Fat-Soluble Vitamin Extraction by Analytical Supercritical Carbon Dioxide

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ABSTRACT: Extraction of fat-soluble vitamins (A, D, E, and β-carotene) by supercritical carbon dioxide (SC-CO_2) was tested to replace conventional liquid extraction methods, which require large volumes of organic solvents. Supercritical fluid extraction (SFE) is a rapid extraction technique for fat-soluble vitamins enabling them to be accurately determined using only small volumes of organic solvents. Extractions were performed on ultra-high-temperature sterilized milk, milk powder, pork, liver pâté (pâté de fois), infant formula, and canned baby food to compare the methods. The proposed method is based on the extraction of fat-soluble vitamins and their esters by using SC-CO_2 with methanol as a modifier. HPLC analysis using photometric detection was used for the vitamin analysis. The results showed no significant differences between extraction methods. The proposed SFE method appears to be useful as a substitute for the traditional organic solvent method, mainly for vitamin A and γ-tocopherol.

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KEY WORDS: Analytical SFE, fat-soluble vitamins, HPLC-UV-VIS, infant products, meat products, milk products, supercritical carbon dioxide.

Food industries such as the milk and meat product industries are always looking for faster and more efficient analytical methods to determine the vitamin content of their raw materials and final products. Conventional analytical methods for vitamins are generally suited for separate vitamin determinations, and at times they require large amounts of reagents, time, and samples (1–5). Vitamin A and E determinations are frequently based on a colorimetric method with purification of the extract on an alumina column before determination; β-carotene is determined by a spectrophotometric method, and vitamin D determination is carried out by HPLC. These methods require 40 g for samples containing 0.3 mg/100 g of vitamin A (6), and vitamin E determinations require 10 g of sample for the most common foods, 40 g for dry foods, and 60 mL for ultra-high-temperature milk (UHT-milk) (7); vitamin D_3 determination in UHT-milk requires 200 mL of sample (8). Hence, a large amount of sample requires the use of several hundred milliliters of traditional organic solvent for extraction and saponification, resulting in increased costs and environmental safety risks. Furthermore, extract purification, for colorimetric determination, requires an increase in analytical time, which causes vitamin degradation. Light and oxygen also degrade vitamins, mainly vitamins A and E.

Analytical supercritical fluid extraction (SFE) has been developed to replace traditional analytical methods using organic solvents (9–11). SFE is a fast extraction technique, and fat-soluble vitamins can be completely extracted and determined using only small volumes of organic solvents (12–17). The reported analytical method uses a sample amount in the range of 0.5–2.0 g. After extraction, the sample is saponified and re-extracted, allowing the extract to be analyzed directly by HPLC (18).

In this study fat-soluble vitamin extractions were conducted by SFE and conventional methods to allow a comparison of these two techniques. α-Tocopherol and vitamin A were determined in UHT-milk, milk powder, pork, liver pâté (pâté de fois), infant formula, and canned baby food; vitamin D_3 was determined only in UHT-milk and infant formula; and γ-tocopherol and β-carotene were determined in infant formula and canned baby food. This research was carried out under an international project (13) to compare analytical SFE with traditional methods for extracting fat-soluble vitamins from food. The results obtained illustrate the benefits of the proposed method.

EXPERIMENTAL PROCEDURES

Equipment. A dual-beam spectrophotometer (UV-vis) Varian DMS 200 (Varian Inc., Palo Alto, CA) with 1-cm quartz cells, capable of measuring absorbance in the range 190–800 nm, was used to determine the purity of the standard solutions. A supercritical fluid laboratory extractor, an FA-100™ (LECO Corporation, St. Joseph, MI), capable of extraction pressures up to 700 bar and temperatures up to 423 K and able to perform static and dynamic extractions, was used in this study. Another supercritical fluid laboratory extractor, an Spe-ed SFE™ (Applied Separations, Lehigh, PA), that allowed extraction pressures up to 700 bar and temperatures up to 523 K, was also used in this study. Extract collection was accomplished in the proper solvent, at room temperature and atmospheric pressure. Both units have no system pump to add the cosolvent, and there is no automatic addition of the collection solvent; therefore, the

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cosolvent was added manually during extractions with both units, and the collection solvent was manually refilled when needed (see the section on SFE conditions).

The HPLC system consisted of a Varian 2010 pump with a Rheodyne 7025 injector and equipped with a UV-vis Varian Varichrom detector. The detector was used at the sensitivity of 0.2 absorbance units full scale. A Varian DS 654 Integrator was used to quantify the peaks.

