Isolation and Separation of Tocopherols from Olive By-products with Supercritical Fluids

E. Ibáñez[a,*], J. Palacios[a], F.J. Señoráns[b], G. Santa-María[a], J. Tabera[b], and G. Reglero[b]

[a]Instituto de Fermentaciones Industriales, C.S.I.C., 28006 Madrid, Spain, and [b]Departamento de Química Agrícola, Geología y Geoquímica, Facultad de Ciencias, Universidad Autónoma de Madrid, Ciudad Universitaria de Cantoblanco 28049 Madrid, Spain

ABSTRACT: Supercritical fluid extraction of olive pomace, the semisolid residue obtained using two-phase olive oil production systems, and supercritical fluid chromatographic separation of the extracts were performed to study the content of tocopherols, a group of compounds of interest for the food industry owing to their antioxidant activity. The developed method consists of supercritical CO_2 extraction at pilot plant scale and subsequent fractionation by two successive depressurizations. Enrichment of α-, β-, and γ-tocopherol was achieved in separator 2 when working at low densities in the first separator. Fractions obtained using high densities in separator 1 contained major proportions of triglycerides, waxes, and sterols. Tocopherols from olive by-products were separated and quantified in an environmentally friendly way by using supercritical fluid chromatography with packed capillary columns coated with polyethylene glycol and neat CO_2 according to a method previously optimized in our laboratory. The studied olive by-products can be considered a natural source of antioxidants because substantial concentration of tocopherols have been obtained in the extracts. The isolation and separation of tocopherols from olive pomace by applying supercritical fluid technology provides an interesting approach to exploit such by-products in an environmentally friendly way.

Paper no. J9319 in *JAOCS 77,* 187–190 (February 2000).

KEY WORDS: Olive by-products, packed capillary columns, pilot plant apparatus, supercritical fluid fractionation, tocopherols.

Olive oil has extraordinary economic and social importance in the Mediterranean basin, especially in Spain and Italy, the main producers in the world. The costs associated with the olive harvest and olive oil extraction are very high. Therefore, achieving optimal use of all the products derived from the olive grove is essential to make its cultivation profitable. Research regarding olive oil and other products derived from olive groves addresses aspects such as olive oil production, olive fruit composition, harvest, storage and processing of olive fruit, by-products of olive fruit processing, factors affecting olive oil quality, methods of olive oil analysis, and nutritional and health aspects (1).

*To whom correspondence should be addressed at Instituto de Fermentaciones Industriales, C.S.I.C., Juan de la Cierva, 3, 28006 Madrid, Spain. E-mail: elena@fresno.csic.es

Tocopherols are well known as components of vitamin E; their presence in olive oil has been extensively described (2) and can be expected in olive by-products (3). The tocopherols α-, β-, γ-, and δ- are widely distributed in natural products, but their biological activities differ from one another. At present, there is increasing interest on the part of the food industry in tocopherols because of their antioxidant activity and other nutraceutical effects (4).

Supercritical fluid extraction (SFE) and preparative supercritical fluid chromatography (SFC) have been recommended for selective isolation of tocopherols from natural products (5–8) owing to their efficiency and mild operating conditions. Residues from palm oil have been studied as a possible industrial source of natural tocopherols (9).

In a previous work (10), separation of the individual tocopherol species with neat CO_2 was accomplished by using packed capillary SFC. The optimization of the separation was based on the study of the column diameter and the type and percentage of the stationary phase coated on the silica particles. The developed method permitted the separation of the tocopherol analogs. This procedure has been used in the present work to study the tocopherol content of extracts obtained on a pilot plant scale with supercritical CO_2 at different fractionation pressures and temperatures.

The main objective of this work is to contribute to the methodology applicable to the reuse of the olive by-products and to the minimization of its environmental impact. Other objectives are to promote the industrial use of olive pomace as a sustainable source of tocopherols and to encourage the application of SFE as a clean processing technology. The work is presented as a first step toward scaling up an SFC column for fractionation of the tocopherol analogs found in olive by-products.

EXPERIMENTAL PROCEDURES

Samples. Samples of olive by-products corresponded to the semisolid residue collected at the outlet of a decanter after two-phase centrifugation of the olive paste for production of olive oil and were obtained from a olive oil factory in southern Spain. The moisture content of the samples was around

60%. These samples were kept at –20°C in the dark until use. Tocopherol standards (α-, β-, γ-, and δ-) were obtained from Merck (Darmstadt, Germany). Hexane used to prepare the solutions was purchased from Labscan (Dublin, Ireland). Tocopherol solutions were maintained at 2°C in dark glass flasks.

