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Supercritical fluid extraction of phenol compounds from olive leaves

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Abstract

A clean, highly selective supercritical fluid extraction (SFE) method for the isolation of phenols from olive leaf samples was examined. Total phenol extracts were determined using the Folin-Ciocalteu reagent. Dried, ground, sieved olive leaf samples (30 mg) are subjected to SFE, using carbon dioxide modified with 10% methanol at 334 bar, 100°C (CO_2 density 0.70 g ml^{-1}) at a liquid flow-rate of 2 ml min^{-1} for 140 min. Diatomaceous earth is used to reduce the void volume of the extraction vessel. The influence of extraction variables such as modifier content, pressure, temperature, flow-rate, extraction time, and collection/elution variables, were studied. Supercritical fluid extracts were screened for acid compounds such as carboxylic acids and phenols using Electrospray-MS (in the negative ionization mode). SFE was found to produce higher phenol recoveries than sonication in liquid solvents such as *n*-hexane, diethyl ether and ethyl acetate. However, the extraction yield obtained was only 45%, using liquid methanol.

Keywords: Supercritical fluid extraction; Phenol; Olive leaves

1. Introduction

The determination of polyphenols in plants is of great interest because of the natural antioxidant activity of these compounds. Phenol compounds are synthesized by plants as a defence mechanism against microorganisms and strong UV radiation [1]. Antioxidants are added to fat-containing foods to prevent the formation of off-flavour and toxic compounds resulting from lipid oxidation. Plant extracts are natural alternatives to synthetic antioxidants as they possess similar or even higher antioxidant activity.

Several studies on the chemical composition of the olive leaf and oil have been carried out [2–7]. Very little information is available on the chemical composition of olive leaves, the primary site of plant metabolism for primary and secondary plant products. Luteolin, luteolin-7-glucoside, rutin, quercitrin and chlorogenic acid have been identified in methanol extracts from the olive leaf [2]. Extraction of highly-hydroxylated flavonoids using supercritical methanol-modified carbon dioxide is difficult [8]. In fact, compounds in SFE extracts that react with the Folin-Ciocalteau

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reagent are more likely to be phenolic acids such as salicylic, hydroxybenzoic, coumaric, caffeic, protocatechuic or ferulic acid. These compounds have been found in aqueous hydrochloric acid and ethyl acetate extracts from olive leaves [3]. Montedoro et al. [4], using HPLC-UV, identified hydroxytyrosol, tyrosol, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid and ferulic acid in methanol extracts from virgin olive oil; the major peaks in the chromatogram, correlated with total phenol, autoxidation stability, could not be assigned. In a subsequent study [5], these compounds were characterized as oleuropeine aglycon and three hydrolysable phenols containing hydroxytyrosol or tyrosol. Angerosa et al. [6], using GC-MS, also detected the presence of linked phenols containing tyrosol and hydroxytyrosol in virgin olive oil.

Polyphenols in leaves are different from those in flowers, stems, roots and fruits. Indeed, the types of phenols present on the surface (e.g. in leaf waxes), are usually different from those occurring within the plant. Surface flavonoids are usually highly methylated and lack sugar substitution [9], so they should be more readily extracted using SC-CO_2.

SFE [10–14] offers special advantages over conventional liquid-solvent extraction such as increased selectivity, expeditiousness, automaticity and environmental safety, in addition to dramatically decreased use of organic solvents. Several SFE-based methods for determining phenol pollutants in environmental samples including waters, dust and waste solids have been proposed [15–17]. Also, SFE with carbon dioxide is the most favored method for the isolation of phenol compounds with antioxidant properties from spices and agricultural by-products [18–21].

The Folin reagent is widely used in this context and is recommended for the determination of total phenols; it reacts with compounds other than the target phenols, as such interfering reductants must be removed prior to the assay [22,23]. Solution-ionizable polar compounds not suitable for gas chromatography analysis can be detected by mass spectrometry following electrospray ionization [24,25]. Electrospray-MS in the ion mode has been used to detect phenolic diterpenes in rosemary SFE extracts [18].

