

GENERIC SFC-MS METHODOLOGY FOR THE QUALITY CONTROL OF VITAMIN D₃ OILY FORMULATIONS

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Abstract

Vitamin D₃ is a key micronutrient whose intakes are inadequate for most populations worldwide. Supplementation with medicines or food supplements is commonly prescribed to correct this imbalance and the quality of these products must be ensured. In this context, a generic methodology for the assay of vitamin D₃ in oily formulations is proposed using supercritical fluid chromatography coupled to mass spectrometry (SFC-MS). It is in line with green analytical chemistry principles and combines the use of i) a fast and robust analytical method (4.0 min analysis time) ii) an easy sample preparation compatible with high throughput analysis (“dilute-and-shoot” approach) and iii) a relevant control strategy. Seventeen products from multiple manufacturers and encompassing a large content range were evaluated in this study. They were classified in four groups to streamline their processing considering the use of a matrix-matched calibration procedure. Matrix effect was thoroughly studied and was found to be low (99–106%), stable intra/inter-series and comparable between the different groups and types of matrices. The implemented control strategy was based on a three-level system suitability tests (SST). Level 1 SST: resolution of the critical pair that was above 1.5 for all analysis series. Level 2 SST: evaluation of the adequacy of the calibration for a QC sample in terms of recovery that was between 97% and 104% with a variability between 1% and 2%. Level 3 SST: method trueness that was between 95% and 102%. Sample analysis highlighted differences in types of products and dosage forms. This is the first study to propose a complete strategy for the quality control of vitamin D₃ oily formulations and should prove useful in QC laboratories.

1. Introduction

Vitamin D₃ (cholecalciferol) is a key micronutrient essential for human health through its involvement in numerous metabolic processes. A well-established role is its function in bone homeostasis via the regulation of calcium and phosphate concentration levels. Other roles, less highlighted but under scientific scrutiny, concern for example cellular maturation and differentiation, cardiovascular health promotion, glucose metabolism, immune system modulation, etc [1], [2]. Vitamin D₃ can either be

synthesized in human skin by a non-enzymatic process that involves ultraviolet radiations from the sun (major source) or be taken up from diet (minor source). A disruption in any of these sources (decreased sun exposure or limited food availability) leads to inadequate vitamin D₃ intake, which is currently the case for most populations worldwide [3]. Therefore, medicines or food supplements are commonly prescribed to correct this imbalance and, for severe cases, to reduce the adverse health consequences of vitamin D₃ deficiency such as osteomalacia or rickets [4].

For this study, we focused on the determination of vitamin D₃ content, as active principal ingredient (API) in oily formulations, which are common supplementation products available as medicines or food supplements. Several analytical challenges are associated with these types of formulations. Firstly, vegetable oil is the main excipient, which is a complex matrix that contains fatty acids, sterols, triacylglycerols, pigments, etc. The presence of so many different compounds can cause interferences with the signal of the API which require the use of separative techniques to achieve its adequate resolution from the matrix. The use of a specific detection technique such as mass spectrometry is also a coherent strategy to improve the overall specificity of the method. In that case, the presence of such matrix requires a rigorous evaluation of the matrix effect. Secondly, formulations are available on the market in a large content range of the API per unit of dosage form (from 200 to 200 000 IU of vitamin D₃ in this study). This represents another serious analytical challenge both in terms of sensitivity and matrix effect control. Such a range can be explained by the fact that the supplementation needs vary with age and groups, and the intended posology (daily, weekly or monthly use). It should be noted that food regulations set a maximum daily amount of vitamins in food supplements based on the tolerable upper intake levels. Since 2012, a scientific opinion from the European Food Safety Authority proposes this amount to be set at a maximum of 4000 UI/day [5] and therefore the majority of low content products are food supplements. Moreover, two main types of dosage forms are encountered: liquid solution or capsule. Sample preparation for liquid forms is straightforward since the oily formulation can directly be accessed and processed. Capsule forms however require special care during sample preparation to ensure complete recovery of the encapsulated oily formulation. Finally, the availability of these products as medicines or food supplements needs to be addressed since the regulatory requirements for medicinal products are not equivalent to those applicable to foodstuffs. Medicines are subjected to tighter controls starting from manufacturing to patient delivery which theoretically translates in products of better quality compared to food supplements. More precisely it can be expected that medicines will fare better for common end-product quality attributes such as uniformity of content, uniformity of dosage forms, batch-to-batch consistency, etc. This has been observed in studies comparing medicines and food supplements [6], [7], [8]. Caution must therefore be exercised when performing assays of food supplements since low quality products can be encountered. It should be noted that regulations impose the following specifications for content determination in finished products: 95–105% for medicines [9] and 80–150% for food supplements containing vitamins [10]. From an analytical point of view, it can be helpful to assess a large dosage range during method validation (e.g. 50–150% or even more) so that low quality products can be measured with accuracy and confidently reported.

Liquid chromatography is widely used for the analysis of vitamin D₃ [11], [12]. NP-LC is routinely used for oily formulations since the sample matrix can be easily dissolved in a non-polar solvent such as hexane, injected in the system and readily eluted with mobile phase (“dilute-and-shoot” approach)

[11], [13]. However, this chromatographic mode has several limitations, notably the consumption of large volumes of (toxic) organic solvents as well as its inadequacy with MS detection commonly used in quality control context. Moreover, NP-LC is increasingly considered outdated compared to current solutions [14]. RP-LC has also been extensively used but in this case the sample preparation must be more thorough. Indeed, an extraction step (e.g. liquid-liquid extraction or solid-phase extraction) is mandatory to limit the presence of lipid residues in the injected sample. This process can be tedious and time-consuming, rendering the strategy incompatible with high-throughput analysis. In this context, supercritical fluid chromatography (SFC) clearly represents an interesting alternative. Indeed, the mobile phase is mostly composed of supercritical carbon dioxide, a green solvent which has a polarity close to n-hexane and can therefore be used as an alternative to NP-LC using a “dilute-and-shoot” approach. Moreover, high-throughput analysis is also achieved because of the high kinetic performances of this technique [15]. Hyphenation to mass spectrometry is straightforward and reliable thanks to the several interface solutions that have been developed for modern instruments [16], [17]. SFC is therefore particularly suitable for these sample types as highlighted by recent studies [18], [19], [20] and its use in quality control laboratories should be promoted.

