Published in : J. Chromatogr. A (2017), vol.1515, pp. 209–217
DOI: 10.1016/j.chroma.2017.07.057
Status : Postprint (Author's version)

LIÈGE université

IS SUPERCRITICAL FLUID CHROMATOGRAPHY HYPHENATED TO MASS SPECTROMETRY SUITABLE FOR THE QUALITY CONTROL OF VITAMIN D3 OILY FORMULATIONS?[†]

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Abstract

Nowadays, many efforts are devoted to improve analytical methods regarding efficiency, analysis time and greenness. In this context, Supercritical Fluid Chromatography (SFC) is often regarded as a good alternative over Normal Phase Liquid Chromatography (NPLC). Indeed, modern SFC separations are fast, efficient with suitable quantitative performances. Moreover, the hyphenation of SFC to mass spectrometry (MS) provides additional gains in specificity and sensitivity. The present work aims at the determination of vitamin D3 by SFC-MS for routine Quality Control (QC) of medicines specifically. Based on the chromatographic parameters previously defined in SFC-UV by Design of Experiments (DoE) and Design Space methodology, the method was adapted to work under isopycnic conditions ensuring a baseline separation of the compounds. Afterwards, the response provided by the MS detector was optimized by means of DoE methodology associated to desirability functions. Using these optimal MS parameters, quantitative performances of the SFC-MS method were challenged by means of total error approach method validation. The resulting accuracy profile demonstrated the full validity of the SFC-MS method. It was indeed possible to meet the specification established by the European Medicines Agency (EMA) (i.e. 95.0 – 105.0% of the API content) for a dosing range corresponding to at least 70.0-130.0% of the API content. These results highlight the possibility to use SFC-MS for the QC of medicine and obviously support the switch to greener analytical methods.

[†]Selected paper from the 12ème Congrès Francophone sur les Sciences Séparatives et les Couplages Analytiques (SEP 2017), 29-30 March 2017, Paris, France.

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Introduction

Supercritical fluid chromatography (SFC) is nowadays expanding in the field of separation sciences [1]. Indeed, the combination of the modern robust instruments and the typical mobile phase of SFC allows fast, effective green analysis, which benefits to many domains and especially the pharmaceutical field [2]. Moreover, the technique is also compatible with the most popular detectors employed in chromatography [3]. So, to specifically detect compounds in complex samples with potential matrix interferences, it is therefore possible to hyphenate SFC to mass spectrometry (MS), which provides new levels of specificity and sensitivity [4–6]. Many application of SFC-MS are currently described for the detection of compounds (e.g. drugs, metabolites, lipids. . .) in challenging matrices having various origins (biological fluid, vegetal extract, . . .) [7–20].

Vitamin D3 (cholecalciferol) is a fat-soluble vitamin having plethora of effects, which are divided in two classes: skeletal and extra-skeletal effects [21,22]. The skeletal effects being in favour to the bone health, while the extra-skeletal effects are related to its beneficial action to e.g. the immune system, the cognition, the cardiovascular system, etc. All these effects are still under fruitful investigation, which explains why the vitamin D3 is the most published vitamin of the century [23]. However, despite these benefits, it appears that the large majority of the population has a deficiency in vitamin D3 [24]. To address this issue, an exogenous supply of vitamin D3 (i.e. by medicines) is usually prescribed to patients [21].

To ensure the quality and safety of the produced medicine, a quality control (QC) is a mandatory step which is performed according to the specifications established by the European Medicines Agency (EMA). Thus, the content of the active pharmaceutical ingredient (API) must be comprised within a ± 5.0% interval centred on the declared content of the drug (i.e. 95.0–105.0%) [25]. In this context, the quantitative performances of the analytical method should be properly established by means of method validation, demonstrating that the method is fit for its intended purpose.

For the analysis of vitamin D3, normal phase liquid chromatography (NPLC) is often employed due to the highly hydrophobic nature of this compound [26–30]. However, SFC is frequently suggested as an alternative to NPLC analysis [31]. Various study reports the use of SFC for the determination of vitamin D3 [32–35] but most of these methods are non or only partially validated [32–34].

With the current expansion of both SFC and MS in the field of separation sciences, the objective of the present work was to evaluate the quantitative performances of SFC-MS method for pharmaceutical quality control. In this context, the more challenging determination of vitamin D3 in a medicine (i.e. complex oily formulation for which ±5.0% specifications apply) was selected as a relevant case study. So, with an assessment of potential matrix effect, the QC of dietary supplements (wider specifications: e.g. −20.0, 50.0%) would be feasible. In a previous project, an analytical SFC-UV method was optimized to separate vitamin D3 and its related impurities [35]. However, the optimization of the hyphenation to MS detector and responses were still required. Also, as this SFC-MS method should be used in routine laboratories, this implementation should be simple and reliable. Consequently, a relatively simple MS device was employed, which consisted of a robust and compact single quadrupole MS detector with ESI ionization source (see Section 2.1 SFC-MS instrumentation). Moreover, only the adjustment of few parameters (i.e. cone voltage, capillary voltage. . .) was possible for the user, which obviously speed up the tuning and ease the routine use of MS detection. Given

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the importance of the make-up solvent in the ionization process and its final impact on the MS signal response, this work started with empirical testing of various classic make-up solvents mixtures. This screening permitted to highlight a preferential make-up solvent composition. Afterward, the Design of Experiments (DoE) methodology was employed to efficiently optimize make-up composition and MS detector parameters. The targeted goal was to increase method sensitivity, which was expressed as the maximization of the signal to noise ratio (S/N) through a desirability function. Once MS detection was optimized, the quantitative capabilities of SFC-MS were challenged by means of a full analytical method validation [36]. This was performed with a total-error approach using ß-expectation tolerance interval and accuracy profiles as a drastic decision tool [37–40]. This methodology permitted to control and appreciate the level of risk linked to future routine application of the method [41,42].