Materials. UHT low-lactose milk (1.5% fat), milk powder (fat content not available), pork (14% fat), liver pâté (23% fat), infant formula (milk powder mixture; 2.5% fat) and canned baby food (2.8% fat) were all market grade, as provided under the EU-FAIR project (13). The fat contents were in the accepted range for all products (19). Products were stored at 277 K before extraction analysis (13). L(+)-Ascorbic acid (p.a.), dichloromethane (HPLC), di-isopropyl ether (p.a.), *n*-hexane (HPLC), isopropyl alcohol (HPLC), methanol (HPLC), petroleum ether (313–333 K) (p.a.), and potassium hydroxide (p.a.) were all purchased from Carlo Erba (Milan, Italy). All-*trans*-retinol (≥99%), DL-α-tocopherol (≥98%), γ-tocopherol (≥97%) (vitamin E), cholecalciferol (≥99%), ergocalciferol (99%), retinol acetate (95% all-*trans* isomer, 5% *cis* isomer), β-carotene, and BHT (99%) were all purchased from Fluka (Buchs, Switzerland). Both acetonitrile (HPLC) and ethanol (99.9%) were purchased from J.T. Baker (Milan, Italy). Ammonium acetate (p.a.) was purchased from Rudi Pont S.p.A. (Torino, Italy). The hydrophilic inert support matrix (LECO-Dry) was purchased from LECO. 6-*O*-Palmitoyl-L-ascorbic acid (p.a.) was purchased from Merck (Darmstadt, Germany). Triethylamine (99%) was purchased from Sigma-Aldrich (Milan, Italy). Carbon dioxide (99.998%) and nitrogen (≥99.9995%) were obtained from Linde-Caracciolossigeno (Perugia, Italy).

Standard solutions. Two standard solutions were prepared. Standard solution A was composed of all-*trans*-retinol (5 mg in 10 mL ethanol), DL-α-tocopherol (10 mg in 10 mL methanol), γ-tocopherol (10 mg in 10 mL methanol), vitamin D_3 (10 mg in 10 mL ethanol), vitamin D_2 (10 mg in 10 mL ethanol), retinol acetate (10 mg in 10 mL ethanol), and β-carotene (3 mg in 100 mL *n*-hexane). Standard solution B (obtained from solution A for use in serial dilution for calibrating standards) was composed of all-*trans*-retinol (1 mL solution A in 10 mL ethanol), DL-α-tocopherol (5 mL solution A in 50 mL ethanol), γ-tocopherol (5 mL solution A in 50 mL ethanol), vitamin D_3 (500 μL solution A in 50 mL ethanol), vitamin D_2 (500 μL solution A in 50 mL ethanol), retinol acetate (500 μL solution A in 10 mL ethanol), and β-carotene (5 mL solution A in 50 mL *n*-hexane).

Purity tests on standard solutions were carried out by measuring the absorbance of solutions in a 1-cm quartz cell with the appropriate solvent in the reference cell. The concentration was then calculated in μg/mL using the corresponding molar extinction coefficient (*The Merck Index,* London, United Kingdom). All standard substances and solutions were stored in amber vials refrigerated at 255 K, and all standards were checked for purity before analysis.

A 50% potassium hydroxide solution was prepared by dissolving 20 g of potassium hydroxide in 20 mL of MilliQ water (Millipore Corporation, Bedford, MA).

Sample preparation. (i) Mixture A: UHT-milk, milk powder, meat, and liver pâté. A 0.5 ± 0.05 g sample (2 ± 0.05 g was used for the determination of all-*trans*-retinol and α-tocopherol, whereas 10 ± 0.05 g was used to determine vitamin D_3 in UHT-milk) was mixed with 1 ± 0.05 g of inert support matrix and 0.5 ± 0.05 g of ascorbic acid in a mortar.

(ii) Mixture B: infant formula. A 0.5 ± 0.05 g sample was mixed with 0.5 ± 0.05 g of water in a mortar with a glass rod until no clumps of sample were visible. The wet sample was mixed with 1.0 ± 0.05 g of inert support matrix and 0.5 ± 0.05 g of ascorbic acid in a mortar.

(iii) Mixture C: canned baby food. A 1.0 ± 0.05 g sample was mixed with 1.0 ± 0.05 g of inert support matrix and 0.5 ± 0.05 g of ascorbic acid in a mortar.

The sample mixtures (A, B, C) were put into a thimble containing 0.4 ± 0.05 g of inert support matrix on the bottom and were gently pressed to set. LECO-Dry inert matrix was then added until the extraction vessel was filled, and 1.5 mL of methanol was added to the top of the extractor vessel when the addition of cosolvent was required and after neat SC-CO_2 extraction when required.