This work is rooted in this context of quality control. The aim is to propose a generic methodology to ensure reliable results during quality control of vitamin D₃ oily formulations using SFC-MS. This methodology was developed to be applicable to a wide array of medicines and food supplements by evaluating products from multiple manufacturers and encompassing a large content range for the API. For this purpose, we used a fast and robust analytical method that was previously developed in the laboratory following analytical quality by design principles (AQbD) [18], [21]. Moreover, a simple “dilute-and-shoot” approach was used as sample preparation technique. To improve method sensitivity and to be able to analyze low-concentrated samples, this method was first transferred from an ESI to an APCI source. This change was performed to take advantage of the APCI source ionization efficiency improvement for non-polar analytes. The optimization of MS settings to reach stable and sensitive signal and the evaluation of matrix effect were therefore considered. Then, aiming routine analysis, a relevant control strategy was evaluated and implemented in order to assess the qualitative and quantitative performance of the method. It was based on a three-level system suitability tests (SST) and represents a key part of the proposed methodology. To the best of our knowledge, this is the first study to describe a complete strategy for the QC of a large panel of vitamin D₃ oily formulations. Additionally, we present a thorough characterization of matrix effect and report routine results for medicines and food supplements.

2. Material and methods

2.1. CHEMICALS AND REAGENTS

Vitamin D₃ (cholecalciferol, 100%) was purchased from EDQM (Strasbourg, France). Vitamin D₂ (ergocalciferol, 99%) was used as internal standard and was obtained from Sigma Aldrich (Overijse, Belgium). 5,6-trans-cholecalciferol (trans-vitamin D₃, 95%) and pre-vitamin D₃ (>80%) were sourced from TRC Inc. (Toronto, Canada). Reference oily matrix was produced in-house and was composed of

refined olive oil, all-*rac*- α -tocopheryl acetate, sweet orange extract and polyglyceryl oleate. All these excipients were kindly provided by an industrial partner.

Carbon dioxide grade 4.5 (99.995%) was obtained from Air Liquide (Liege, Belgium). Ethanol (HPLC gradient grade), n-heptane (LC-MS grade) and ammonium formate (AmFm, 98.5%) were supplied by VWR Chemicals (Leuven, Belgium). Methanol (HPLC gradient grade) was purchased from J.T. Baker (Deventer, Netherlands). Water and 2-Propanol (LC-MS grades) were sourced from Biosolve BV (Valkenswaard, Netherlands).

2.2. SFC-MS INSTRUMENTATION AND ANALYTICAL CONDITIONS

SFC analysis was performed using a Waters Acquity UPC² (Waters, Milford, MA, USA). This system is equipped with a binary solvent delivery system, an autosampler with a 10 μ L loop, a column oven with an active preheater, a PDA detector and a two-stage backpressure regulator. The SFC system was coupled to a Waters SQ Detector 2 (Waters, Milford, MA, USA), equipped with an APCI source for mass spectrometry detection. Hyphenation to mass spectrometry was achieved using a dedicated SFC-MS splitter interface and a Waters Acquity Isocratic Solvent Manager (Waters, Milford, MA, USA) as make-up solvent delivery system.

The analytical conditions used for this study were previously developed in the laboratory. They are thoroughly described in ref. [18] and summarized here. An SFC-dedicated column was employed for the separation, the Acquity UPC² BEH (3.0 $\times$ 100 mm; 1.7 μ m), acquired from Waters (Milford, MA, USA). Ethanol was used as modifier. The analyses were performed in gradient mode, starting directly with a linear increase in modifier proportion from 2.0% to 10.5% in 2.8 min. Then, an isocratic step for 0.7 min and finally a fast return to initial condition in 0.01 min and column re-equilibration for a total analysis time of 4.0 min. Mobile phase flow rate was 1.5 mL min⁻¹. Column temperature was set to 41 °C and back-pressure to 110 bar. The autosampler temperature was set to 4 °C and the injection volume to 3 μ L. Methanol and 2-propanol were used as strong and weak needle wash, respectively.

MS conditions were optimized after transfer to the APCI source and were set as follows: cone voltage 25 V, corona discharge 10 μ A, probe temperature 400 °C, cone gas flow rate 50 L h⁻¹, desolvation gas flow rate 500 L h⁻¹ and source temperature 150 °C. Pure methanol was used as make-up solvent at a flow rate of 0.2 mL min⁻¹. Mass spectrometer was operated in positive mode and single ion recording (SIR). Targeted *m/z* values were 385.36 for vitamin D₃ and 397.34 for the internal standard. Peak areas of vitamin D₃ and pre-vitamin D₃ (if detected in sample) were summed for data analysis.

Empower 3.0 (Waters, Milford, MA, USA) was used for instrument control, data acquisition and processing. Data analysis was performed with Microsoft Excel 2019.

2.3. SAMPLE OVERVIEW AND CLASSIFICATION

Eight medicines and nine food supplements were evaluated in this study. These products were bought from a pharmacy for the purpose of this study and were from multiple manufacturers with vitamin D₃ content ranging from 200 to 200 000 IU (1 IU = 0.025 μ g). The starting point was to classify the samples in 4 groups as summarized in Table 1. This classification was performed to streamline the processing of samples. The dosage form content was selected as criteria to devise the groups with limits fixed at

a ratio of ± 2 between the highest and lowest dosage form content in a group. A notable exception is group 1 for which the lowest content forms were batched together.

Table 1. Sample classification (total number of samples $n = 17$). IU = international unit, 1 IU = 0.025 μg of vitamin D₃.