This study constitutes a further step in our work devoted to the implementation of the SFC for pharmaceutical QC [35,43]. To the best of our knowledge, among all the SFC-MS method available in the literature, the present paper reported in literature a first fully validated SFC-MS method for QC of medicines.

2. Material and method

2.1. SFC-MS INSTRUMENTATION

A Waters Acquity UPC^{2TM} system (Waters Corp., Milford, USA)was used to carry out the experiments. Compared to previous description of this system [35], the PDA detector was shunt and a dedicated interface (Waters Isocratic Solvent Manager − ISM) was employed for the hyphenation to a Waters Acquity QDa mass detector (performance version). The ISM interface module comprised a SFC dedicated flow splitter (SFC ratio = 1/5) and an isocratic pump, which ensured the post-column make-up solvent addition (flow rate=0.5 mL min^{-1}).
The analytical column employed in this study was an Acquity UPC^2 BEH column (3.0×100mm; 1.7µm) provided by Waters.

2.2. CHEMICALS AND REAGENTS

Cholecalciferol (vitamin D3; 99.0%) and Ergocalciferol (vitamin D2; 99.2%) were purchased from Sigma Aldrich (St Louis, MO, USA). 5,6-trans-cholecalciferol (95%) and pre-vitamin D3 (> 80%) were sourced from TRC Inc. (Toronto, Canada). Batches of oily matrix of the medicine (i.e. not containing any API; confidential composition) were produced and used to reconstitute the various samples required in this study.

Ethanol (HPLC gradient grade) and ammonium acetate (AA; >98.0%) were supplied by Merck Millipore (Darmstadt, Germany). Ammonium formate (AF; 98.7%) was bought from VWR chemicals (Leuven, Belgium). Methanol and n-heptane (HPLC grades) were purchased from J.T. Baker (Deventer, Netherlands). Water (MS grade) and 2-propanol (MS grade) were sourced from Biosolve

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BV (Valkenswaard, Netherlands). Carbon dioxide grade 4.5 (99.995%) was obtained from Westfalen BVBA (Aalst, Belgium).

2.3. ANALYTICAL METHOD

2.3.1. SFC METHOD

SFC method for the separation of vitamin D3 and its related impurities was previously optimized using DoE-DS methodology [35]. Briefly, this method employed ethanol as co-solvent with gradient mode elution on a hybrid bare silica column (Acquity UPC^2 BEH: 3.0×100mm; 1.7µm). Validated optimal conditions included a mobile phase flow rate equal to 2.0 mL min^{-1}, 2.0 µL of sample injected, active back pressure regulator (BPR) set to 110 bar and heating of the column to 41°C. In these conditions and with a mobile phase composed of CO_2 and ethanol (98:2 v/v), the pressure recorded at the beginning of the run was equivalent to 230 bar (head of the pump).

2.3.2. MAKE-UP SOLVENT SCREENING

Various make-up solvents were tested and the mean signal intensity was monitored (ESI, positive mode, single ion record (SIR)). Experiments consisted in triplicate injections of vitamin D3 (m/z = 385.48; 2.0 µL injected; 1 µg mL^{-1} in pure n-heptane), under given SFC conditions. This allowed to directly consider potential effects of the carbon dioxide and organic modifier in actual analytical conditions. The make-up solvents tested were composed of i) methanol + ammonium formate (0.06% *m/v*), ii) methanol + ammonium formate (0.1% *m/v*), iii) methanol + ammonium formate (0.2% *m/v*), iv) methanol + ammonium formate (0.4% *m/v*), v) methanol: H_2O (9:1) + ammonium formate (0.2% *m/v*), vi) ethanol + ammonium formate (0.2% *m/v*), vii) methanol + ammonium acetate (0.2% *m/v*).

For this screening, MS parameters were set as following: cone voltage (25 V), capillary voltage (1.5 kV), probe temperature (600°C) and acquisition frequency (8 pt sec^{-1}). All make-up solvents were added post-column at a flow rate fixed to 0.5mL min^{-1}; split ratio being constant and equal to 1/5.

2.3.3. MS OPTIMIZATION: DESIGN AND COMPUTATION

The make-up screening allowed selecting a make-up composition, the second step was to optimize MS parameters to improve method sensitivity. Thus, a four factors Central Composite Design (CCD) involving triplicate of the central point, as described in Table 1, was employed.

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Table 1
Summary of the experimental parameters and their levels, as defined in the DoE.