The aim of this work was to develop an SFE-based method for the determination of total phenols in plant samples. Olive leaves were chosen as the plant model because they are by-products of olive farming, one of the most important agricultural activities in the Mediterranean region, and because phenols with a high pharmacological activity are particularly commonplace in plants of warm, dry regions.

2. Experimental

2.1. Apparatus

All SFE experiments were performed on a 7680T Hewlett Packard supercritical fluid extractor equipped with a Hewlett Packard 1050 isocratic modifier pump and furnished with a 7 ml extraction vessel, an automated variable restrictor and a solid-phase trap packed with octadecylsilica (ODS) or PorapackQ material. A Hewlett Packard 8453 diode array spectrophotometer was used to determine the total phenol content in olive leaf extracts. A Fisons VG Platform electrospray and a Fisons VG Autospec mass spectrometer were used to screen acid compounds in the SFE extract.

2.2. Chemicals

Caffeic acid and diatomaceous earth (acid-washed, approximately 95% SiO_2) were purchased from Sigma and used as received. All solvents and reagents were HPLC-grade and analytical reagent grade, respectively. SFC-grade carbon dioxide from Air Liquide (Paris, France) was used as extraction fluid.

Calibration solutions containing 1, 2, 4, 6, 8 and 10 μg ml^{-1} caffeic acid were prepared from a 1 g l^{-1} stock solution in ethanol by appropriate dilution in *n*-hexane. Appropriate volumes of ethanol were also added in order to equalize its content in all calibration solutions. Since no significant differences were found between the signals produced by hexane, diethyl ether or ethyl

acetate solution containing the same amount of caffeic acid, calibration solutions prepared in *n*-hexane were used to determine total phenol in water-immiscible solvents. For methanol extracts, calibration solutions containing 10, 25, 50, 75 and 100 μg ml^{-1} caffeic acid were prepared from 1 g l^{-1} methanol stock solution by appropriate dilution in methanol.

An aqueous solution of 2% (v/v) Folin-Ciocalteu phenol reagent (Merck) and a 0.2 M aqueous solution of sodium bicarbonate (Merck) adjusted to pH 12.4 with sodium hydroxide (Merck) was also used.

Olive (*Olea europaea* L.) leaves were collected from trees in the surrounding countryside, dried at 100°C for 2 h, ground and sieved ≤ 500 μm).

2.3. Supercritical fluid extraction

CO_2 was aspirated from a cylinder furnished with a dip tube, pressurized to 155–334 bar (corresponds to 0.35–0.70 g ml^{-1} density at 100°C) and mixed on-line with 0–20% (v/v) methanol. Ethanol was also assayed as CO_2 modifier. The flow-rate of liquid carbon dioxide was varied from 1 to 4 ml min^{-1}. The sample amount used was 30–200 mg. Extractions were conducted in 7 ml thimbles that were filled with diatomaceous earth in most cases in order to reduce the void volume. Each extraction was carried out in duplicate and the extraction recoveries reported are the averages of the two. Samples were subjected to dynamic extraction for 5–140 min, depending on the particular experiment. A static extraction period of 1 min was used. Extraction curves at different temperatures were obtained by performing consecutive extractions of the same sample and plotting the cumulative concentration obtained. Extracted analytes were collected on an ODS or PorapackQ trap. After extraction, the analytes were eluted from the trap at 20°C with 1.5 ml of HPLC-grade methanol or *n*-hexane. When the rinse solvent was methanol, an additional rinse with *n*-hexane was necessary in order to remove methanol non-soluble coextractives from the trap. The trap temperature was raised to 70°C in order to avoid undesirable modifier condensation on the trap during extraction/collection.