Calibration curves were prepared by analyzing different concentrations of tocopherol standards and by representing peak area vs. concentration (correlation coefficients between 0.95 and 0.98).

SFE. A pilot-scale supercritical fluid extractor (Iberfluid, Barcelona, Spain) was used to obtain olive by-product extracts (see Fig. 1). For all experiments, the 400-mL extraction cell was of 316 stainless steel, with a stainless-steel frit. Each extraction cell was filled with 300 g of olive pomace. Extraction time was 3 h, and each experiment was performed in duplicate. Extracts obtained by SFE at the different conditions tested (see Table 1) were diluted 50% (vol/vol) with hexane and injected directly into the SFC system. The extracts were maintained at 2°C in dark glass flasks.

SFC analysis. An SFC system (Carlo Erba, Milan, Italy) equipped with a flame-ionization detection system was used. Sample was loaded by a time-controlled, rotating-valve injection device (Vici, Houston, TX) containing a 1-µL internal loop. Injection time was 1500 ms, which correspond to the complete injection of the contents of the loop. The injector temperature was maintained at 30°C. Detector temperature was kept at 350°C. SFC-grade CO_2 (Liquid Carbonic, Madrid, Spain) was pumped by using an SFC 300 pump (Carlo Erba). A 13 µm i.d. × 25 cm linear restrictor made of fused-silica tubing (Composite Metal Services, Worcestershire, United Kingdom) was used to control the flow rate through the column. A packed capillary column that was prepared, as previously described (10), by using 500 µm i.d. × 25 cm deactivated stainless-steel tubing (Chrompack, Middelburg, The Netherlands) packed with 10-µm silica particles coated with 10% (w/w) Carbowax 20 M (polyethyleneglycol, Supelco, Bellefonte, PA) was used as the optimal chromatographic column to perform separation of tocopherol analogs with neat CO_2. The column was connected to the injection valve *via* a flow splitter. Fused-silica tubing (10 µm × 15 cm) was used as a flow split. Conditions of analysis were isothermal at 60°C and 320 bar constant pressure.

Gas chromatographic/mass spectrometric analysis. A Hewlett-Packard model HP-5890 gas chromatograph (Palo Alto, CA) with a mass spectrometer, detector model 5971A (electron impact, 70 eV) and a 30 m × 250 µm i.d. SE-30 (methylsilicone) (d_f = 0.25 µm) fused-silica capillary column was used. Injection was carried out at 250°C. Helium was the carrier gas (10 psi). The oven temperature was programmed from 230 to 275°C at 8°C min^{-1}. The final temperature was maintained for 30 min.

Two microliters of extracts obtained by SFE and diluted 50% (vol/vol) with hexane were injected.

The target compounds were identified by mass spectrometry in both SCAN (total number of ions) and SIM (selected ion monitoring) mode. SCAN mode was performed by using a