DoE Parameter:	Water	Cone voltage	Capillary voltage	Acquisition frequency
	% (v/v)	*V*	*kV*	*pt/sec*
Level:				
Minimum	0	5	0.3	5
Low intermediate	2	6	0.42	5.5
Central	10	10	0.9	7.5
High intermediate	18	14	1.38	9.5
Maximum	20	15	1.5	10

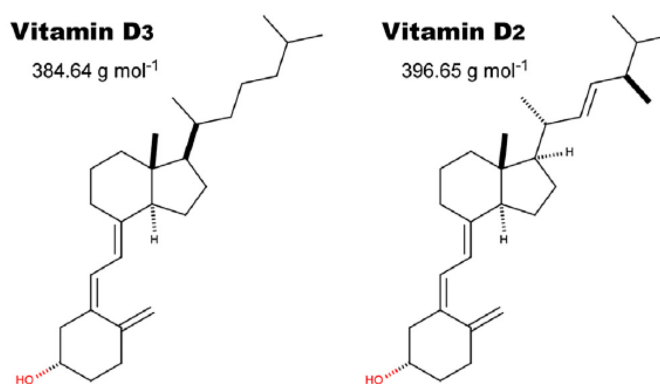

Fig. 1. Structure of the compounds involved in this study. Vitamin D3 is the API and Vitamin D2 is the internal standard for MS quantification.

2.4. SAMPLE PREPARATION

2.4.1. SFC METHOD ADAPTATION

A solution containing pre-vitamin D3, trans-vitamin D3 and vitamin D3 in pure *n*-heptane (1 ppm each), was employed to assess the baseline separation of all compounds when the system was hyphenated to a MS detector.

2.4.2. MS OPTIMIZATION

Two different kind of samples were prepared and employed for the MS optimization process. These solutions were prepared with pure *n*-heptane or with a solution containing the medicine's matrix (0.1% v/v in *n*-heptane). In both cases, the concentration levels of vitamin D2 and vitamin D3 were 0.5 ppm and 1 ppm, respectively. To avoid any undue ionization suppression from competing ions, the concentration of the internal standard was purposely set to 50.0% of the API target concentration (Fig. 1).

2.4.3. VALIDATION PROTOCOL

All the standards employed (i.e. for validation and calibration) were prepared with a solution of *n*-heptane containing 0.1% v/v of the matrix, which permitted to take account of potential matrix effect. So as, it corresponded to a dilution (factor = 1000) of the original medicine. All samples were prepared with 1.25 ppm (50 UI mL^{-1}) of vitamin D2,which was used as internal standard (IS) for MS quantification. For the validation, the monitored responses were the calculated ratios between the areas of the peaks

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corresponding to vitamin D3 and vitamin D2 (i.e. ratio = $Area_{D3}/Area_{D2c}$) for each experiment. For all series, a correction factor was applied to the area of the peak of vitamin D2. This factor was directly linked to the freshly prepared IS solution (i.e. correction factor = C_{D2mean}/C_{D2-S1}; where C_{D2mean} and C_{D2-S1} are respectively the mean concentration of D2 employed in the validation process and the concentration of IS employed for e.g. the validation serie 1) and enabled comparison of the ratios obtained between series. To ensure correct and precise handling of viscous, oily and/or volatile organic solutions, positive displacement pipettes were employed.

As the vitamin D3 is subject to thermal instability and photosensitivity, all solutions were prepared in absence of direct light in a room with controlled temperature (18°C).
Four independent series were performed to validate the determination of vitamin D3 by SFC-MS. The monitored response was the peak area ratio between the API and the IS. For all the validation experiments, 3.0 μL of the samples were injected (peak area RSD=0.94% for 6 injections of vitamin D3 (100 UI mL^{-1} in n-heptane-matrix (999:1)).

2.4.3.1. Calibration standards

For each series, a new stock solution was freshly prepared for calibration standards (CS). It was produced by accurate weighting of 25 mg of vitamin D3 and 12.5 mg of vitamin D2, which were dissolved in a 20.0 mL volumetric flask, fulfilled of pure n-heptane. The stock solution was then diluted to get concentrations levels corresponding to 1.25, 2.50 and 3.75 μg mL^{-1} in 10.0 mL volumetric flasks. Prior to volume adjustment, all these solutions were spiked with 1.0 mL of a solution containing 1.0% (v/v) of reconstituted matrix in n-heptane. So, the total content of matrix is equal to 0.1% v/v in all samples.

2.4.3.2. Validation standards.

Validation standards (VS) of vitamin D3 were prepared freshly and independently (i.e. independent weightings for each solution) in triplicate for each validation series. An equivalent protocol as described for the vs was employed, providing samples having concentrations of 1.25, 2.50 and 3.75 μg mL^{-1} and containing 0.1% v/v of reconstituted matrix.

2.4.4. IMPURITY TEST

To assess the possibility to detect and potentially quantify a specified impurity with the SFC-MS method, a solution containing 0.025 μg mL^{-1} of trans-vitamin D3 (=1.0% of relative API target content) was prepared in n-heptane containing 0.1% v/v of matrix. Also, for these experiments, the same volume of sample was injected (3.0 μL).

2.5. SOFTWARE

UPC^2 and QDa mass detector were piloted through Empower 3 (build 3471) (Waters Corp., Milford, USA), which also served for data handling. JMP 12.0 Pro (SAS institute, Cary, NJ, USA) for Apple Mac OS X was used to create the design of experiments and to carry out the statistical analysis. The validation of the SFC-MS method and accuracy profile were computed using Enoval 3.0 (Arlenda Sa, Liege, Belgium).