2.4. Sonication with a liquid-solvent

Ground, sieved olive leaf sample (100 mg) was extracted four times with *n*-hexane (4 ml + 3 × 2 ml). The extraction was performed using ultrasonication at room temperature and the duration of the first and subsequent steps was 30 and 15 min, respectively. The same procedure was followed for ethyl acetate, diethyl ether and methanol extractions; the amount of sample and volume of solvent used in the first step was 30 mg and 6 ml, respectively. After each step, the mixture was centrifuged and the supernatant separated. The amount of total phenol was determined using the water-immiscible solvent calibration procedure for *n*-hexane, diethyl ether and ethyl acetate, and the water-miscible solvent calibration procedure for methanol.

2.5. Photometric determination

Total phenol in the SFE and liquid–solvent extracts was quantified using the Folin-Ciocalteu reagent and either of two procedures depending on the particular rinse/extraction solvents used. Amounts of phenols are given in micrograms of caffeic acid.

Water-immiscible solvents (*n*-hexane, diethyl ether and ethyl acetate): Two aliquots of Folin-Ciocalteu and buffer solutions (2.5 ml) were mixed in a test-tube and 1 ml of *n*-hexane solution containing the phenols was added. The mixture was stirred for 1 min and allowed to stand for 30 min. The absorbance of the aqueous phase was measured at 750 nm after centrifugation.

Water miscible solvent (methanol): Two aliquots of Folin-Ciocalteu and buffer solutions (2.5 ml) were mixed in a test-tube and a volume of 0.5 ml of methanol solution containing the phenols was added. The mixture was stirred for 1 min and allowed to stand for 30 min before its absorbance at 655 nm was measured.

2.6. Mass spectrometric screening of the SFE extract

The SFE extract was screened by electrospray MS (in the negative ionization mode) in order to

detect acid compounds such as carboxylic acids and phenols from their M-1 peaks. A volume of 10 μl of SFE extract was injected into a 50:50 methanol/water carrier solution at 20 μl min^{-1}. The source temperature and cone voltage were 60°C and 30 V, respectively. The voltage of the capillary and high-voltage lenses were 3 kV and 0.5 kV, respectively. The M-1 peaks obtained by Electrospray MS of an SFE extract are listed in Table 1. Those compounds previously found in olive leaf or virgin olive oil [2–6] that could have provided an M-1 signal at each m/z ratio are also tabulated. Obviously, tentative assignments are only an approximation and should be further investigated by HPLC fractionation followed by electron impact mass spectrometry analysis.

Table 1
MS screening (M-1 peaks) of SFE extract from olive leaves

m/z (relative abundance (%))	Tentative compound(s)
101 (10), 107 (12), 117 (29), 119 (13), 123 (9), 127 (100), 129 (9)	
137 (9)	Tyrosol, hydroxybenzoic acid[a]
143 (16)	
147 (10)	Cinnamic acid[a]
151 (24)	
153 (9)	Hydroxytyrosol, protocatechuic acid[a]
157 (14), 159 (17), 161 (9), 163 (9), 173 (8), 177 (10)	
179 (33)	Caffeic acid[a]
181 (43)	Homovanillic acid[a]
185 (9), 191 (13), 195 (9)	
197 (15)	Syringic acid[a]
213 (33), 223 (9)	
241 (12)	Elenolic acid[a]
265 (24), 287 (19)	
297 (32)	4′-Methoxytectochysin[c]
309 (17)	
311 (91)	Caftaric acid[b]
312 (14)	
313 (17)	Cirsimaritin[c]
325 (70)	Fertaric acid[b]
326 (9), 339 (34)	
353 (13)	Chlorogenic acid[a]
403 (42)	
523 (9)	Ligstroside[a]
539 (25)	Oleuropeine[a]

[a] Present in olive.
[b] Present in wine.
[c] Flavones present in rosemary.

