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Supercritical fluid extraction of t-resveratrol and other phenolics from a spiked solid

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Abstract Supercritical fluid extraction of spiked phenolics including gallic acid, (+)-catechin, (–)-epicatechin, caffeic acid, p-coumaric acid, myricetin, t-resveratrol, quercetin and salicylic acid from an inert support using pure CO_2 and methanol-carbon dioxide mixtures was studied. Extraction and collection variables including modifier percentage, extraction temperature, flow rate, extraction time, trap packing and rinse solvent were optimized. The study revealed that the use of methanol as modifier was mandatory. Only the less hydroxylated compounds such as p-coumaric acid, t-resveratrol and salicylic acid could be quantitatively recovered (mean recovery ≥ 95%) from spiked diatomaceous earth. Mean recoveries of more polar phenolic acids and flavonoids such as gallic acid, caffeic acid, catechins and quercetin were between 30% and 70%. Myricetin was not recovered at all.

Introduction

There is an urgent need for rapid and clean methods for the extraction and determination of highly valuable natural products. Supercritical fluid extraction [1–6] poses several advantages over traditional liquid-solvent-based extraction methods including improved selectivity, expeditiousness, automation and environmental safety. The avoidance of organic solvents is a major goal in the isolation of natural products which may be commercialized as food additives.

Plant phenols are substances with the same metabolic origin; they are derived from the shikimate pathway and phenylpropanoid metabolism [7]. The study of plant phenolics has gained special interest because of their pharmacological and, in some instances, cancer-preventive properties. Flavonoids are potent antioxidants, free radical scavengers and metal chelators; they inhibit lipid peroxidation and exhibit various physiological activities including antiinflammatory, antiallergic, anticarcinogenic, antihypertensive and antiarthritic activities [7]. These compounds represent an important source of antioxidants in the human diet. Resveratrol has been correlated with serum lipid reduction and inhibition of platelet aggregation [8] and its cancer chemopreventive activity has been recently reported [9]. Phenolic compounds may be synthesized by plants for the defence against microorganisms or strong UV radiation. For instance, t-resveratrol concentrations have been correlated with resistance to fungal infections [10].

Most of the methods reported to measure wine phenolics are based on high-performance liquid chromatography [11–15] or capillary electrophoresis [16–18], and usually involve a previous fractionation by liquid-liquid extraction or solid phase extraction. t-Resveratrol has been determined in wine, juices, berries and by-products of the grape juice industry including pomace, purees and seeds by HPLC [19, 20] or gas chromatography [21, 22] in order to establish the amount of resveratrol incorporated into the human diet and to evaluate the by-products as potential ingredients in food [20]. A combination of geographical origin, variety, growing methods and winemaking procedures seems to affect the resveratrol concentration in wines [19, 22]. The content of t-resveratrol in berries steadily decreases during fruit ripening, being practically undetectable in mature berries [21].

The present work was designed to study the suitability of supercritical CO_2 and methanol-CO_2 mixtures to extract a selection of phenolics from spiked diatomaceous earth and to find the optimum conditions for the extraction process. In order to cover the existing classes, compounds selected were among the best known unconjugated phenolics. In addition, the influence that different functional groups had on the final recoveries from a spiked support (diatomaceous earth) using supercritical methanol-CO_2 mixtures is also discussed.

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Experimental

Apparatus

A Hewlett Packard 7680T supercritical fluid extractor equipped with a Hewlett Packard 1050 isocratic modifier pump, an automated variable restrictor and an assortment of packed traps, including octadecylsilane (ODS), diol, cyano, PorapackQ and Florisil packings, was used.

A 1050 Hewlett Packard liquid chromatograph equipped with a 20 µl loop injector, and Ultrabase-C_{18} (250 × 4.6 mm, 5 µm) column, and an HP 1040A diode array detector were used for the separation/detection of phenolics in the extracts.

Reagents

Diatomaceous earth (acid-washed, approximately 95% SiO_2) and phenolic compounds, including gallic acid (water content 0.5 mol/mol), (+)-catechin (> 98%, water content 1.5 mol/mol), (–)-epicatechin, caffeic acid, gentisic acid, p-coumaric acid (predominantly trans isomer), myricetin (85%), t-resveratrol (99%), quercetin (dihydrate) and salicylic acid, were obtained from Sigma and used as received. Chemical structures of phenolic compounds are shown in Fig. 1. Individual standard solutions of concentrations ranging from 1.0 to 1.6 mg/mL were prepared in methanol and subsequently used to prepare a stock solution mixture containing 100 µg/mL of each compound in methanol. All solutions were stored at –4° C in dark conditions.