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3. Results and discussions

3.1. ADAPTATION OF THE CHROMATOGRAPHIC METHOD

Using the MS configuration of the system, a partial co-elution of the critical pair of peak was observed in the optimal conditions described for UV detection [35]. As vitamin D3 and its related compounds (pre-vitamin D3 and trans-vitamin D3) have the same m/z ratio (all = 385.48), baseline separation of the compounds is mandatory. Thus, adaptation of the chromatographic method was required to achieve a total separation of the API and its related compounds.

For the previously reported UV experiments and for these MS experiments, the same kind of instrument was employed. However, in the second case, several modifications were brought to the system to permit its hyphenation to the MS detector. As depicted by Fig. 2, a make-up pump and a pre-BPR split were added to the system. As expected, the presence of the splitter and additional pump led to an increase of the pressure measured in the chromatographic conditions(≈15% higher at the head of the pump).

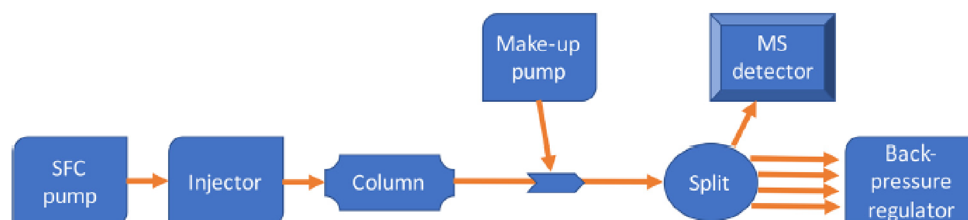

Fig. 2. Schematic representation of the SFC-MS system employed in this study.

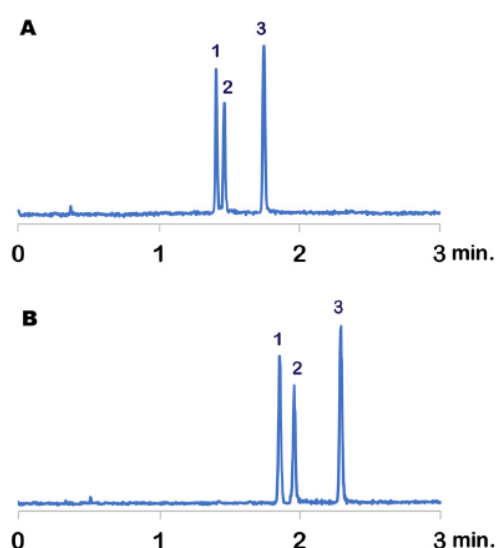

Fig. 3. Chromatograms obtained with (**A**) direct application of previously determined chromatographic parameters (flow rate – 2 mL min-1, column temperature – 41 °C, BPR – 110 bar, ethanol gradient slope – 3.0% min^{-1}; as presented in [35]); (**B**) adaptation of the chromatographic conditions to cope with the addition of MS to the system (SFC flow rate – 1.5 mL min^{-1}, make-up flow rate – 0.5 mL min^{-1}; other parameters being kept constant). Both chromatograms benefit from MS detection (ESI+, SIR, m/z – 385.48) and compounds are 1) pre-vitamin D3, 2) trans-vitamin D3 and 3) vitamin D3 (each 1 µg mL^{-1} in pure n-heptane).

However, as previously reported by Andri et al., the density and eluotropic strength of the mobile phase must be controlled and lowered to ensure baseline separation of these lipophilic compounds [43]. Consequently, as suggested by Tarafder et al. [44] in the context of method transfer in SFC, isopycnic conditions should be considered, which means that system pressure should be lowered to the original values (i.e. approximately 230 bar in the starting conditions). For this purpose, several alternatives could be investigated: changing the temperature, modifying the back-pressure setup or even changing the mobile phase flow rate. However, because they were already optimally defined by a Design Space approach [35], the temperature and gradient profile were not altered. The active back-pressure regulator of the instrument was already set to the lowest settable value (i.e. 110 bar). Thus, a reduction of the mobile phase flow rate was performed to reach initial pressure and mobile phase conditions. When reduced to 1.5 mL min^{-1} (originally 2.0 mL min^{-1} in the SFC-UV method), system pressure was similar as the one optimally defined by the DoE-DS methodology leading to use isopynic conditions (SFC-UV and SFC-MS method parameters are summarized in Supplementary Table 1). As presented by Fig. 3, baseline separation of all compounds was achieved in the optimized conditions, therefore showing the relevance of the robust method optimization already applied and its interest in the case of method transfer [45].

3.2. OPTIMIZATION OF THE MS DETECTION

3.2.1. ASSESSMENT OF VARIOUS MAKE-UP SOLVENTS

To obtain the higher signal intensity for the vitamin D3, various make-up solvents were tested. A permanently charged additive (e.g. ammonium formate or ammonium acetate) was added to the make-up solvent, which permitted to enhance the ionization process of the fat-soluble vitamin (i.e. only a ESI source was available)[11,29,46,47].