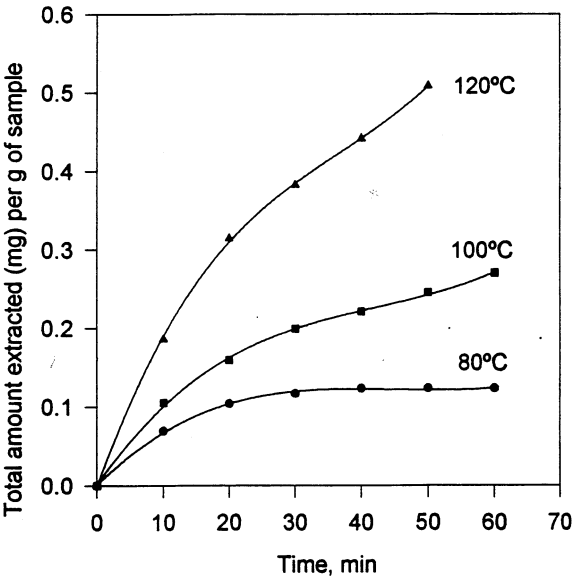

Fig. 1. Effect of the extraction temperature on the SFE efficiency of phenols. Amount of sample, 100 mg. SFE conditions: CO_2 modified with 10% methanol; CO_2 pressure, 334 bar; flow-rate, 2 ml min^{-1}; ODS trap; nozzle and trap temperature during collection 45 and 70°C, respectively; rinse solvent, *n*-hexane; rinse volume, 1.5 ml; nozzle and trap temperature during rinse, 20°C. Amounts of phenols per gram of sample expressed as milligrams of caffeic acid.

3. Results and discussion

Preliminary experiments demonstrated that the addition of a modifier to CO_2 was mandatory in order to extract phenols, the extraction yield was strongly influenced by pressure and, especially, the extraction temperature. The restrictor was plugged and overloaded by samples larger than 100 mg (especially at high temperatures) which caused overpressure problems.

3.1. Influence of the extraction temperature on the SFE efficiency

The effect of the extraction temperature on the amount of phenols extracted was studied at con-

stant pressure (334 bar). Fig. 1 shows the extraction curves obtained at three different temperatures (80, 100 and 120°C), corresponding to CO_2 densities of 0.78, 0.70 and 0.63 g ml^{-1}, respectively. Although the solvating power of methanol-modified carbon dioxide decreased with decreasing density, raising the temperature increased both the extraction rate and extraction efficiency through increased diffusion and desorption. The amount of phenols extracted at 80°C increased with increasing extraction time up to 40 min beyond which it remained constant. However, greater amounts were extracted at 100 or 120°C and no plateau was observed over the period studied (1 h). The datum for 60 min at 120°C could not be obtained owing to the short lifetime of the thimble cap. Indeed, the manufacturer recommends that thimble caps be used only once, if the extraction is performed at 120°C. In fact, caps were dramatically deformed and became useless after five extractions. An extraction temperature of 100°C was selected for further experiments as a compromise between extraction efficiency and thimble cap lifetime.

3.2. Void volume

Since the extraction vessel volume (7 ml) was much larger than the sample size (< 0.5 ml), an inert solid (diatomaceous earth) was added to the vessel in order to fill any extra void volume. The void volume was thus reduced and no additional extraction time was required to sweep the SC-extract out of the vessel. Diatomaceous earth was placed at the thimble edge of the CO_2 inlet. Diatomaceous earth (1 g) was extracted under the same conditions in order to check for the absence of interferents extracted from this material. The amount of phenols extracted was markedly increased by the addition of 1 g of diatomaceous earth to the thimble (see Table 2).

