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Technical Report

Optimization of Supercritical Fluid Extraction Parameters for Vitamins D2, D3, and K1 from Pharmaceutical Preparations

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Abstract:

The objective of this report was to optimize the supercritical fluid extraction (SFE) of fat-soluble vitamins (D2, D3, and K1) from pharmaceutical formulations followed by High-Pressure Liquid Chromatography-Photo diode array (HPLC-PDA) analysis. Various formulations with differing vitamin content were evaluated. The results indicated that, in addition to CO_2, the use of a water-rich modifier significantly enhanced the extraction yields of vitamins D2 and D3.

Keywords: Supercritical fluid extraction, fat-soluble vitamins, pharmaceutical preparation

1. Introduction

Vitamins are organic compounds, essential in small amount, crucial for the normal functioning of the human body, facilitating numerous enzymatic and metabolic functions[1]. Since the human organism cannot produce vitamins, the diet represents the main route of ingestion of these compounds. As consumer awareness grows, reliable information about the vitamin content in foods is becoming a critical issue. However, considering that vitamins could be losses during food processing and storage, the ready-to-use multi-vitamin pharmaceutical preparations are becoming widely employed among consumers. These facts, together with the introduction of labelling regulations, introduced the need for ensure the quality control of these types of formulation.

2. Experimental

2.1 Chemicals and reagents

Methanol (MeOH, grade ≥ 99.9 %), ethanol (EtOH, grade ≥ 99.5 %), acetonitrile (ACN, grade ≥ 99.9 %), ergocalciferol (vitamin D2), cholecalciferol (vitamin D3), and phylloquinone (vitamin K1) were purchased from Merck Life Science (Darmstadt, Germany).

2.2 Samples and sample preparation

Three commercial samples, one capsule (sample 1: S1) and two tablets (samples 2: S2 and 3: S3), were purchased in pharmacies located in Messina (Italy).

According to the supplement labels, the active ingredients content were: 25 µg of vitamin D3 and 50 µg of K1, 50 µg of vitamin D2, and 50 µg of vitamin D3, for S1, S2, and S3, respectively. Regarding the capsule sample, the capsules were broken, the powder portion was used, while the capsule one was discarded. The tablet samples were ground in a mortar and blended in a powder. The 0.2 mL extraction vessels were directly filled with the supplement powder without other additional steps.

At the end of the dynamic step, to remove the remaining solvent contained in the extraction vessel, 1 min of emission with 100 % of CO_2 was employed. Furthermore, in order to avoid analytes carryover, both the system and the SFE unit were washed for 3 min using 100 % of modifier at a flow rate of 1 mL/min. A gas-liquid separator allowed the elimination of the decompressed CO_2, while the liquid fraction was directly dripped and collected into a 32 mL glass vial without dispersing. To compare and determine the extraction yields for the target compounds, all the extracts obtained by SFE were diluted in volumetric flasks of 5, 10 mL, or 20 mL to have a controlled dilution volume and directly analyzed by HPLC-PDA. Each sample was analyzed in triplicate.

The HPLC-PDA analysis were carried out with a Prominence LC-20A system (Shimadzu, Kyoto, Japan) composed by: a CBM-20A, a DGU-20A on-line degasser, two LC-20AD dual-plunger parallel-flow pumps, a SIL-20AC autosampler, a CTO-20A column oven, and an SPD-M30A diode array detector. Separation was achieved on a Shim-pack GIS C18-P with the following dimension: 25 cm × 4.6 mm ID, 5.0 µm *dp* (Shimadzu). The elution was performed in isocratic mode with MeOH (100 %). The flow rate was 1.5 mL/min, injection volume: 5 µL; the column temperature was maintained at 40 ˚C. The UV-vis spectra were acquired in the range of 190-460 nm, while the chromatograms were extracted at 265 nm (sampling frequency: 4.1667 Hz; time constant: 0.480 s).

The analytical conditions are reported in Table 1. Figure 1 reports the HPLC-PDA chromatogram of the vitamins standard mixture at a concentration of 5 mg/L. The standard stock solutions of each vitamin D2, D3, and K1 (10000 mg/L) were prepared in EtOH and stored in amber vials at 4 ˚C until use. Six-point calibration curves were constructed by analyzing standard solutions at the following

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concentration range 0.2-50 mg/L. The concentration ranges were chosen according to vitamins content in the commercial dietary supplements.