As depicted by Fig. 4, it was observed that ammonium acetate and ethanol might inhibit the ionization process, their presence providing the lower signal intensity for vitamin D3. In the other hand, intensity of the response was enhanced by the combination of methanol and ammonium formate. Indeed, in all cases, this combination provided the best response, regardless of the concentration of the salt present in the make-up. The difference of ionization between ethanol and methanol might be related to the proton affinity of these compounds (methanol = 754 kJ/mol, ethanol = 777 kJ/mol [48]). In presence of this mixture (i.e. methanol and AF), 10% v/v of water didn't provide an enhancement of signal intensity. However, presence of water might help to prevent salt precipitation inside the system (e.g. head of the pump or tubings). So, even if its impact seems not actually beneficial or detrimental for the signal's intensity (at 10% v/v level), water is expected to at least improve the reliability of the method in the case of intensive routine use. From the various AF concentration assessed, it resulted that the gain of signal intensity was not directly proportional to the concentration of the salt in the make-up solvent. As can be observed from Fig. 4, an improvement of only 20% of the signal intensity was noticed when AF concentration was nearly 7 times greater. Consequently, to ensure the reliability of the MS coupling, the lower concentration of ammonium formate (i.e. 0.06% m/v: 10 mM) was selected for further investigations.

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Status : Postprint (Author's version)

3.2.2. OPTIMIZATION OF MS DETECTION AND MAKE-UP COMPOSITION

Optimization of MS parameters was performed using DoE. This one also studied the proportion of water in the make-up. A desirability function was set to provide the maximal S/N ratio for all the case studied (e.g. all compounds in presence or absence of matrix).

Fig. 5 and Table 2 present the results of the optimization for all cases studied. Regarding the make-up composition, the higher proportion of water (i.e. 20% v/v) was found finally beneficial to signal to noise ratio. This observation is indeed related to the enhancement of ESI ionization process in presence of protic solvents (e.g. methanol/water mixture).

Regarding detectors settings, a cone voltage superior to 9–10 V was found detrimental for the signal to noise ratio. Past this value, S/N tends to decrease as cone voltage is augmented. This observation could be related to an "in-source" fragmentation of the studied molecule, which is known as quite fragile and can easily be degraded with light and/or temperature. Contrarily, the increase of capillary voltage provided a nearly proportional augmentation of S/N, leading to the selection of the upper value studied was defined as optimal working condition. According to Fig. 5, the definition of the lowest acquisition frequency seems mainly driven by the samples containing matrix of the medicine. However, even if this frequency is the lowest, it still allows the collection of enough data points under the chromatographic peak (i.e. >10 points) for quantitative purpose.

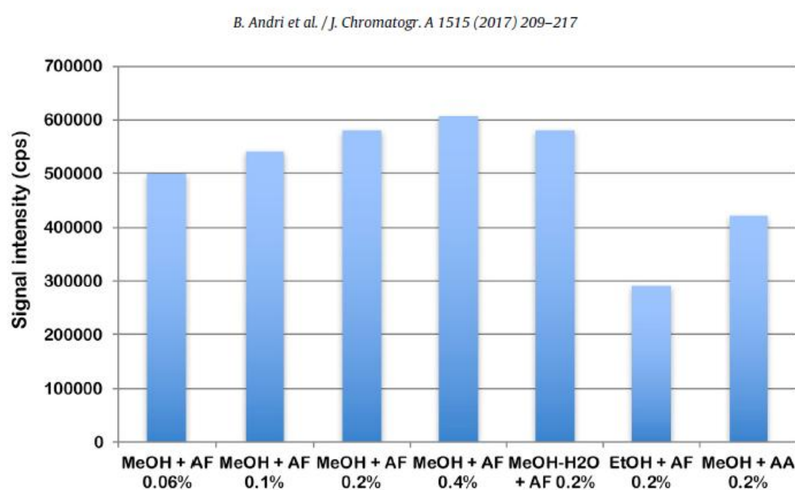

Fig. 4. Graphical representation of the mean signal intensity monitored (ESI+, SIR, m/z = 385.48) with triplicate injection (2 µL) of vitamin D3 (1 µg mL^{-1}) in presence of 0.5 mL min^{-1} of different make-up solvents.

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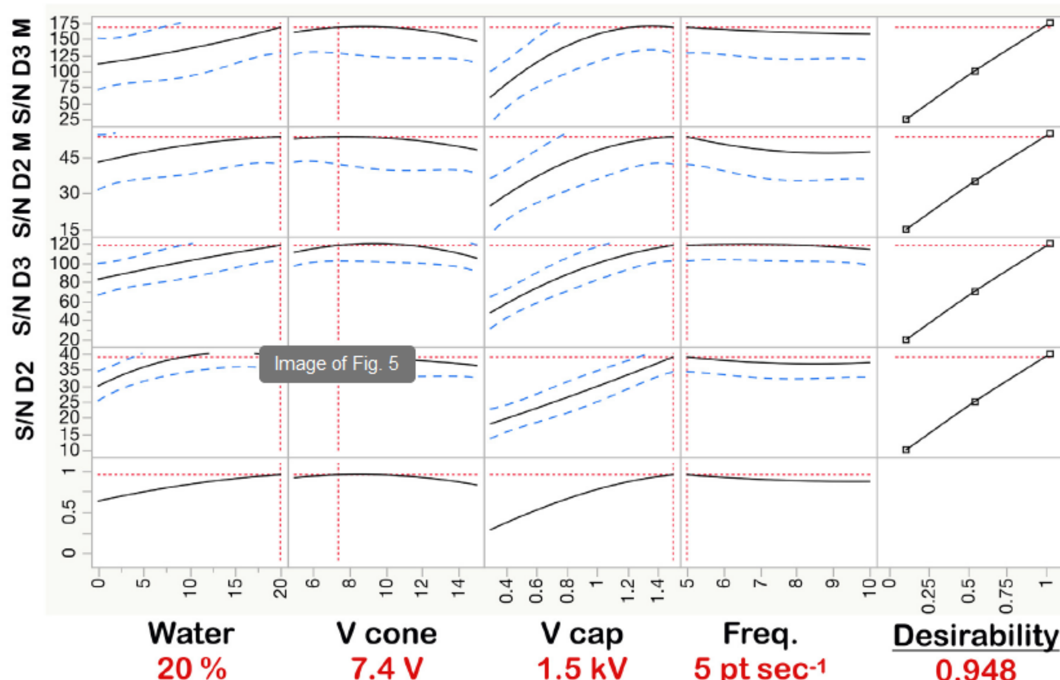