3.3. Influence of collection variables

ODS and PorapackQ were tested as packing materials for the analyte trap. Methanol and *n*-hexane were used as rinse solvents with both packings. The amounts of phenols found in the SFE extracts obtained under the same conditions with the four collection/elution systems are shown in Table 2. As can be seen, the amounts eluted from ODS and PorapackQ trap were much larger (34–21 fold) with methanol than with *n*-hexane. Methanol proved to be a better rinse solvent than *n*-hexane with both traps. However, methanol was not efficient enough to completely remove other extracted substances (yellow/green pigments), so a subsequent rinse using *n*-hexane was mandatory in order to make the trap ready for a new extraction. Indeed, the use of an ODS/methanol or PorapackQ/methanol collection system to extract phenols allowed on-line clean-up of the supercritical extracts. It seems that methanol dissolves the more polar phenols, which may be insoluble in *n*-hexane. A volume of 1.5 ml of rinse solvent (*n*-hexane or methanol) at 2 ml min^{-1} and 20°C was sufficient to remove all soluble phenols from the trap. No analytes were detected after a subsequent rinse. The nature of the trap packing affected the amount of phenols to a lesser extent than the nature of the rinse solvent. Slightly greater amounts of phenols (about 10%) were obtained with ODS than with PorapackQ. However, PorapackQ provided cleaner extracts, so it was selected for subsequent experiments.

Table 2
Influence of the collection system (trap/rinse solvent) and void volume used on the SFE efficiency of phenols from olive leaves

SFE conditions	Concentration in olive leaves[a] (mg g^{-1})
PorapackQ/MeOH, no DE	2.6
PorapackQ/MeOH, DE	3.8
PorapackQ/*n*-Hexane, DE	0.11
ODS/MeOH, DE	4.2
ODS/*n*-Hexane, DE	0.20

Amount of sample, 30 mg. SFE conditions: CO_2 modified with 10% methanol; pressure, 334 bar; extraction temperature, 100°C; flow-rate, 2 ml min^{-1}; extraction time, 20 min; nozzle and trap temperature during collection, 45 and 70°C, respectively; rinse volume, 1.5 ml; nozzle and trap temperature during rinse, 20°C. DE, addition of 1 g of diatomaceous earth.
[a] Expressed as caffeic acid.

Table 3
Influence of the extraction pressure on the SFE efficiency of phenols from olive leaves

Pressure (bar)	(CO_2 density (g ml^{-1}))	Mean concentration[a] (mg g^{-1})	rsd (%)
155	(0.35)	0.59	13
207	(0.50)	1.9	12
256	(0.60)	2.6	9
334	(0.70)	3.4	5

Amount of sample, 30 mg. SFE conditions: CO_2 modified with 10% methanol; extraction temperature, 100°C; flow-rate, 2 ml min^{-1}; extraction time, 20 min; PorapackQ trap; nozzle and trap temperature during collection, 45 and 70°C, respectively; rinse solvent, methanol; rinse volume, 1.5 ml; nozzle and trap temperature during rinse, 20°C. Addition of 1 g of diatomaceous earth.
[a] Expressed as caffeic acid.

3.4. Influence of pressure

The effect of extraction pressure on the amount of phenols dynamically extracted from olive leaves using CO_2 modified with 10% methanol for 20 min was studied at a constant temperature of 100°C. The amounts extracted increased linearly with increasing CO_2 density (Table 3, intercept -2.2 mg g^{-1}, slope 8.1 mg ml g^{-2}, r^2 0.9996). An extraction pressure of 334 bar and a temperature of 100°C (viz. a CO_2 density of 0.70 g ml^{-1}) were chosen for subsequent extractions.

3.5. Influence of the modifier

The amount of total phenols extracted from olive leaves with pure CO_2 and various methanol–CO_2 mixtures is shown in Fig. 2. The effect of the modifier content on the extraction yield was examined at 100°C and 344 bar. As can be seen, the addition of methanol to CO_2 was mandatory in order to fully extract these compounds. A 10% methanol–CO_2 mixture provided the highest recovery of phenols while a modifier content of 20% produced undesirable methanol condensation on the analyte trap.