Type	Content per unit of dosage form in IU	Number of samples	Sample group	% of injected matrix
Food supplements	200	1	1	0.75
	800	2		
	1520	2	2	0.3
	2000	1		
	3000	2		
Medicines	3200	1	3	0.024
	20000	2		
	25000	2		
	50000	2		
	100000	1	4	0.006
	200000	1		

Each group was further defined with a percentage of injected matrix as reference value used for sample preparation (calibration / reconstituted samples) and matrix effect evaluation. For this purpose, a median content was first selected to be representative of the groups as follows: 800 IU (group 1), 2000 IU (group 2), 25 000 IU (group 3) and 100 000 IU (group 4). Then, from these values, the percentage of injected matrix was calculated considering the dilution factor needed to reach the target concentration for injection.

2.4. SAMPLE PREPARATION

Since vitamin D₃ is sensitive to light all solutions were prepared in a room with safe light. Amber vials were used to store the samples in the autosampler. A positive displacement pipetting system (Eppendorf multipette, Eppendorf, Hamburg, Germany) was used to handle the viscous oily samples and organic solutions. An example of injection sequence table is given in Table S1 (Supplementary material).

2.4.1. STOCK MATRIX SOLUTIONS

Stock matrix solutions were prepared for each sample group by diluting the reference oily matrix with n-heptane to achieve the following concentrations of matrix: 30% v/v (group 1), 12% v/v (group 2), 0.96% v/v (group 3) and 0.24% v/v (group 4). These solutions were prepared once and stored at 2–8 °C.

2.4.2. STANDARD SOLUTIONS AND CALIBRATION SOLUTION

Stock standard solutions of vitamin D₃ and D₂ (internal standard) were prepared by weighting accurately 12.5 mg (500 000 IU) of each vitamin in a 25.0 mL volumetric flask and dissolving with n-

heptane. The internal standard solution was sonicated for 2 min prior to filling to the mark to ensure the complete dissolution of the compound. The solutions were then diluted to reach a concentration of $6 \mu\text{g mL}^{-1}$ (equivalent to 240 IU mL^{-1}).

A single point external calibration model was used. The calibration solution was reconstituted in matrix to match the final percentage of injected matrix for each sample group as explained in Section (2.3). It was prepared by mixing adequate volumes of D_3 stock solution, internal standard stock solution and stock matrix solution in a 10.0 mL volumetric flask to reach a concentration of 150 ng mL^{-1} (equivalent to 6 IU mL^{-1}) of vitamin D_3 , 75 ng mL^{-1} (equivalent to 3 IU mL^{-1}) of internal standard and the final percentage of matrix for the considered sample group. A standard solution at the same concentration levels but without matrix was also prepared for MS optimization and matrix effect evaluation.

All the solutions were prepared freshly for each series of analysis.

2.4.3. PREPARATION PROTOCOL FOR CAPSULE DOSAGE FORMS

Individual capsules were directly cut in a volumetric flask with a scalpel. The scalpel was repeatedly rinsed with n-heptane and an adequate volume of the diluent was added to the flask. The solution was shaken for 15 min using an orbital shaker at 250 RPM (IKA Yellow Line OS 10 Control, IMLAB bvba, Boutersem, Belgium) then filled to the mark. This solution was diluted and mixed with the internal standard stock solution to reach the concentration levels of the calibration solution. Three independent replicates were prepared for each capsule dosage form product.

2.4.4. PREPARATION PROTOCOL FOR LIQUID SOLUTION DOSAGE FORMS

Liquid solutions (included drops) were recuperated from their dosing container, pipetted into a volumetric flask and diluted with n-heptane. They were further diluted and mixed with the internal standard stock solution to reach the concentration levels of the calibration solution. Three independent replicates were prepared for each liquid solution dosage form product.

2.4.5. SAMPLE PREPARATION FOR SST

A three-level SST formed the basis of the control strategy. The sample preparation for each SST was as follows:

Level 1 SST: a standard solution was prepared by mixing adequate volumes of stock standard solutions of pre-vitamin D_3 ($10 \mu\text{g mL}^{-1}$), trans-vitamin D_3 ($10 \mu\text{g mL}^{-1}$) and vitamin D_3 ($50 \mu\text{g mL}^{-1}$) to reach a concentration of 100 ng mL^{-1} of each compound in n-heptane. This solution was prepared once and stored at -20°C .

Level 2 SST: a reconstituted solution was prepared by independently weighting 12.5 mg (500 000 IU) of vitamin D_3 in a 25.0 mL volumetric flask. It was diluted, mixed with the internal standard stock solution and the appropriate stock matrix solution for the considered sample group to reach the concentration levels of the calibration solution. This reconstituted solution was prepared freshly for each series of analysis.

Level 3 SST: a quality control sample (reference sample of known content) was prepared as described in Section 2.4.4. This sample solution was prepared freshly for each series of analysis.

3. Results and discussion

3.1. MS SOURCE OPTIMIZATION AND SYSTEM STABILIZATION

Following the previous studies, preliminary experiments were performed using a make-up solvent composed of MeOH-H₂O (80:20, v/v) + 10 mM AmFm. This composition was optimized previously to maximize signal intensities for the analytes using ESI source. However, when transferred to the APCI source, a high peak area variability was observed with RSD (%) up to 8% for vitamin D₃ and the internal standard. This make-up solvent was replaced with pure methanol and we observed that peak areas doubled and variability decreased to 4%. This evaluation was repeated and confirmed, highlighting that the water content in the make-up solvent was an important factor. A first hypothesis could be that it diminishes the desolvation efficiency in the ionization source. It might also be that a high content of water (20%) can disrupt the decompression process in the connector tubing directed to the MS. This could result in phase separation inside the connector which, even if moderate, can lead to analytes precipitation [17]. This could be translated in loss of signal and increased variability. For this second hypothesis, switching to pure methanol could have resolved this issue in two parts i) better miscibility of methanol to sub/supercritical CO₂ decreases the probability of phase separation ii) the solvent composition is better suited to dissolve the non-polar analytes. The fact that it was not observed for ESI operation might be related to differences in splitter configuration and connector geometry.