Fig. 5. Summary of the optimal parameters for MS detection obtained through the joint application of DoE and desirability function.

3.3. VALIDATION OF THE SFC-MS METHOD

As a first step of the validation process, the selectivity of the SFC-MS method was assessed by injections of i) *n*-heptane (blank solution with only the injection solvent), ii) matrix of the medicine diluted with *n*-heptane (absence of the active ingredient), iii) a solution of vitamin D3 in pure *n*-heptane and iv) a solution of vitamin D2 in pure *n*-heptane. No interferences were observed among the API, internal standard, medicine matrix and injection solvent (data not shown).

In this study, the quantitative performances of the optimized SFC-MS method were assessed for the quality control of vitamin D3 in an oily drug formulation. For this purpose, method validation by total error approach using accuracy profiles based on ß-expectations tolerance intervals was employed to challenge the analytical method. This predictive approach focused the validation process on the reliability of the future results of routine analysis. So, when the beta-expectation tolerance limits are included in the defined acceptance limits, it means that future analytical results will fall inside this acceptance interval with a pre-defined risk. In this case, the analytical method is therefore recognised as suitable for its intended purpose and is obviously considered as valid. For a medicine, the European Agency for Medicines defines a general acceptation criterion ±5.0% of the active ingredient (i.e. an interval of 95.0-105.0% of the declared content of the API) [25]. On this basis, accuracy profile was computed with acceptance limits set to ±5.0% regarding the target concentration of the medicine, which was set to 2.5 µg mL^{-1} or 100 UI mL^{-1} (i.e. 1000 folds dilution of the medicine). In this study, the dosing range investigated corresponds to50.0% of the target concentration (1.25–3.75 µg mL^{-1} or 50–150 UI mL^{-1}) which is larger than the dosing range required by ICH recommendations (i.e. interval = 80.0–120.0% for the assay of a drug substance or a finished (drug) product [49]).

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Table 2
Summary of the optimal chromatographic and MS parameters.

Chromatographic method			
Column	Acquity UPC2 BEH (3.0 × 100 mm; 1.7 μm)		
Temperature	41 °C		
Mobile phase	CO_2 + ethanol (organic modifier)		
Flow rate	(1.5 mL/min.)		
Gradient elution profile	Time (min.)	CO_2 (%)	EtOH (%)
	0	98	2
	2.8	89.5	10.5
	3.5	89.5	10.5
	3.51	98	2
	4.00	98	2
Back-pressure	110 bar		
Injection volume	3 μL		
Wash solvent	weak: propan-2-ol; strong: methanol		
MS detection parameters			
Ion source	Electrospray (positive mode)		
Cone voltage	7.4 V		
Capillary voltage	1500 V		
Acquisition rate	5 pt/sec.		
Make-up solvent	MeOH-H_2O (80:20) + 10 mM AF – flow rate = 0.5 mL/min.		
Targeted m/z ratio	385.48		
	397.64	- vitamin D3, trans-D3, pre-D3 - vitamin D2	

Response of the MS detector was found linear at these concentration levels and a regression model through 0 fitted to the higher level was employed as calibration model. Thus, only "one point" calibration curves were required, which is an obvious advantage for routine use of the method. The Table 3 makes the summary of the different validation criteria assessed.

3.3.1. TRUENESS

Trueness is calculated based on the calibration curve and expresses the mean relative bias (%) observed for the validation results at the targeted levels. Trueness of the SFC-MS method was studied on the three concentration levels and found satisfactory. Indeed, the relative bias at the target concentration is equal to 0.51%. The slightly higher bias observed for the lower concentration level may be associated to some extents to matrix effect and higher variability of the response due to the calibration model (regression through 0 fitted to the highest level).

3.3.2. PRECISION

Precision reflects the ability of the analytical method to provide similar results while experiments are repeated on the same samples and in the same experimental conditions. It is usually appreciated with relative standard deviation (RSD (%)) at two different levels: repeatability (intra-series) and intermediate precision (inter-series).

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Table 3
Validation criterion assessed for vitamin D3.