All solvents were HPLC grade. SFC-grade carbon dioxide from Air Liquide (Paris, France) was used as extraction fluid. An aqueous solution of acetic acid (1%, v/v) and acetonitrile were used as components of the HPLC mobile phase.

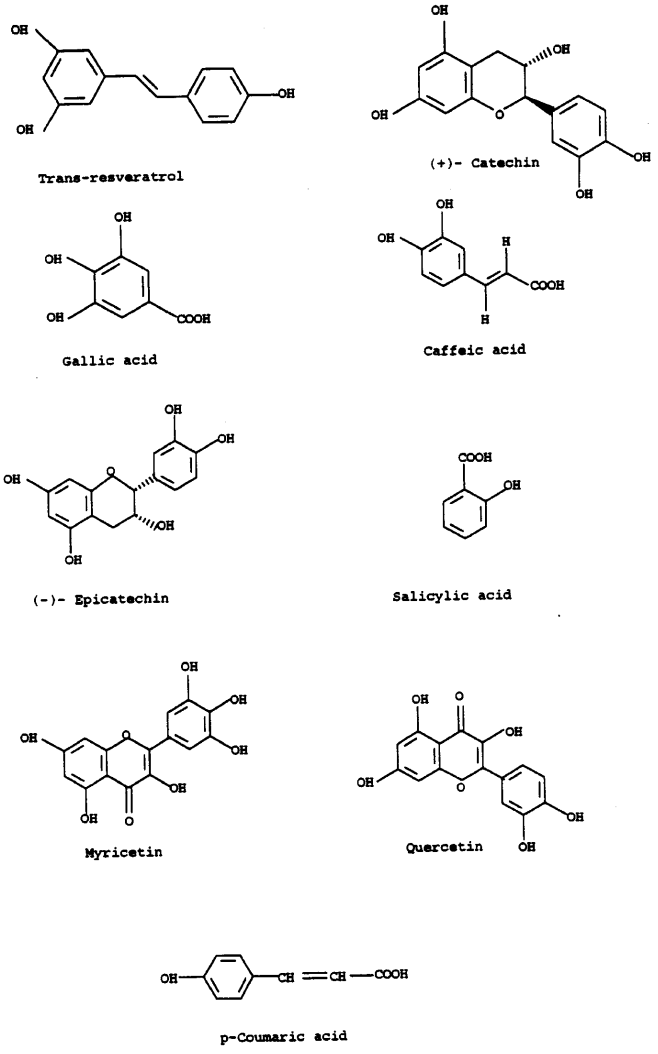

Fig. 1 Chemical structures of the nine phenolic compounds studied

Chromatography

Dilution of the methanolic solutions and extracts with aqueous acetic acid was necessary in order to obtain sharp phenolic peaks, avoiding peak broadening. Calibration solutions in methanol and extracts were diluted (1:2, v/v) in aqueous acetic acid and the resulting solution filtered through a 0.45 µm Nylon syringe filter (Micron Separations, Inc. Westboro, MA). The injected volume was 20 µL and the flow rate of the mobile phase was maintained at 1 mL/min. After the injection, the phenolics were eluted from the Ultrabase-C_{18} (250 × 4.6 mm, 5 µm) column with a 75:25 aqueous acetic acid (1%, v/v)/acetonitrile mobile phase for 2 min, which was then linearly increased to 50:50 for 2 min, and kept at that composition for a further 6 min. A post-run time of 5 min was chosen to ensure the initial conditions were restored (dead time, 2 min).

Chromatograms were recorded at 280 nm and the peak area used for quantitation. Calibration curves obtained were linear