Further optimization was performed to reach a stable signal with high sensitivity using a one-factor-at-a-time approach. The parameters and levels evaluated as well as the results are reported in Table S2 (Supplementary material). The optimization started with the cone gas flow rate with other parameters fixed at 400 °C for the probe temperature, 10 µA for the corona discharge, 25 V for the cone voltage. The make-up flow rate was initially set to 0.5 mL min⁻¹. A standard solution was injected in triplicate at each point tested. Results show that variability was decreased progressively with each parameter set to reach adequate levels around 1–2%. Regarding signal intensity, peak areas were mostly affected by the cone voltage and makeup flow rate. For this last parameter, the highest intensities were observed at lower flow rates with an optimum at 0.2 mL min⁻¹. Higher flow rates were associated with a decrease of signal intensity and increase of variability which could be related to a diminution of desolvation efficiency. In this final setup, we observed at least a more than ten-fold improvement of sensitivity using the APCI source (LOD<50 ppb) compared to the ESI source (LOD± 500 ppb, QDa detector from previous study) for the analysis of vitamin D₃.

Following the MS optimization, preliminary analysis tests were performed and some stability issues were observed after several injections. Indeed, a gradual decrease of recoveries and increase of matrix effect were highlighted. These observations also seemed to be more important from series to series. The progressive contamination of the ion source by matrix components was highlighted as a potential cause. Therefore, a source component cleaning procedure was implemented between each analysis series (one analysis series per day so one cleaning procedure per day was performed). It should be stated that such a high frequency of cleaning is unusual and was evaluated as a troubleshooting measure considering the nature of the application. Indeed, the combination of a complex matrix and a “dilute-and-shoot” approach can accelerate the contamination of the ion source which would require to increase the frequency of cleaning. The procedure consisted in the cleaning of the cone assembly

with the recommended wash cycles of the instrument manufacturer. It took a total of 30 min and was mostly automated since the cycles were performed in an ultrasonic bath. This systematic source cleaning between analysis series was found to be beneficial for overall system stability.

3.2. MATRIX EFFECT STUDY

Matrix effect is an important parameter to consider in quantitative MS analysis since it can affect the accuracy and sensitivity of the method [22]. The main strategy to compensate its effect consists in using an internal standard. The standard addition method can also be applied however it is not suitable for the analysis of multiple samples considering the high throughput requirements. Alternatively, if a representative matrix is available, then a matrix matched calibration procedure can be used. This latter approach was selected for the proposed methodology along with the use of an internal standard and the evaluation of matrix effect was then considered to ensure the reliability of the results. Matrix effect percentage (%ME) was calculated by dividing peak area of the analyte in sample matrix to peak areas of the analyte in neat solvent (standard solution), multiplied by 100. In practice, a standard solution was injected before the calibration solution (reconstituted sample) which permitted to evaluate %ME across the sample groups. Results are summarized in Table 2. %ME values less than 100% indicates ionization suppression and %ME above 100% indicates ionization enhancement.

Table 2. Matrix effect study summary.

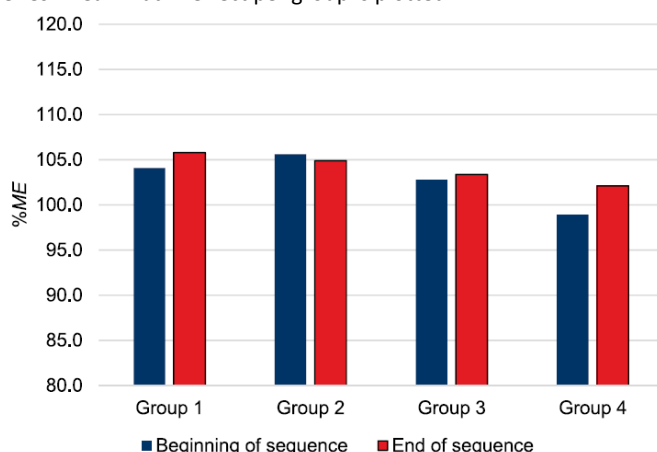
Matrix effect variation during analytical run (vitamin D ₃ and internal standard)			
Analyte	Number of analysis series (n)	Beginning of sequence (mean $\pm$ SD for n $\geq$ 3)	End of sequence (mean $\pm$ SD for n $\geq$ 3)
Vitamin D ₃	11	103.7 $\pm$ 2.3	104.2 $\pm$ 2.0
Internal standard	11	103.8 $\pm$ 2.0	104.1 $\pm$ 2.1
Matrix effect variation during analytical run per sample group (vitamin D ₃)			
Sample group	Number of analysis series (n)	Beginning of sequence (mean $\pm$ SD for n $\geq$ 3)	End of sequence (mean $\pm$ SD for n $\geq$ 3)
Group 1	2	104.1	105.8
Group 2	4	105.6 $\pm$ 0.8	104.9 $\pm$ 1.5
Group 3	4	102.8 $\pm$ 0.5	103.4 $\pm$ 0.5
Group 4	1	98.9	102.1
Matrix effect per matrix type (internal standard)			
	Number of samples (n)	Mean $\pm$ SD for n $\geq$ 3	
Reference matrix	(11) ^a	102.4 $\pm$ 3.2	
Olive oil	8	102.5 $\pm$ 2.7	
Glycerides	5	102.4 $\pm$ 4.1	
Corn oil	3	104.2 $\pm$ 5.6	
Chia seed oil	1	97.9	

^a 1 QC sample per analysis series for a total of 11 determinations.