Vitamin D3				
Response Function	($s=4$; $l=3$; $r=3$; $n=3$)			
Model	Linear regression through 0 fitted to higher level			
	[1.25 –2.50 – 3.75 µg/mL]			
Series	S1	S2	S3	S4
Slope	1.413	1.406	1.430	1.410
Intercept	0	0	0	0
R^2	ND	ND	ND	ND

Vitamin D3		
Trueness	($s=4$; $r=3$; $n=3$)	
Level	Concentration (µg/mL)	Relative bias (%)
1	1.25	−1.333
2	2.50	0.506
3	3.75	0.0508

Vitamin D3		
Precision	($s=4$; $r=3$; $n=3$)	
Level	Repeatability %RSD	Intermediate precision %RSD
1	1.685	1.920
2	1.106	1.487
3	0.738	1.214

Vitamin D3	
Accuracy	($s=4$; $r=3$; $n=3$)
Level	Relative β-expectation tolerance limits (%)
1	[−5.938; 3.271]
2	[−3.314; 4.327]
3	[−3.336; 3.438]

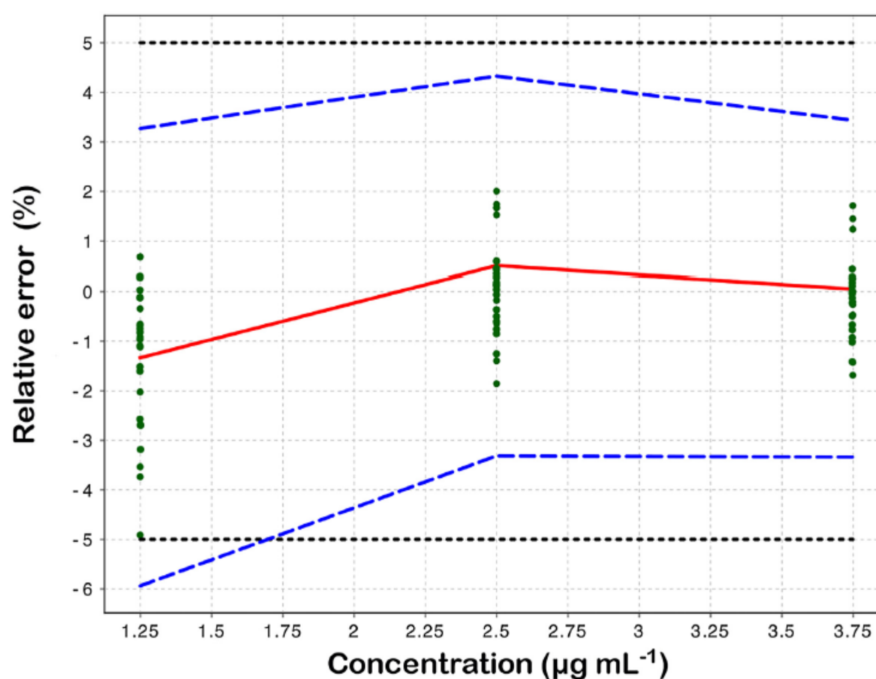
Vitamin D3	
Linearity	($s=4$; $l=3$; $r=3$; $n=3$)
Range	[1.25: 3.749 µg/mL]
Slope	1.007
Intercept	−0.0192
R^2	0.999

Vitamin D3		
LOD (µg/mL)	LLOQ (µg/mL)	ULOQ (µg/mL)
0.513	1.696	3.749

s = number of series of experiments.
l = number of concentration levels.
r = number of replicates per series and per levels.
n = number of experiments per replicate

Published in : J. Chromatogr. A (2017), vol.1515, pp. 209–217
DOI: 10.1016/j.chroma.2017.07.057
Status : Postprint (Author's version)

For each of the concentration levels assessed, the precision of the method was found satisfactory. As expected, the higher RSD values were found for the lower level of concentration. However, for repeatability and intermediate precision, these values were respectively not greater than 1.69% and 1.92%. Moreover, considering that these results were obtained with MS detection, which may be considered as more variable than UV, these RSD values (i.e. lesser than 2% for intermediate precision) really highlight the great quality of the SFC-MS method proposed and the use of relevant internal standard.



3.3.3. ACCURACY

Accuracy expresses the closeness of agreement between the test results and an accepted reference value, which is by convention accepted as a true value. Accuracy depicts the total error associated to the analytical results, which corresponds to the sum of random and systematic errors.

From 70.0–150.0% of the target concentration of vitamin D3, the lower and upper limits of the ß-expectations tolerance intervals fall inside the acceptance limits defined by EMA (95.0–105.0%) (Fig. 6). As a result, the SFC-MS method reported in this work is considered valid for this range. Given the validity of the method, it was employed to perform some QC on actual samples (supplementary Table 2).

Additionally, for results below ≈70.0% (i.e. <67.8%) of the targeted concentration, the ß-expectations limits are falling outside the ±5.0% acceptance limits. So, with these ±5.0% specifications, the method is not suitable for the determination of samples having vitamin D3 concentrations lower than 1.70 µg mL^{-1} (Table 3).