Table 1: HPLC-PDA analytical conditions.

Column	Shim-pack GIS C18-P (25 cm × 4. 6 mm ID, 5.0 μm d_p)
Column oven	40 °C
Mobile phase	MeOH
Elution	Isocratic
Flow rate	1.5 mL/min
Injection volume	5 μL
UV detection wavelength	265 nm

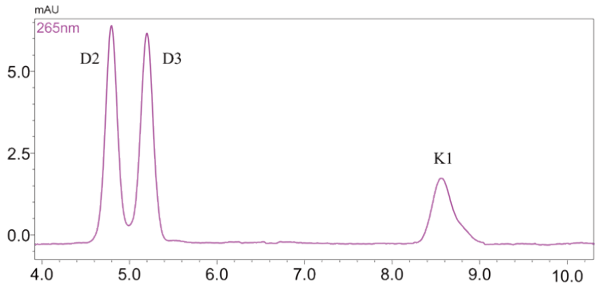

Figure 1: HPLC-PDA chromatogram of the vitamins standard mixture (5 mg/L).

2.3 Instrumentation

The supercritical fluid extraction (SFE) was carried out by using a SFE pretreatment system (Shimadzu Nexera UC) equipped with: a CBM-40 controller, an SFE-30A module, an LC-30AD$_{SF}$ CO_2 pump, an LC-40D XR modifier pump, an LC-40D make-up pump, an SFC-30A back pressure regulator, a DGU-40 on-line degasser, an FRC-40 fraction collector. A combination of static and dynamic extraction was employed (Figure 2).

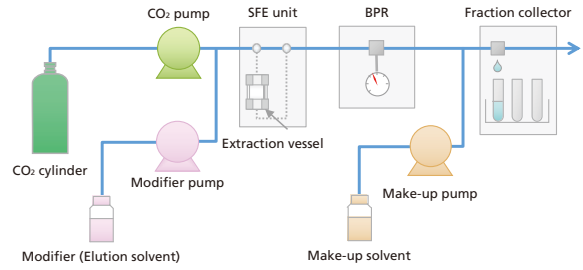

Figure 2: Flow diagram of the supercritical fluid extraction system.

3. Results and discussion

As initial experiment, we started from S1 (capsule with vitamins D3 and K1) with the extraction conditions for M1 shown in Table 2. At these conditions, the vitamins recovery was equal to 0 % and 69 % for vitamin D3 and K1, respectively. By increasing the extraction temperature to 50 ˚C, the flow to 2 mL/min, the dynamic extraction up to 18 min, and the pressure to 350 bar, the conditions for M2 in Table 2 resulted in a recovery of 78.5 % for vitamin K1(log k_{ow} 10.9). However, considering its slightly higher polarity (log k_{ow} 7.9), vitamin D3 was not extracted under the conditions for M2 (using MeOH) and M3 (using EtOH, a greener alternative solvent). Additionally, no improvements were observed neither using a higher modifier concentration (20 %), increasing the total extraction time from 4 to 19 min, or with higher extraction temperature or pressure. Considering the poor recovery obtained values, a comparison with a previously published study was performed[2].

Table 2: SFE methods employed for vitamin D3 extraction in S1, along with recovery percentage values.