Results show that overall matrix effect is very low, encompassing a range of 99–106%. This can be explained by i) the fatty components of the oily matrix are theoretically eluted early in SFC and therefore cause less interference with the signal of the analytes ii) the high dilution of the samples. The latter required a high sensitivity which was achieved by switching to the APCI source. Matrix effect was evaluated at the beginning and end of the analysis sequence in order to study its variation during the analytical run. As seen in Table 2 for results across series, the stability of matrix effect is highlighted as the difference in %ME between the beginning and end is less than a percent. Matrix effect is also comparable between vitamin D₃ and the internal standard (vitamin D₂). This is an important aspect since a key property of a suitable MS internal standard is that it must exhibit a similar ionization response as the analyte of interest. Isotopically labeled internal standards represent the gold standard but are generally expensive and difficult to source. In contrast, vitamin D₂ is cheap and readily

available. Conversely, it also means that vitamin D₃ can be used as the internal standard for formulations containing vitamin D₂ as the API.

Fig. 1. Matrix effect variation during routine analysis per sample group. Data reported for vitamin D₃. Total number of series $n = 11$ with a breakdown per sample group as follows: Group 1 = 2 series, Group 2 = 4 series, Group 3 = 4 series, Group 4 = 1 series. Mean matrix effect per group is plotted.



The same observations of %ME stability is made when the data is categorized per sample group (Table 2 and Fig. 1). Another interesting observation is that %ME is comparable between the sample groups. Indeed, the difference of %ME between groups is around 2–3%. Considering that the only difference between the sample groups is the percentage of matrix, it means that there is no significant change in %ME across the matrix range studied (0.006–0.75%) which is directly tied to the large sample content range. This can be expected considering the high dilution of the samples and highlights the suitability of the sample classification approach. Indeed, a typical matrix matched calibration procedure would require matching the matrix content in the calibration to each specific sample. This is challenging for samples with a large content range. It would impose to limit each analytical run to samples with the same or relatively close content. Here it is demonstrated that the samples are diluted enough to exhibit a similar matrix effect and therefore they can be grouped. The proposed classification could even be more streamlined. For example, only two groups could be made to target low content samples (group 1 + group 2) and high content samples (group 3 + group 4), which would simplify even further the analysis protocol. A comparison can be made with a study by Feng *and al.* [20], in which they analyzed two oily formulations of vitamin D₃ by a “dilute-and-shoot” approach. In that study, the expected percentage of injected matrix was 1% (samples dilution factor = 100) which is comparable to the reference value of injected matrix for group 1 in our study (0.75%). At these levels, they observed significant matrix effect which impacted recoveries up to 30–70% (SFC-MS mode). In contrast, we observed very low matrix effect for group 1 (104.1%). This could be explained by i) the analytical conditions in our study could limit more the co-elution of matrix component ii) the use of APCI which generally exhibits less matrix effect compared to ESI [22].

Finally, matrix effect was evaluated per type of sample matrix. Indeed, different types of vegetable oils are used for the formulations which could have an influence on matrix effect. The different oily matrices and number of samples evaluated are reported Table 2 and representative chromatograms in Fig. 3. For each analysis series, %ME was compared between the QC sample (Level 3 SST) containing the reference matrix and the assayed samples since they were injected in the same timeframe. This

evaluation was not directly performed for vitamin D₃ since variations in sample content would bias the results. Therefore %ME was calculated for the internal standard which was added at the same concentration in all the solutions. The calculated values per matrix type were averaged across analysis series to give a general overview and reported in Table 2 and Fig. 2. Results show that %ME is comparable between matrices and centered around 102%. The lower value for chia seed oil is attributed to the low number of samples. Indeed, only one analysis series was performed and, looking at the specific data for this series, the %ME was equal between the reference matrix and the chia seed matrix as seen in Table S3 (Supplementary material).

In summary, matrix effect is low, stable, comparable across sample groups and matrix type. Although a wide array of products was assessed to propose a generic approach, these observations can only be formally considered as valid for the sample matrices investigated. Therefore, the authors advise to carefully assess the matrix effect for the specific case studied.

Fig. 2. Matrix effect per type of sample matrix. Number of samples (*n*) per type of matrix as follows: Reference matrix = 11, Olive oil = 8, Glycerides = 5, Corn oil = 3, Chia seed oil = 1. Mean matrix effect per matrix type is plotted. Error bars (SD) are only displayed for *n* samples ≥ 3 .

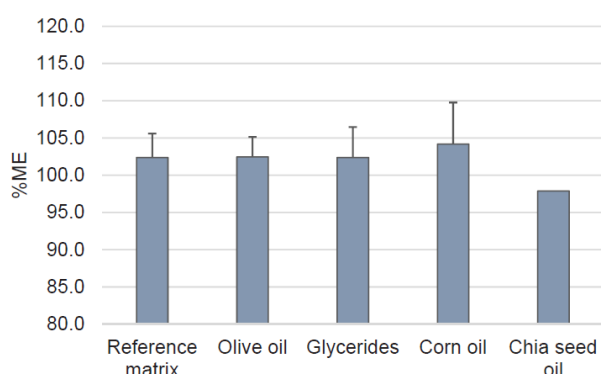
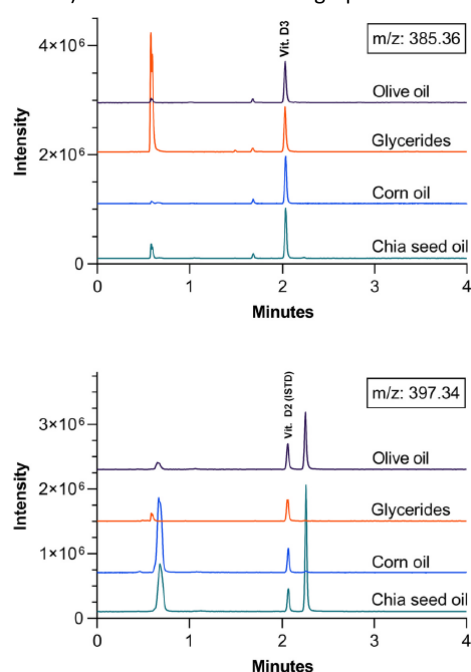


Fig. 3. Representative sample chromatograms in different matrices. Top: vitamin D₃ SIR channels. Bottom: vitamin D₂ (internal standard) SIR channels. Chromatographic conditions in text.