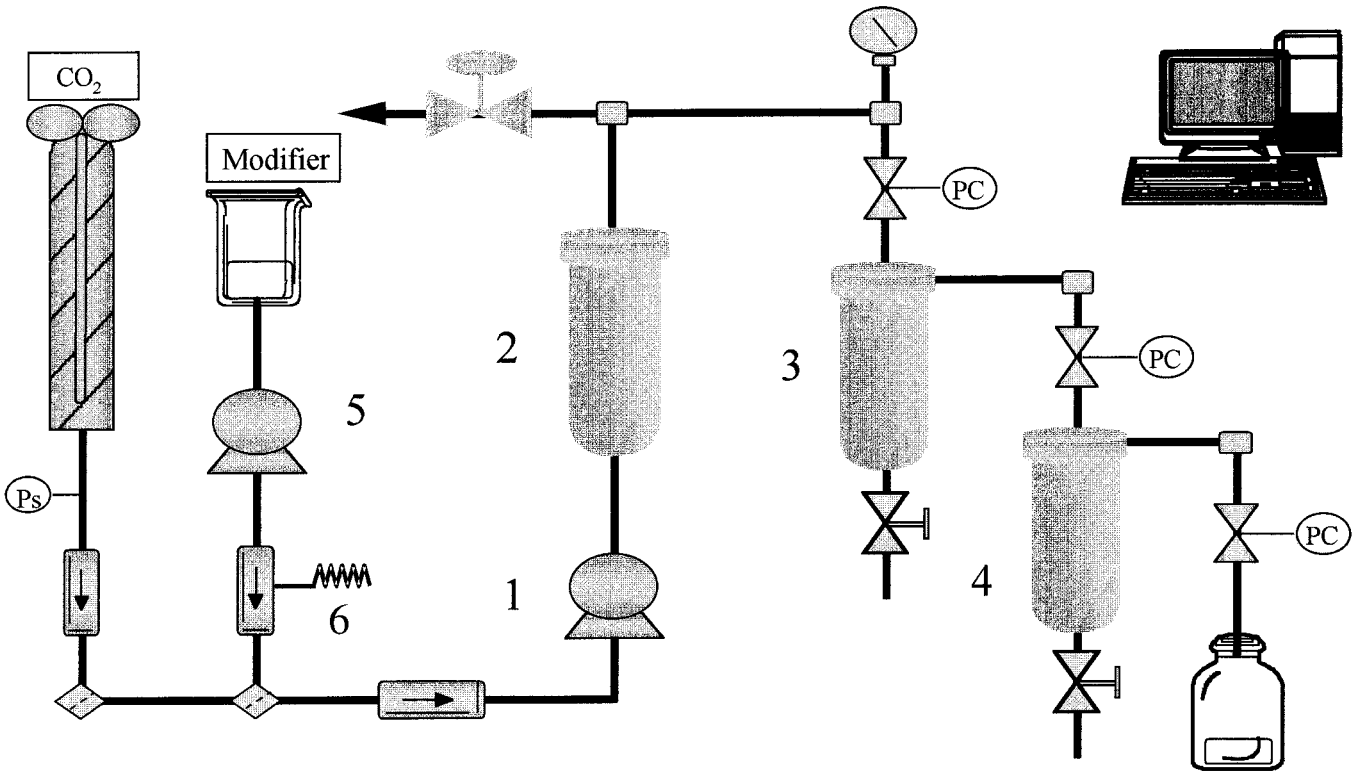

FIG. 1. Supercritical fluid extraction (SFE) pilot plant scale employed. (1) CO_2 pump, (2) extraction cell, (3) first separator, (4) second separator, (5) modifier pump, (6) pre-heater; PC, pressure controller; Ps, pressure switch.

TABLE 1
Experimental Conditions for the Pilot Plant Scale Supercritical Fluid Extractions

Experiment no.	EtOH[a] (mL/h)	P_{ext} (bar)	T_{ext} (°C)	ρ_{ext} (g/mL)	P_{s1} (bar)	T_{s1} (°C)	ρ_{s1} (g/mL)	P_{s2} (bar)	T_{s2} (°C)	CO_2 flow rate (mL/h)
1	100	350	50	0.9	100	40	0.63	10	25	2000
2	100	350	50	0.9	100	60	0.30	10	25	2000
3	100	350	50	0.9	135	40	0.75	10	25	2000
4	100	350	50	0.9	135	60	0.55	10	25	2000
5	100	350	50	0.9	165	47	0.77	10	25	2000
6	100	350	50	0.9	165	54	0.69	10	25	2000
7	100	350	50	0.9	200	47	0.81	10	25	2000
8	100	350	50	0.9	200	54	0.75	10	25	2000

[a]Ethanol was added as a modifier to increase the polarity of the CO_2, favoring the extraction of tocopherols. P_{ext}, extraction pressure; T_{ext}, extraction temperature; ρ_{ext}, extraction density; P_{s1}, pressure in separator 1; T_{s1}, temperature in separator 1; ρ_{s1}, density in separator 1; P_{s2}, pressure in separator 2; T_{s2}, temperature in separator 2.

mass interval ranging from 40 to 500 *m/z*. Analysis by SIM was used in order to confirm the tocopherol analogs in the extracts obtained by SFE. The ion at *m/z* 430 was selected for α-tocopherol identification, *m/z* 416 for β- and γ-tocopherol, and *m/z* 402 for δ-tocopherol. Spectra of the compounds were obtained and compared with those in the U.S. National Institute of Standards and Technology (NIST) library.