Published in : J. Chromatogr. A (2017), vol.1515, pp. 209–217
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3.3.4. Limit of detection, limit of quantification, dosing range

The limit of detection (LOD) corresponds to the lowest quantity of a compound which can be detected with certitude but not accurately quantified. For vitamin D3, LOD was determined according to Eq. (1) and was equal to 0.38 µg mL^{-1}.

$$LOD = \frac{LLOQ}{3.3} \quad (1)$$

The limit of quantification (LOQ) is the quantity of the substance in a sample that can be determined with a given accuracy in the prescribed conditions. It actually exists two different LOQ: the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). These limits are represented on the accuracy profile and corresponds to the lower and higher concentration for which ß-expectation tolerance interval fall outside the acceptance limits. In the present case, ß-expectations are comprised within all the acceptance limits defined for the whole range studied. So, LLOQ and ULOQ are respectively equal to 1.70 and 3.75 µg mL^{-1}, which are also defining the dosing range of the method: 67.8–150.0%.

3.3.5. Linearity

Linearity accounts for the capacity of the method to provide results which are proportional to the concentrations. In this case, regression analysis was performed among the true values and the estimated values yielded using the calibration model. A regression coefficient (R^2) equal to 0.999 was retrieved, strongly highlighting the relevance of the single point calibration model employed.

3.4. PERSPECTIVES ASSOCIATED TO THE METHOD

Thanks to the increased specificity and sensitivity associated to MS detection, it is possible to retrieve vitamin D3 impurities at low levels. This was indeed experimented for trans-vitamin D3 at a concentration corresponding to 1.0% of the API (i.e. 25 ng mL^{-1}). As depicted by Fig. 7, the peak corresponding to trans-vitamin D3 is easily detected. Moreover, although it was not the purpose of the present report, the S/N ratio of this peak (i.e. superior to 10) would potentially allow quantification of the impurity.

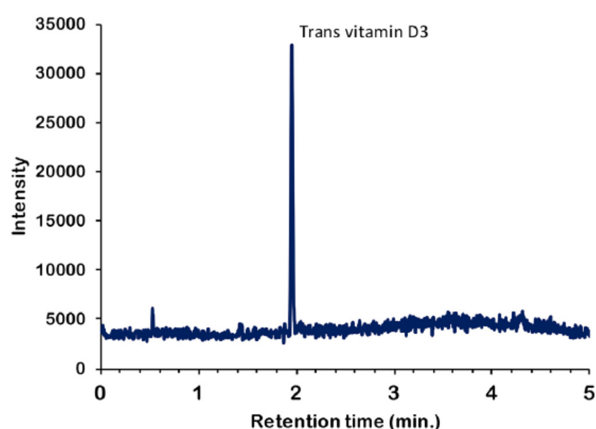

Fig. 7. Chromatogram obtained with the injection of 3.0 µL of a solution containing 25 ng mL^{-1} of trans-vitamin D3 (toxic impurity) in a solution of *n*-heptane-medicine matrix (999:1); MS detection (ESI$^+$, SIR, *m/z* = 385.48).

4. Conclusion

The present work demonstrates the possibility to use SFC-MS to perform some QC of medicines. Indeed, a robust analytical SFC method was adapted to deal with the hyphenation of an MS detector to the system by means of isopycnic methodology. A design of experiments was employed to optimize MS parameters and to adjust the make-up solvent composition. Then, the quantitative performances of the SFC-MS method were validated by a total error approach methodology using accuracy profile as decision tool. It permitted to demonstrate the validity of the method, while controlling the risks associated to further routine use. The SFC-MS analytical method meet the requirements established by the EMA, which mean that the method is adequate for the determination of vitamin D3 following the 95.0–105.0% specifications; over a dosing range equivalent to at least 70.0–130.0% (ICH recommendation = 80.0–120.0%). Also, the present SFC-MS method provides faster and greener analysis than NPLC method generally used to analyse theses oily formulations of lipophilic vitamins. Indeed, in SFC-MS, the retention time for vitamin D3 is equal to ≈2.3 min, while it is assumed superior to 19 min with the NPLC method described in monographs [50], which makes a noticeable difference in terms of analytical throughput (increased by a factor ≈8; and even greater if NPLC column re-equilibration is considered), solvent consumption (80 mL of n-hexane based mobile phase vs ≈10 mL of SFC mobile phase are required per analysis) and associated waste generation (2–3 mL/analysis: ≈ 65% of the mobile phase is released as CO2 at the end of the system and can be recycled for further analyses). Moreover, the additional value of the MS detection (i.e. sensibility, specificity and orthogonality to chromatography) and the validity of the hyphenated method reported clearly support the switch to greener analytical technique for the QC of vitamin D3. So, the pharmaceutical industry may benefit of a faster, cleaner, more selective and more sensitive way to perform the mandatory QC for medicines having currently a growing demand.

As these works were conducted with a single quadrupole mass detector, one can also expect further improvements of e.g. sensitivity when tandem mass spectrometers are hyphenated to a SFC system.

Acknowledgements

B. Andri kindly acknowledges the research grants from the Walloon region (project First International In-Scrit, n°1217860).

Appreciations and thanks are expressed to B. Streel and R. Klinkenberg (Galephar Research Center M/F, Belgium) for their stimulating discussions.

Special funds from the University of Liege (Fonds spéciaux pour la Recherche, number C-14/72) supporting the purchase of the MS equipment employed are kindly acknowledged.

Anonymous reviewers are also acknowledged for their useful comments.

Published in : J. Chromatogr. A (2017), vol.1515, pp. 209–217
DOI: 10.1016/j.chroma.2017.07.057
Status : Postprint (Author's version)

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.2017.07. 057.

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