Because the proposed SFE of phenols could be implemented by the food, cosmetic and pharmaceutical industry provided the modifier used is non-toxic, the feasibility of replacing methanol with ethanol as modifier was investigated. Ethanol was found to be useful as a modifier, but less effective than methanol: the extraction yield of phenols obtained with 10% ethanol as modifier was 2.0 mg g^{-1} (rsd 2.5%), the yield obtained with 10% methanol under the same conditions was 3.6 mg g^{-1} (rsd 5.6%).

3.6. Influence of the flow-rate and extraction time

There was no significant difference between the results obtained at a flow-rate of 1 and 2 ml min^{-1} at 80°C; however, the extraction yield in-

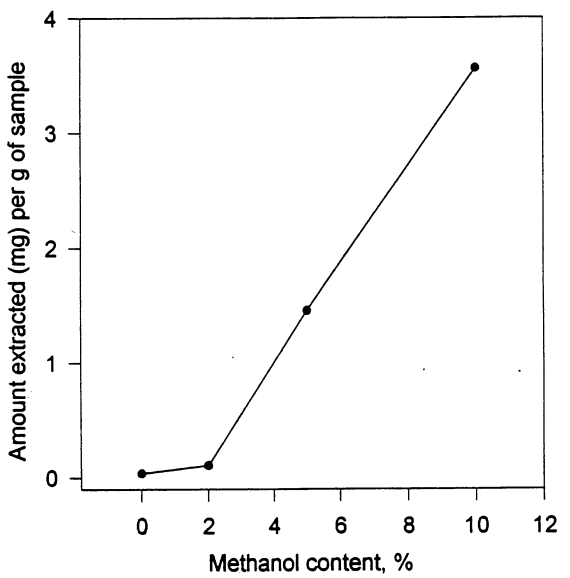

Fig. 2. Influence of methanol content on the SFE efficiency of phenols from olive leaves. Amount of sample, 30 mg. SFE conditions: pressure, 334 bar; extraction temperature, 100°C; flow-rate, 2 ml min^{-1}; extraction time, 20 min; PorapackQ trap; nozzle and trap temperature during collection 45 and 70°C, respectively; rinse solvent, methanol; rinse volume, 1.5 ml; nozzle and trap temperature during rinse, 20°C. Addition of 1 g of diatomaceous earth. Amounts of phenols per gram of sample expressed as milligrams of caffeic acid.

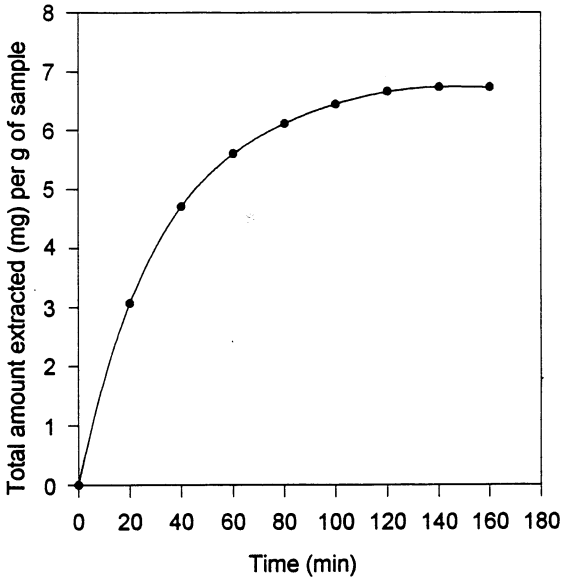

Fig. 3. Influence of the extraction time on the SFE efficiency of phenols from olive leaves. Amount of sample, 30 mg. SFE conditions: CO_2 modified with 10% methanol; pressure, 334 bar; extraction temperature, 100°C; flow-rate, 2 ml min^{-1}; PorapackQ trap; nozzle and trap temperature during collection, 45 and 70°C, respectively; rinse solvent, methanol; rinse volume, 1.5 ml; nozzle and trap temperature during rinse, 20°C. Addition of 1 g of diatomaceous earth. Amounts of phenols per gram of sample expressed as milligrams of caffeic acid.