3.3. CONTROL STRATEGY

A formal method validation was not performed in this study as it requires a dedicated validation protocol for each sample (i.e. each matrix) which is not relevant nor realistic considering the large panel of samples. In this context, our strategy was to evaluate method performance and ensure results consistency by means of a control strategy. A control strategy is defined as “a planned set of controls, derived from current product and process understanding that assures process performance and product quality” [23]. It is based on the definition of relevant quality criteria that must be satisfied in order to accept a set of results. In this study, a three-level SST was used for the control strategy.

3.3.1. LEVEL 1 SST

Level 1 SST consisted in the evaluation of the chromatographic separation as a control derived from the AQbD framework. Indeed, this approach was applied during method development [18], [21] and consisted in optimizing relevant method parameters to separate vitamin D₃ and several related substances. The challenge lied in the separation of the critical pair pre- and trans-vitamin D₃, which could be achieved with the conditions defined following the optimization phase. Therefore in this study the separation of the critical pair was monitored by injecting a standard mix solution at the beginning of the analytical run. It should be noted that since the separation criterion (S) was used during the optimization phase, it should also be considered for this SST. However, considering that the aim of this study is to propose a generic methodology that could be implemented in QC laboratories, the resolution (Rs) was computed since it is a more common metric. A minimal resolution of 1.5 was selected as limit value to assess the separation of the critical pair. As seen in Table 3, this specification was met for all analysis series with a minimal resolution value equal to 1.7 for the third analysis series. Following this observation, it can be seen that the first few analysis series had the lowest resolution values as better illustrated in Fig. 4. The chart shows the gradual increase in resolution starting from series 4 which stabilizes at 1.9. This observation is further discussed in Section 3.3.3. as the QC sample (Level 3 SST) exhibits a similar pattern. Overall, these routine results highlight the adequate chromatographic performance of the method achieved thanks to the robust optimization of the AQbD approach. An in-depth description of this strategy can be found in publication [24].

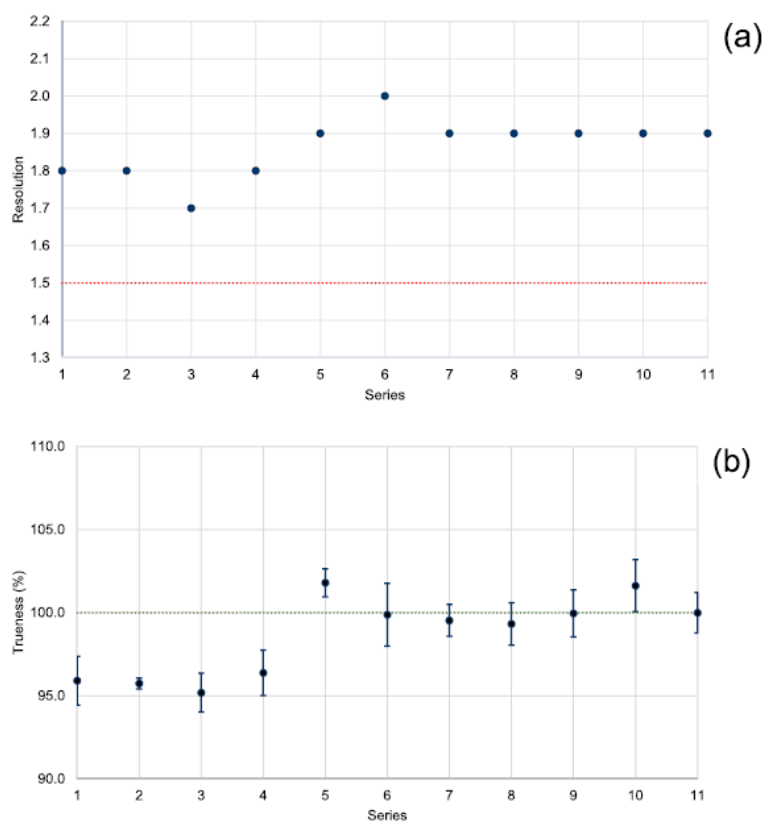
Table 3. SST results summary. Acceptance limits for Level 1 SST: Resolution ≥ 1.5 .

Series	Level 1 SST	Level 2 SST					Level 3 SST	
	Resolution	Beginning of sequence RSD (%)	Middle of sequence RSD (%)	End of sequence RSD (%)	Global RSD (%)	Global recovery (%)	Trueness (%)	RSD (%)
1	1.8	1.1	1.6	3.9	2.7	97.4	95.9	1.5
2	1.8	1.6	1.3	2.5	2.1	99.0	95.7	0.3
3	1.7	1.4	0.6	1.0	1.6	101.3	95.2	1.2
4	1.8	0.7	2.2	0.6	1.4	99.9	96.4	1.4
5	1.9	1.4	1.5	1.6	1.4	101.9	101.8	0.8
6	2.0	1.3	1.6	0.4	1.3	100.1	99.9	1.9
7	1.9	0.6	1.8	0.7	1.3	100.0	99.5	1.0
8	1.9	2.0	2.0	1.4	1.7	100.0	99.3	1.3
9	1.9	0.7	1.4	0.3	1.0	104.0	100.0	1.4
10	1.9	1.5	0.5	0.6	1.0	101.4	101.6	1.6
11	1.9	1.2	0.3	1.8	1.4	99.7	100.0	1.2

This SST should be implemented at least at the very beginning of the analytical run to ensure that the system is able to separate the compounds. Should this control fail, root cause analysis must be performed and in priority target a potential issue with the mobile phase and/or column state, assuming of course that the equipment is properly maintained and qualified.

Going further, the continued monitoring of this SST during routine analysis can bring several benefits. For example, the resolution could be evaluated at the beginning and end of each analysis sequence and continuously monitored over a long period of time. A control chart can be used to aggregate the data and to perform statistical analysis [25].

Fig. 4. Series charts for Level 1 SST - Resolution (a) and Level 3 SST - Trueness (b). Red line for Level 1 SST represents the acceptance limit. Green line for Level 3 SST represents the 100% level.