RESULTS AND DISCUSSION

SFE of olive pomace were performed in a pilot-scale plant at 350 bar and 50°C to achieve a high density (around 0.9 g/mL), which favors the extraction of most nonpolar compounds; the mild temperature is an additional desirable feature. For this reason, the maximum working pressure of the pilot plant was used, i.e., 350 bar, and a mild temperature (50°C) was selected to avoid degradation of thermally labile compounds such as tocopherols. Ethanol was added as a modifier to increase the polarity of the CO_2, thus favoring the extraction of tocopherols. Because the main goal of this study was the isolation and separation of tocopherols, fractionation conditions were varied so as to selectively enrich one of the fractions with tocopherols. Therefore, in using the conditions described in Table 1, two fractions of different composition were obtained. The fraction containing the tocopherols was collected in separator 2, whereas the fraction collected in separator 1 contained the low-polarity and high molecular weight compounds, mainly triglycerides, waxes, and sterols. Tocopherol content of the samples obtained by fractionation was analyzed by SFC using a packed capillary column with silica particles coated with polyethylene glycol, which allowed the separation of the different tocopherol analogs with neat CO_2. A detailed study of the different types and percentages of stationary phases for tocopherol separation has been published (10).

The developed method allowed the separation of tocopherol analogs at low temperatures, avoided solute decomposition, and used neat CO_2. The tested columns were robust, more than 100 injections were carried out without loss of efficiency. For the real samples analyzed, polyethylene glycol, used as stationary phase, achieved a satisfactory resolution among the different tocopherol analogs, with the separation between β- and γ-tocopherol (that is, the isomers most difficult to separate) higher than 1.5 (this separation refers to baseline separation as measured experimentally in the chromatogram). The dimensions of the columns have demonstrated their usefulness for easy interface SFE/SFC because sample capacity and loadability allowed their use without the need of an intermediate trap or accumulation device. These columns are presented as a first step toward scaling up an SFC process for fine fractionation of tocopherol analogs found in olive by-products.

Figure 2 shows the chromatograms corresponding to a tocopherol standard solution and to the extract obtained in the second separator at conditions of experiment 2. In the extracts obtained from the olive by-products, α-, β- and γ-tocopherol were detected, but no δ-tocopherol was found in any of the analyzed extracts. Identity confirmation was obtained by analyzing the samples with gas chromatography–mass spectrometry (GC–MS). By using the SIM mode, it was possible to confirm the presence of α-, β- and γ-tocopherol in the extracted samples.

The peak area corresponding to γ-tocopherol was abnormally high, indicating possible co-elution with another compound tentatively identified as squalene; therefore, the area attributable to γ-tocopherol was corrected using data obtained by GC–MS.

In Table 2, the tocopherol content in the supercritical fluid extracts from olive pomace obtained in separator 2, quantitative data for α-, and in some experiments, β-tocopherol and semiquantitative data for β-tocopherol in other experiments and γ-tocopherol are presented. The greatest concentration of tocopherols was found when pressures set in separator 1 were low (100–135 bar), i.e., when the differences between the pressures of the extraction cell and of the separator were highest. This fact could be due to the lower solubility of the triglycerides at lower extraction pressures, which resulted in accumulation of triglycerides under lower pressure in the first separator, and an enrichment of tocopherols in separator 2. On the other hand, the effect of the separator temperature seemed to be less under these extraction conditions, and similar results were found for the two temperatures tested at each pressure of the first separator. The major tocopherol found in