creased with increasing flow rate at 100°C (from 6.4 mg g^{-1} at 1 ml min^{-1} to 8.0 mg g^{-1} at 2 ml min^{-1}). Extraction from plant materials at low temperature seems to be limited primarily by desorption/diffusion since the flow rate has no effect on the extraction rate. However, the process is solubility-controlled at increased extraction temperatures. The behaviour of spiked phenols [8] was different at 50°C; increasing the flow-rate resulted in increased recoveries because solubilization was the limiting step. Undesirable methanol condensation on the analyte trap that could result in analyte loss (by ejection of methanol drops) was observed above 2 ml min^{-1}.

In order to ensure complete methanol evaporation, the trap temperature was raised from 70 to 100°C, which reduced collection efficiency. The extraction yields fell from 7.6 to 6.7 mg g^{-1} as a result.

Fig. 3 shows the extraction curve obtained under the optimal SFE conditions. Exhaustive extraction was achieved after 140 min of dynamic extraction. A comparison of the cumulative amount of phenols per gram of sample obtained after seven 20-min extractions (6.7 mg g^{-1}) with that obtained in a single 140-min extraction (8.0 mg g^{-1}) revealed the absence of analyte losses after a long extraction time. In absence of losses, the amount obtained by seven 20-min extractions or by a single 140-min extraction must be the same. Determination of low phenol concentrations was difficult, particularly in extracts obtained from the last 20-min steps. It may explain the decreased result of 6.7 mg g^{-1}.

The precision of the whole method (SFE + colorimetry), expressed as percent relative standard deviation (extraction time = 20 min, $n = 5$) was 5.6%.

3.7. Comparison of SFE and sonication in a liquid solvent

SFE and liquid solvent extraction were compared in terms of phenol yields (Table 4). The data for liquid solvents was obtained in four successive extractions of triplicate samples. Only

Table 4
Concentration of total phenols found in olive leaves using the Folin-Ciocalteu reagent after SFE or sonication in a liquid solvent

Solvent	Concentration in olive leaf ± SD[a] ($n=3$) (mg g^{-1})
n-Hexane	0.18 ± 0.03
Diethyl ether	1.1 ± 0.3
Ethyl acetate	1.6 ± 0.4
Methanol	16.8 ± 0.8
CO_2 + 10% Methanol	7.6 ± 0.5

Sonication time: 75 min, SFE conditions: pressure, 334 bar; extraction temperature, 100°C; flow-rate, 2 ml min^{-1}; extraction time, 140 min; PorapackQ trap; nozzle and trap temperature during collection, 45 and 70°C, respectively; rinse solvent, methanol; rinse volume, 1.5 ml; nozzle and trap temperature during rinse, 20°C. Addition of 1 g of diatomaceous earth. Amount of sample, 30 mg.
[a] Expressed as caffeic acid.

with methanol was the olive leaf sample exhaustively extracted. The results reveal that the higher the solvent polarity, the higher the phenol extraction yield. Thus, SFE provided much greater amounts of phenols than extraction with *n*-hexane, diethyl ether or ethyl acetate; however, carbon dioxide modified with 10% methanol recovered 45% of the amounts extracted by liquid methanol.

4. Conclusions

A clean, highly selective, automated method for the isolation of phenols from olive leaves was investigated. Because solvent polarity increases extraction rate and efficiency, the addition of a polar modifier (e.g. methanol or ethanol) to non-polar supercritical CO_2 is essential. Modifier contents and flow-rates higher than 10% and 2 ml min^{-1}, respectively, give rise to methanol condensation on the trap. Ethanol was found to be a less efficient modifier than methanol. PorapackQ packing and methanol was the best combination for collecting and eluting extracted analytes. This collection system affords on-line clean-up of plant extracts. Supercritical CO_2 modified with 10% methanol was found to be a more efficient solvent than *n*-hexane, diethyl ether and ethyl acetate, but was surpassed by liquid methanol in terms of yield.

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