For each analysis series (intra-run monitoring), the variation of the resolution between the beginning and end of the sequence will give an indication of its stability during the run. This can highlight the need for (increased) system equilibration time before starting the run.

Over the long term (inter-run monitoring), the control chart can be used to evaluate the state of the column. Indeed, repeated injections and aging will inevitably lead to column deterioration. This will translate in a progressive loss of resolution which can be highlighted on a control chart. Furthermore, a resolution limit can be derived as proposed by Declerck and al. [26] to state that a column is considered deteriorated and needs to be preemptively replaced.

3.3.2. LEVEL 2 SST

Level 2 SST consisted in the evaluation of the adequacy of the calibration in terms of recovery and variability of a control sample. For this purpose, a reconstituted sample (QC calibration) was injected in triplicate at the beginning, middle and end of the analysis sequence (total number of injections $n = 9$). The adequacy of the calibration was evaluated by calculating the percentage recovery (%) between the concentration value obtained from the calibration and the theoretical concentration

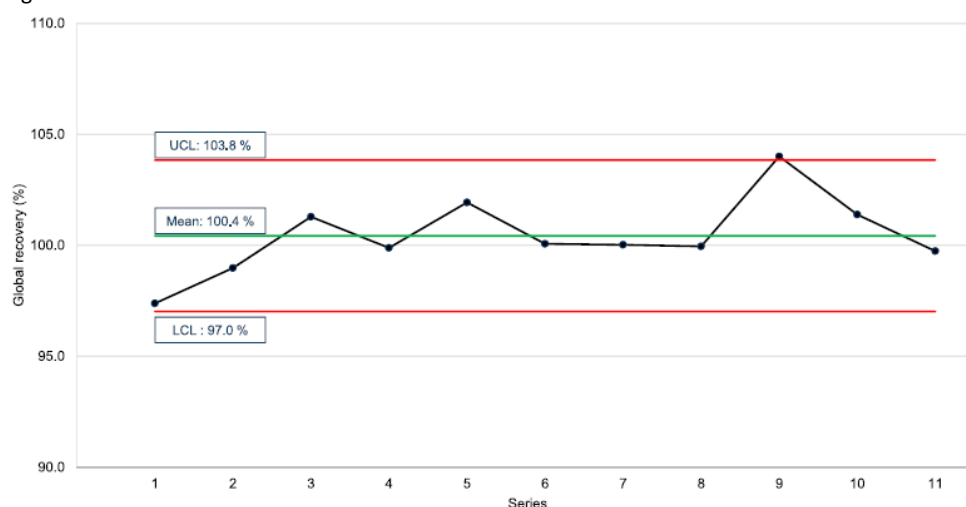
value of the reconstituted sample. RSD (%) were calculated for the evaluation of variability. Data analysis was performed for each set of triplicate injection and globally considering all the injections. Global results are reported in Table 3 and extended results are available in Table S4 (Supplementary material).

Adequate results were obtained as the global minimal and maximal recoveries calculated were 97.4% and 104.0% respectively. The variability was also adequate as it was between 1% and 2% globally. For each series of analysis, the suitability of the calibration over the whole analysis sequence is therefore highlighted.

The interest of evaluating the recovery and variability throughout the whole analysis sequence by performing injections periodically allows to identify a potential signal drift. As seen in Table S4 (Supplementary material), for each analysis series, the mean recoveries were similar at each time point and therefore no signal drift can be highlighted. For the variability, no trend can be observed during the analysis sequences as it also is similar at each time point. This was an important verification to perform considering the stability issues encountered in the preliminary studies (explained in Section 3.1.).

Setting acceptance limits for this SST is not as straightforward compared to the Level 1 SST for which an achievable performance was known a priori. This information can be obtained after method validation which was of course out of the scope of this paper considering that we aimed at proposing a generic methodology applicable to a large panel of products. The differences in specification between medicines and food supplements also poses a challenge in setting the limits. Nevertheless, routine data can give valuable insights on the long-term performance of the method which could in turn help to set the acceptance limits [29]. Also, this data (11 analysis series) was acquired over the course of a month which is a representative timeframe. It was plotted on a control chart in Fig. 5 and control limits (2-sigma) were computed. Results show that these control limits can fit in a range of 95%– 105%.

Fig. 5. Control chart for SST level 2 (global recovery). UCL = Upper Control Limit, LCL = Lower Control Limit. Control limits at 2-sigma level = $\text{mean} \pm k \cdot \text{SD}$ with $k = 2$.



As recommendations, this control should be performed at the very least at the beginning of the analytical run to ensure the calibration is adequate before analyzing the samples. For long sequences,

verifying the adequacy of the calibration periodically is also recommended to ensure that signal drift does not impact the results. It should be added that this risk is mitigated by the use of an internal standard.

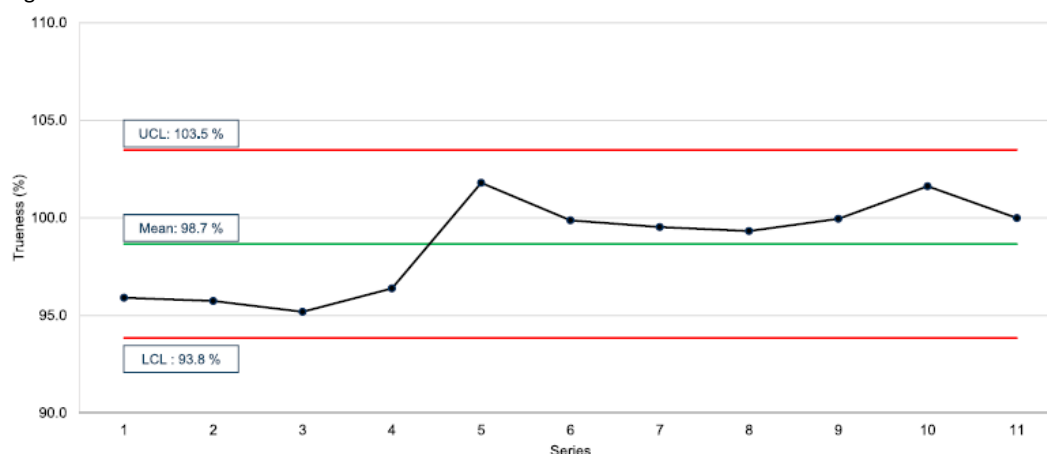
3.3.3. LEVEL 3 SST

Level 3 SST consisted in the evaluation of method trueness by analyzing a “real-life” sample with a known content in vitamin D₃ (QC sample). This QC sample was injected 3 times in total and interspaced between the assayed samples. Method trueness was expressed as the mean percentage recovery between the calculated content (the analytical result) and the real content value of the sample. Results are presented in Table 3 and Fig. 4.

