & 2.3 $\mu\text{g mL}^{-1}$ and 17 & 23 $\mu\text{g mL}^{-1}$ were employed for TRANS and PRO, respectively). The different results are summarized in Table 3.

4. Conclusion

The present study demonstrates the possibility to use SFC for the quality control of vitamin D as a pharmaceutical raw material. Thanks to DoE-DS methodology, it was possible to define robust optimal experimental conditions. The quantitative performances of the method and the UV correction factors were validated by means of the total error approach using accuracy profile methodology as decision tool. The SFC method was demonstrated to be suitable for the intended quantitative analysis of vitamin D3 (see Supplementary materials Table 1). Indeed, the method was able to match the acceptance criteria defined for vitamin D3 by the USP ($\pm 3.0\%$) and the Eur. Ph. (97.0–102.0%) monographs. In addition, using UV correction factors, the SFC method is also able to quantify the impurities according their specifications (0.1 and 1.0%).

The SFC method reported in this paper allows green and very fast analysis, which is really valuable in industrial framework. Indeed, for routine use, high throughput and eco-friendly robust analytical techniques are highly sought-after. For instance, in comparison to NPLC, the reported method is more than ten times faster (1.7 min vs. ≥ 19 min for the peak of vitamin D3). Practically, if runtime is defined as twice the retention time of vitamin D, one analysis with the SFC method requires less than 8 mL of mobile phase. The same analysis with NP-LC would require more than 76 mL of *n*-hexane, regardless of the re-equilibration of the column. Moreover, as the SFC mobile phase is mostly composed of carbon dioxide, it has a lower solvent cost per analysis and generates a significantly lesser amount of liquid organic waste. Indeed, at the outlet of the system, gaseous CO₂ is released and only the residual ethanol is retrieved as a waste. With the SFC method, it is estimated that ≈ 0.4 mL of liquid waste may be collected per analysis, which is approximately 200 folds lesser than NP-LC. The performances and advantages of this validated SFC method suggest considering the switch from NP-LC to SFC for the QC of vitamin D. Indeed, the adoption of this technique would be a good answer to the demand of QC, which grows with the increased needs of vitamin D. Doing so, the pharmaceutical industry would make a leap forward in the framework of green analytical chemistry for the QC of vitamin D3.

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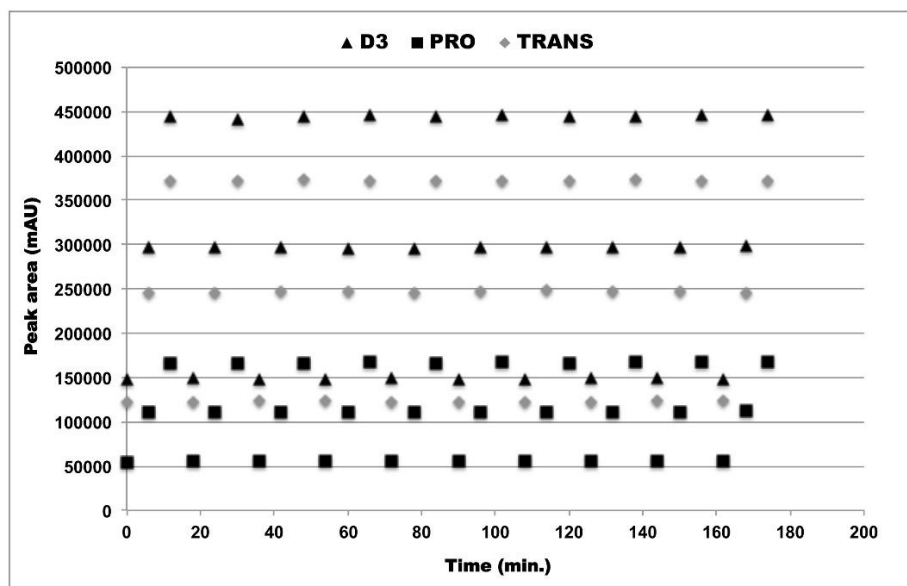
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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at

<http://dx.doi.org/10.1016/j.chroma.2017.01.090> or see below :



Supplementary Figure 1

Graphical representation of the peak area vs time for the three compounds studied.

Supplementary Table 1

Summary of the QC results for two batches of vitamin D3 raw material (industrial origin) using the SFC method reported. Testing was performed according to the specifications of the European Pharmacopeia: vitamin D3 = 97.0-102.0%; trans-vitamin D3 = max. 0.1% and pro-vitamin D3 = max. 1.0%. Both batches were found conform to the specifications.

	Vitamin D3	Trans-vitamin D3	Pro-vitamin D3	Decision
Specifications	97.0 - 102.0%	max. 0.1%	max. 1.0%	European Pharmacopeia 8.0
Batch 1 mean result	99.09%	Not detected *	0.04% **	Compliant to EP specifications
RSD (n =3)	0.43%	/	1.98%	
Batch 2 mean result	99.52%	Not detected *	0.08% **	Compliant to EP specifications
RSD (n =3)	0.33%	/	3.52%	
n= number of replicates * Below LOD ** Below LLOQ : estimated value				

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