Method	Extraction Temperature	Flow	BPR	static + dynamic extraction (min)	Mobile Phase (v:v(:v))	Recovery (%)
M1	30 ˚C	1 mL/min	150 bar	1+3	CO_2:MeOH (90:10)	0
M2	50 ˚C	2 mL/min	350 bar	1+18	CO_2:MeOH (90:10)	0
M3	50 ˚C	2 mL/min	350 bar	1+18	CO_2:EtOH (90:10)	0
M4	30 ˚C	1 mL/min	150 bar	1+3	CO_2:MeOH:H_2O (90:9:1)	0
M5	30 ˚C	1 mL/min	150 bar	1+18	CO_2:MeOH:H_2O (90:9:1)	0
M6	30 ˚C	1 mL/min	150 bar	1+18	CO_2:EtOH:H_2O (90:9:1)	0
M7	30 ˚C	2 mL/min	150 bar	1+18	CO_2:MeOH:H_2O (90:9:1)	51
M8	50 ˚C	2 mL/min	350 bar	1+10	CO_2:MeOH:H_2O (90:9:1)	90
M9	30 ˚C	2 mL/min	350 bar	1+18	CO_2:MeOH:H_2O (90:9:1)	70
M10	50 ˚C	2 mL/min	350 bar	1+18	CO_2:MeOH:H_2O (90:9:1)	98
M11	50 ˚C	2 mL/min	350 bar	1+18	CO_2:EtOH:H_2O (90:9:1)	99

Particularly, a salting-out assisted solid-liquid extraction using 1 g of sample, 10 mL of H_2O, and 10 mL of ACN was conducted. The mixture was sonicated for 15 min, followed by the addition of 7.5 g of NaCl for the salting-out purpose. The final mixture was vortexed (2 min at 1500 rpm) and centrifuged for 5 min at 5000 rpm. In such conditions, vitamin D3 was recovered at 78 %.

Consequently, it was decided to use a water-rich modifier, keeping the modifier concentration unchanged while adding 10 % water (MeOH:H_2O, 90:10, *v/v*). It was shown that flow rate, temperature, and pressure were significant variables for the extraction of such compound.

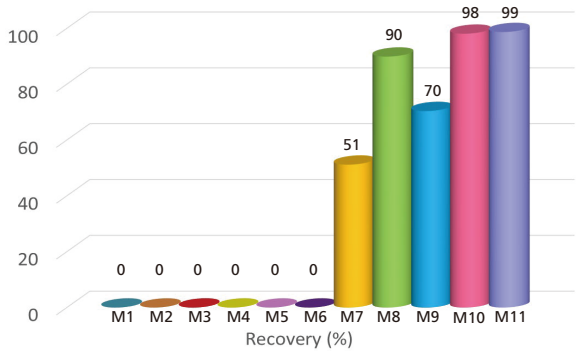

Figure 3: Bar-graph reporting the vitamin D3 recovery values obtained for S1 (with methods of Table 2).

Respect to the initial test of M5 (1 mL/min, 30 ˚C, 150 bar with water-rich modifier), where the recovery rate was 0 %, we changed the conditions to M7 through M10 (each combination of 2 mL/min, 50 ˚C, 350 bar), resulting in an increase in vitamin D3 recovery from 51 % to 98 %. A similar recovery value (99 %) was measured using EtOH:H_2O, 90:10, *v/v*, as a greener solvent. All the methods tested for S1 are reported in Table 2, while the obtained recovery values are reported in Figure 3.

Regarding S2 (tablet containing only vitamin D2), the extraction was performed at the same initial conditions employed for S1. Despite its slightly more polar nature compared to vitamin D3 (log k_{ow} 7.4 vs 7.9), using MeOH or EtOH as modifier, vitamin D2 was poorly recovered with values lower than 15 %. No recovery increments were observed neither by increasing the extraction time and temperature nor the modifier amount. Therefore, also in this case, a water-rich (MeOH:H_2O, 90:10, *v/v*) modifier was employed. As observed for S1, it was shown that both temperature and pressure were significant variables for the extraction of such compound. Also, the flow rate was increased from 1 to 2 mL/min.

The best recovery value (77 %) was obtained at the following conditions: 1+18 min of static and dynamic extraction, 2 mL/min, 50 ˚C, and 250 bar. The same recovery (77 %) was measured using EtOH:H_2O, 90:10, *v/v*.

Finally, for S3 (capsule containing only vitamin D3), the best recovery value (87 %) was obtained at the following conditions: 1+18 min of static and dynamic extraction, 1 mL/min, 50 ˚C, and 250 bar. A similar recovery (84 %) was measured using EtOH:H_2O, 90:10, *v/v*.

Conclusion

Considering the obtained results, the vitamins extraction revealed as strictly matrix dependent, according to the formulation excipients. Therefore, detailed study needs to be performed to better understand the vitamin formulation.

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