The results encompass a range of 95–102% but it can be seen that the values were lower for series 1–4 at around 95%. It increased starting from series 4 and centered around 100% for the subsequent series. This observation was also made for the resolution criterion (Level 1 SST) as explained in Section 3.3.1. and illustrated with the series charts in Fig. 4. Root cause analysis did not highlight any issues related to the sample. A possible explanation might be related to the system instability due to contamination occurring during MS optimization and preliminary tests as discussed in Section 3.1. Indeed, the four series concerned were directly launched after beginning to implement the systematic source cleaning. Consequently, several series were required to reach an in-depth cleaning of the system resulting in a completely stabilized system. It can also be seen that for Level 2 SST, series 1 and 2 had the lowest recoveries and highest variability (RSD (%)) and is most likely related to this general observation.

The same difficulties in setting a priori acceptance limits expressed in Section 3.3.2 are applicable for this SST. Therefore, a control chart with control limits (2-sigma) was plotted in Fig. 6. The limits encompass a range between 93% and 104% but their computation is highly impacted by the lower values of the first four series, for which an assignable cause was highlighted. It should be expected that they would become narrower and focused around 100% when more data is available with the system in complete stabilization. As recommendations, the long-term monitoring of method trueness via a control chart can help to detect systematic errors and changes in performance during long term routine analysis [25].

Fig. 6. Control chart for SST Level 3 (method trueness). UCL = Upper Control Limit, LCL = Lower Control Limit. Control limits at 2-sigma level = mean $\pm$ k.SD with k = 2.



3.4. SAMPLE ANALYSIS RESULTS

Three independent preparations were tested for each sample and the results of the assays are reported in Table 4 for food supplements and Table 5 for medicines. As seen in the chromatograms in Fig. 3, samples contained vitamin D₃ and pre-vitamin D₃. This last compound is formed in solution by isomerization depending on temperature and time. When analyzing the data, the peak areas of the two compounds were summed as recommended by the pharmacopoeias [27], [28] since the biological activity is due to both compounds.

Table 4. Food supplements content determination.

Sample number	Content (<i>n</i> = 3 independent replicates)			Mean	RSD (%)	Type
1	107.2	105.1	105.8	106.0	1.0	Capsule
2	119.0	114.1	114.1	115.7	2.4	
3	107.4	101.6	108.0	105.7	3.3	
4	113.3	114.0	108.3	111.9	2.8	
5	101.6	103.8	100.9	102.1	1.5	
6	91.0	94.5	93.0	92.8	1.9	
7	115.9	113.1	117.5	115.5	1.9	
8	98.9	97.9	101.8	99.5	2.0	Solution

Assays results for food supplements are between 80% and 150% as recommended by the regulatory agencies. However, it can be seen that content variation is high between the samples, encompassing a range of 92–115%. Moreover, higher RSD (%) are observed for capsules compared medicines which suggests that the uniformity of content is better for medicines and can be attributed to manufacturing and quality control differences.

Table 5. Medicines content determination.

Sample number	Content (<i>n</i> = 3 independent replicates)			Mean	RSD (%)	Type
1	102.8	105.0	104.5	104.1	1.1	Capsule
2	99.7	99.7	101.4	100.3	1.0	
3	100.1	101.0	101.6	100.9	0.7	
4	98.3	102.0	100.7	100.3	1.9	
5	111.2	111.8	112.1	111.7	0.4	Solution
6	98.7	97.1	99.0	98.3	1.0	
7	98.5	97.7	98.0	98.1	0.4	
8	95.3	96.6	96.8	96.2	0.9	
9	101.4	103.3	102.3	102.3	0.9	

The results for medicines are in the expected range of 95–105% except for sample 5 which is around 112%. This sample was retested and the results were confirmed. Lower RSD (%) are observed for solutions compared to capsules, which is expected since the sample preparation is easier.

4. Conclusion

A generic methodology to perform assays of vitamin D₃ oily formulations by SFC-MS was evaluated in a routine analysis context. To be applicable to a broad array of products, this evaluation was performed on samples from multiple manufacturers and over a large content range. The operating procedure is comprised of robust analytical conditions and easy sample preparation technique (“dilute-and-shoot” approach) that permits high throughput sample processing and analysis. Matrix effect was thoroughly studied and its adequate control was highlighted. Moreover, a control strategy based on a three-level SST was implemented which demonstrated the adequate chromatographic and quantitative performance of the method.

This strategy could be expanded to any type of oily formulation provided that the analytical conditions are properly defined and that matrix effect is carefully assessed since it can have a strong impact on the results. Furthermore, it is clear that the benefits provided by SFC in terms of cost per analysis and environmental impact make the case for its adoption in QC laboratories. In a broader context, considering that the push for ecological alternatives and sustainable processes has also reached the analytical chemistry community, SFC is undoubtably proving to be the lead technology in the separative and preparative field.

CRediT authorship contribution statement

Hugues Jambo: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Amandine Dispas:** Conceptualization, Supervision, Writing – review & editing. **Cédric Hubert:** Conceptualization, Supervision, Writing – review & editing. **Frédéric Lecomte:** Writing – review & editing. **Éric Ziemons:** Supervision. **Philippe Hubert:** Project administration, Writing – review & editing, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2021.114492

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