SFE conditions. Vitamin A, D_3, α-tocopherol, and γ-tocopherol extractions were carried out using a single extraction process for UHT-milk, milk powder, meat, and liver pâté. The extraction lasted 15 min (static) plus 60 min (dynamic); a liquid CO_2 flow rate of 1.082 g/min was used while performing the extraction. Methanol was added as a cosolvent at a 5% mass ratio (mole fraction = 0.066). Extraction pressure and temperature were 366 bar and 353 K, respectively (extraction solvent density, 0.823 g/mL). The restrictor temperature was held at 358 K. Collection solvent was 15 mL of isopropyl ether/ethanol (50:50 vol/vol) containing approximately 5–10 mg of palmitoyl ascorbic acid.

A dual extraction was conducted to extract the above-mentioned vitamins using neat SC-CO_2 (first extraction) as well as to complete the β-carotene extraction for infant formula and canned baby food by adding methanol as a cosolvent (second extraction). The first extraction was conducted with neat SC-CO_2 using a static extraction time of 5 min and a dynamic extraction time of 40 min, a pressure of 380 bar, an extraction temperature of 313 K (CO_2 density 0.954 g/mL), a restrictor temperature of 318 K, and dichloromethane/*n*-hexane (50:50 vol/vol) containing 5–10 mg of BHT as the collection solvent. The second extraction was conducted by adding methanol as a modifier; extraction pressure and temperature were 380 bar and 353 K, respectively (extraction solvent density, 0.830 g/mL; mole fraction = 0.066). The restrictor temperature was 358 K, and the collection solvent was 15 mL of isopropyl ether/ethanol (50:50 vol/vol) containing 5–10 mg of palmitoyl ascorbic acid.

The LECO FA-100 extractor described above was used to determine vitamin A, E, and β-carotene concentrations. To obtain the required CO_2/methanol ratio, 2 mL of methanol

was added manually every 10 min during the extraction, depressurizing each time. To reach the total extraction time, four successive 10-min dynamic extractions were conducted. Only vitamin D_3 extractions from UHT-milk and infant formula were carried out with the Applied Spe-ed SFE extractor, and a 50-mL vessel and 10 g of sample were used to increase the vitamin concentration. Methanol was added manually, as when using the LECO FA-100.

Saponification. SFE extracts were concentrated under nitrogen and then saponified by adding 4 mL of ethanol, 10–20 mg of ascorbic acid, and 1 mL of 50% KOH solution to the extract. When vitamin D_3 was determined in UHT-milk, 100 μL of vitamin D_2 (approx. 10 μg/mL) was added as internal standard to correct for losses during saponification. The sample tube containing the extract was gently flushed under nitrogen and then maintained in a water bath (313 K) for 30 min. The sample was then shaken vigorously every 10 min during the saponification process.

When saponification was completed, the sample was cooled under tap water (light was excluded during the entire process). Then 6 mL of MilliQ water and 5 mL of petroleum ether were added to the collection tube, which was shaken vigorously for at least 1 min. After equilibration, the tube was cooled under tap water to separate the phases, and 4.5 mL of the organic phase was decanted off and washed twice with MilliQ water. This was followed by removal of 4 mL of the organic phase which was evaporated to dryness under nitrogen. Ethanol (500 μL) was then added as solvent and the resultant solution was filtered, followed by direct injection into the HPLC system. When the vitamin concentration was close to the detection limit, 50 μL of retinol acetate solution (*ca.* 25 μg/mL) was added as internal standard, whereas only 200 μL of ethanol (instead of 500 μL) was added as solvent to improve detection.

HPLC. Vitamins were identified by comparing the retention times of individual peaks with the chromatograms obtained using the standard solutions. Vitamin concentrations were calculated with two mathematical formulas (see Equations 1 and 2 below) using a concentration curve established with standards.

All HPLC determinations were carried out at least in duplicate; for UHT-milk and milk powder samples, four determinations were made.

HPLC conditions were as follows: flow rate, 1.0 mL/min; temperature, 291–296 K; injection volume, 20 μL; column, Alltima C_{18} (Alltech Italia, Sedriano MI, Italy), film thickness 5 μm, 250 × 4.6 mm (for retinol, vitamin D_3, γ-tocopherol, and α-tocopherol), and VYDAC 201TP54 C_{18} (Grace-Vydac, Hesperia, CA), 250 × 4.6 mm (for β-carotene). The HPLC mobile phase was methanol/water (96:4) for the vitamin A, vitamin D_3, γ-tocopherol, and α-tocopherol assays, and acetonitrile/0.05 M ammonium acetate in methanol/dichloromethane (75:20:5), containing 50 mg BHT/L and 0.5 mL/L triethylamine, for the β-carotene assay. Detection wavelengths for the above analytes were 325 nm for vitamin A, 265 nm for vitamins D_2 and D_3, 295 nm for γ- and α-tocopherol, and 450 nm for β-carotene.