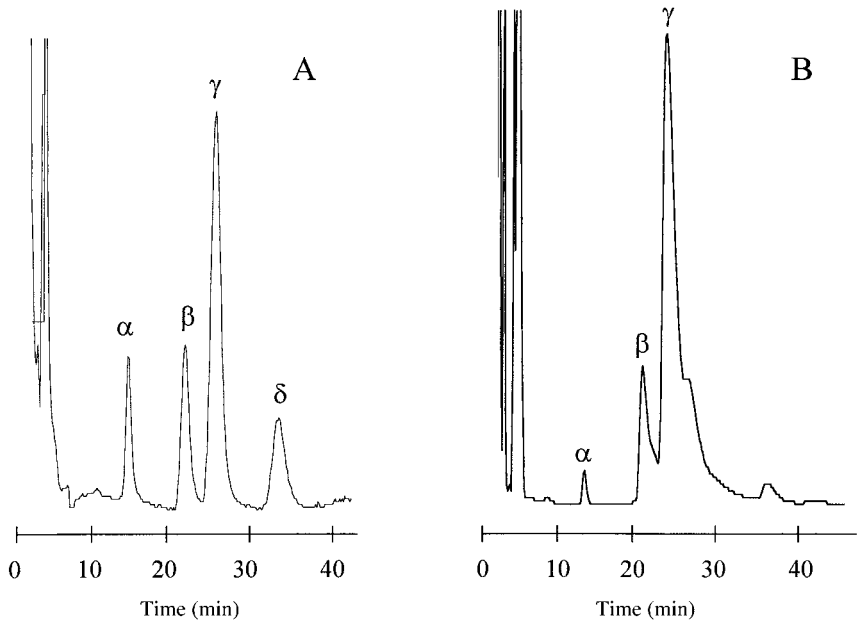

FIG. 2. Chromatogram of tocopherols, derived using supercritical fluid chromatography, in (A) a standard solution and (B) an SFE extract of olive by-product. Column 25 cm × 500 µm i.d.; 10% Carbowax 20 M (Supelco, Bellefonte, PA) coated silica particles (10 µm), 60°C, 320 atm.

olive pomace was γ-tocopherol, followed by β- and then α-tocopherol (Table 1). Even though these results are distinctly different from those found in olive oil, we cannot conclude that this is a general trend for all olive pomaces. Further studies will be needed to determine whether these differences are due to the processing conditions that produced the olive oil and olive pomace or to the selective precipitation of α-tocopherol in the first fraction owing to the nature of the extracted sample at the conditions used.

Further purification for γ-tocopherol would be possible when scaling up the SFC column to perform the SFE and column fractionation of the tocopherols.

Fraction 1, corresponding to the extracts in separator 1, was also analyzed by using the method described, but its tocopherol content could not be quantified because of the high content of triglycerides, waxes, and sterols.

TABLE 2
Tocopherol Content in Supercritical Fluid Extracts from Olive Oil By-products

Experiment no.	α-Tocopherol (ppm)	β-Tocopherol (ppm)	γ-Tocopherol (ppm)
1	<500	>8,000	>40,000
2	1,747	>6,000	>40,000
3	778	>4,500	>30,000
4	2,084	>4,000	>25,000
5	288	576	2,818
6	468	885	>4,000
7	185	2,196	>10,000
8	ND[a]	365	1,830

[a]ND, not detected.

ACKNOWLEDGMENTS

This research was supported by the Comisión Interministerial de Ciencia y Tecnología, Project OLI96-2161-C04-01. The authors greatly acknowledge Julián Pastor for performing the SFE at pilot plant scale.

REFERENCES

1. Kiritsakis, A.K., *Olive Oil: From the Tree to the Table*, Food & Nutrition Press Inc., Trumbull, CT, 1998.
2. Sherwin, E.R., Antioxidants for Vegetable Oils, *J. Am. Chem. Oil Soc. 53*:430–436 (1976).
3. Madhavi, D.L., R.S. Singhai, and P.R. Kulkarni, Technological Aspects of Food Antioxidants, in *Food Antioxidants*, edited by D.L. Madhavi, S.S. Deshpande, and D.K. Salunkhe, Marcel Dekker, New York, 1996, pp. 180–188.
4. Garewal, H.G., *Antioxidants and Disease Prevention*, CRC Press, Boca Raton, 1997.
5. King, J.W., F. Favati, and S.L. Taylor, Production of Tocopherol Concentrates by Supercritical Fluid Extraction and Chromatography, *Sep. Sci. Technol. 31*:1843–1857 (1996).
6. García, A., A. de Lucas, J. Rincón, A. Alvarez, I. Gracia, and M.A. García, Supercritical Carbon Dioxide Extraction of Fatty and Waxy Material from Rice Bran, *J. Am. Oil Chem. Soc. 73*: 1127–1131 (1996).
7. Modey, W.K., D.A. Mulholland, and M.K. Raynor, Analytical Supercritical Fluid Extraction of Natural Products, *Phytochem. Anal. 7*:1–15 (1996).
8. Choo, Y.M., A.N. Ma, H. Yahaya, Y. Yamauchi, M. Bounoshita, and M. Saito, Separation of Crude Palm Oil Components by Semipreparative Supercritical Fluid Chromatography, *J. Am. Oil Chem. Soc. 73*:523–525 (1996).
9. Birgith, A., M. Johannsen, G. Brunner, and N. Nair, Supercritical Fluid Extraction of Oil-Palm Components, *J. Supercrit. Fluids 8*:46–50 (1995).
10. Ibañez, E., J. Palacios, and G. Reglero, Analysis of Tocopherols by On-line Coupling SFE/SFC, *J. Microcol. Sep. 11*:605–611 (1999).

[Received July 19, 1999; accepted September 28, 1999]