Calculations. For all-*trans*-retinol, α-tocopherol, γ-tocopherol, and β-carotene,

$$C = \frac{C^{\text{std}} A^s V^1 V^2 100}{A^{\text{std}} V^3 m} = \frac{C^{\text{std}} A^s 250}{A^{\text{std}} 4m} \quad [1]$$

where C = the calculated concentration of vitamin (mg/100 g); C^{std} = concentration of the standard solution (mg/mL); A^s = vitamin peak area of the sample; A^{std} = vitamin peak area of the standard solution; m = weight of the sample (g); V^1 = final volume of the sample extract before HPLC analysis (mL); V^2 = volume of extraction solvent added to the saponified extract (mL); and V^3 = volume of washed organic phase evaporated with nitrogen (mL).

For vitamin D_3,

$$C = \frac{V^1 C^e 100}{mQ} \quad [2]$$

where C = calculated concentration of vitamin (mg/100 g); C^e = concentration of vitamin in the extract (mg/mL); V^1 = final sample volume (V = 0.250 mL); m = weight of the sample (g); and Q = correction coefficient for vitamin, expressed as

$$Q = \frac{\text{area } D^s}{\text{area } D^i} \quad [3]$$

where D^s = area of internal standard (vitamin D_2) in the sample; and D^i = area of internal standard (vitamin D_2) in the internal standard mixture.

Conventional methods. Analyses were conducted under the EU-FAIR project using liquid extraction methods (13). The concentrations of vitamin A and vitamin E in UHT-milk, milk powder, meat, liver pâté, infant formula, and canned baby food were determined by colorimetric methods (6,7). The concentrations of vitamin D_3 in UHT-milk and infant formula were determined by the AOAC liquid chromatographic method (8). The concentrations of β-carotene in infant formula and canned baby food were determined by the AOAC spectrophotometric method (20).

Statistical analysis. Data from the analyses were processed using Excel spreadsheets (Microsoft, Redmond, WA). They were then evaluated for SD and reported as percentage relative standard deviation (RSD%) for the intralaboratory repeatability assessments.

RESULTS AND DISCUSSION

Data comparing the vitamin concentrations obtained by the SFE method and traditional organic solvent methods are reported in following tables. The concentration of vitamin A in all samples is reported in Table 1. Generally, SFE gave recoveries comparable to the traditional method and with better repeatability (RSD%) (21,22). The recovery of vitamin A in liver pâté was 103%. The concentration found for vitamin A in milk powder was moderately high compared to the traditional method, and its recovery was good (119%). Vitamin A in liquid milk and infant formula extracted by SFE was lower

TABLE 1
Determination of Vitamin A by Supercritical Fluid Extraction (SFE) and the Conventional Method[a]

	SFE		Conventional (13)		Literature (19)
	(mg/100 g)	RSD%	(mg/100 g)	RSD%	(mg/100 g)
UHT-milk	0.040	0	0.055	11	0.020
Milk powder	0.167	2	0.140	4	0.350
Pork	ND	—	0.005	9	Traces
Liver pâté	4.859	1	4.700	2	7.330
Infant formula	0.234	1	0.430	1	0.395
Canned baby food	<0.0005	—	0.001	7	—

[a]$n = 4$ for canned baby food; $n = 2$ for other samples; RSD%, percentage relative standard deviation; ND, not detected; UHT, ultra-high-temperature.

than the traditional method. The recovery was only 67% for liquid milk. It is possible to predict the influence of high water content to contrast the extraction. The restrictor can be plugged with polar compounds (proteins, carbohydrates, etc.) dragged out with water during the vitamin extraction. The recovery of vitamin A from infant formula was 54%. In this case, degradation of the sample could explain the low efficiency of this particular extraction. We did not find any vitamin A in pork because of the very low amount of this vitamin in pork, as reported in the literature (19) and as detected by the conventional methods. The concentration of vitamin A in canned baby food was lower than 0.0005 mg/100 g. To increase the recovery of vitamin A, either an increase in extraction time or pretreatment of the sample with acids to free the fat and fat-soluble vitamins from muscle tissues may help.

The α-tocopherol extracted by SFE compared with that extracted by conventional methodology is reported in Table 2. Good agreement was shown between SFE and the literature values, and the RSD% was generally lower for SFE than for the conventional methods. The recovery from pork was 80% compared with the traditional method. SC-CO_2 enabled the extraction of 69% of that found in UHT-milk by extracting with an organic solvent. The recovery of α-tocopherol from liver pâté was 59%, and that from milk powder was 55% compared to the traditional method. The recovery from canned baby food was only 50%, whereas it was only 17% from infant formula. The content of vitamin E in SFE extracts was generally lower than for the conventional methods. This

TABLE 2
Determination of α-Tocopherol by SFE and the Conventional Methods[a]

	SFE		Conventional (13)		Literature (19)
	(mg/100 g)	RSD%	(mg/100 g)	RSD%	(mg/100 g)
UHT-milk	0.022	1	0.032[b]	—	0.030
Milk powder	0.219	0	0.400	3	0.270
Pork	0.193	0	0.240	10	0.000
Liver pâté	0.238	1	0.400	6	—
Infant formula	1.338	3	8.000	3	1.320
Canned baby food	0.207	4	0.410	4	—

[a]$n = 2$. For abbreviations see Table 1.
[b]$n = 1$.

TABLE 3
Determination of γ-Tocopherol by SFE and the Conventional Methods[a]

	SFE		Conventional (13)		Literature (19)
	(mg/100 g)	RSD%	(mg/100 g)	RSD%	(mg/100 g)
Infant formula	1.724	1	1.600	3	—
Canned baby food	0.423	2	0.460	1	—

[a]$n = 2$. For abbreviations see Table 1.

may have been a consequence of considerable degradation of this vitamin, and certain changes in the procedure suggested by the EU project (13) are required (e.g., the samples should be stored at 193 K instead of 277 K).

The concentrations of γ-tocopherol in the various foodstuffs are reported in Table 3. This concentration was 108% for infant formula using SFE compared with the traditional method, and in canned baby food it was 92%; both results were repeatable. The different behavior of γ-tocopherol compared to α-tocopherol was probably due to their different antioxidant activities, which are proportional to the speed of degradation (23).

The concentration of vitamin D_3 in UHT-milk (Table 4) was found to be lower than the instrumental detection limit when extracted using SC-CO_2. Vitamin D_3 in infant formula was very high in comparison with both the official method and the literature data. This was probably due to the effectiveness of the SFE procedure, which is very rapid, thus preserving vitamin D_3 from oxidative degradation and light exposure. The recorded RSD% was below the official requirements.

The concentration of β-carotene determined by SFE and the official method is reported in Table 5. Although not detected in infant formula by SFE, it was shown by the conventional method. In canned baby food the β-carotene concentration found by using SFE was very low, with a high RSD% (6%). Similar to vitamin E, the very low recovery of β-carotene is probably due to considerable degradation in the stored sample before the extraction. Conventional extractions were conducted several months earlier on samples from the same batch.

The RSD% with conventional methods was comparable to that obtained for SFE. In any case, RSD% were below the 30% target performance parameter for analysis under the U.S. Environmental Protection Agency analyte recovery control limits, at mg/kg or μg/kg concentration range (21). The

TABLE 4
Determination of Vitamin D_3 by SFE and the Conventional Methods[a]

	SFE		Conventional (13)		Literature (19)
	(mg/100 g)	RSD%	(mg/100 g)	RSD%	(mg/100 g)
UHT-milk	<0.00008	—	0.0004[b]	—	0.00001
Infant formula	0.021	10	0.009	4	0.011

[a]$n = 2$. For abbreviations see Table 1.
[b]$n = 1$.

TABLE 5
Determination of β-Carotene by SFE and the Conventional Methods[a]

	SFE		Conventional (13)		Literature (19)
	(mg/100 g)	RSD%	(mg/100 g)	RSD%	(mg/100 g)
Infant formula	N.D.	—	0.009	8	0.015
Canned baby food	0.030	6	1.120	5	—

[a]$n = 2$. For abbreviations see Table 1.

RSD% requirements in the AOCS SFE official method for total fat determination indicate a variance between 1.13 and 4.67% for differing concentrations of analytes (22).

It is possible to make both positive and negative appraisals regarding the described method. The method is compatible with different matrices for meat, meat products, dairy products, and infant products, as demonstrated in this paper. Similar results were obtained by others authors (13–17), and more positive results were shown in other studies on Mediterranean food products, such as salami and Parmigiano cheese (15). Although it is a fast and efficient method, limitations also exist. For example, α-tocopherol and β-carotene are inadequately extracted by SFE and, under these conditions, are highly sensitive to sample degradation. Further studies are in progress to investigate the best way to increase the extraction of α-tocopherol and to optimize the extraction of vitamin D_3.

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