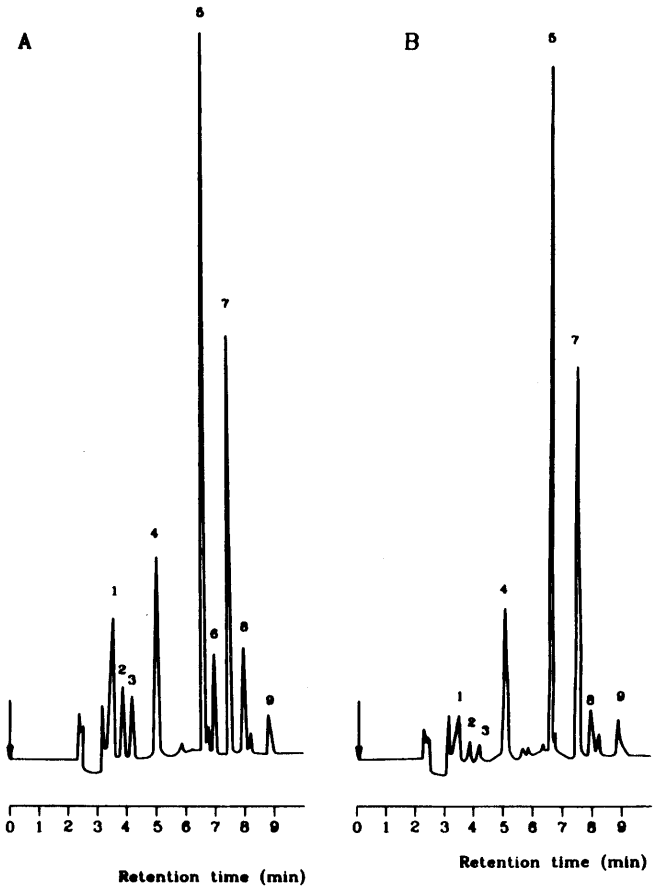

Fig. 2 A, B HPLC-chromatograms corresponding to a 2 µg/ml standard solution (**A**) and a SFE extract (**B**). Peak identification: Gallic acid (*1*), (+)-Catechin (*2*), (–)-Epicatechin (*3*), Caffeic acid (*4*), p-Coumaric acid (*5*), Myricetin (*6*), t-Resveratrol (*7*), Quercetin (*8*), Salicylic acid (*9*)

throughout the studied range (0.5–13 μg/mL for caffeic acid, p-coumaric acid, t-resveratrol and quercetin, and 1–13 μg/mL for gallic acid, (+)-catechin, (–)-epicatechin, myricetin and salicylic acid). Detection of gentisic acid within those ranges was difficult due to its low molar absorpivity at 280 nm. Typical chromatograms are shown in Fig. 2. The precision of the HPLC method expressed as percent relative standard deviation (n = 11) was between 1.7% and 5.7% at a concentration of 2 μg/mL.

Supercritical fluid extraction

Extractions were conducted in 7 mL thimbles. Each extraction was completed in triplicate, the extraction recoveries reported being the average of three extractions. Samples were subjected to dynamic extraction for 2–30 min, depending on the particular experiment. No static extraction period was used. All extractions were accomplished at 350×10^5 Pa using CO_2 or methanol-modified CO_2. Methanol modifier (5–20%) and CO_2 were mixed in line. For this study, the extraction temperatures were 50°C and 90°C. Extracted analytes were collected in a solid sorbent trap of various packings. For modified-fluid extraction the trap temperature was increased to 70°C. After completion of each extraction, the analytes were eluted from the trap at 20°C with 1.5 mL of HPLC-grade methanol or acetonitrile.

Sample preparation

The spiking procedure was as follows. Firstly, 0.6 g of diatomaceous earth was weighed in the extraction thimble. Secondly, a volume of 100–200 μL of stock solution of the phenolics was added to the diatomaceous earth in the thimble. Finally, the spiked solid was allowed to stand for at least 24 h in order to evaporate methanol.

Results and discussion

Stability of p-coumaric acid and t-resveratrol

Methanolic solutions of t-resveratrol and p-coumaric acid were stable at room temperature, whilst some decomposition occurred when they were dissolved in aqueous acetic acid/methanol. t-Resveratrol and p-coumaric acid concentrations diminished with time. It was observed that p-coumaric acid and t-resveratrol peaks decreased giving rise to the appearance of additional peaks at 6.75 min and 8.26 min, respectively. Degradation of p-coumaric acid and t-resveratrol is illustrated in Fig. 3. As can be seen, peak areas of p-coumaric acid and t-resveratrol decreased up to a 16% and 30% in 2 h, respectively. In order to avoid losses of these compounds, methanolic calibration and extract solutions were diluted prior to HPLC injection.

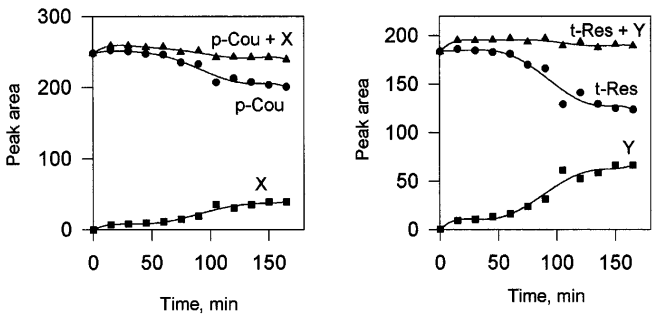

Fig. 3 Stability of p-Coumaric acid (*A*) and t-Resveratrol (*B*) in mobile phase. Absorbance monitored at 280 nm. X and Y denotes peak at 6.75 and 8.26 min, respectively

Optimization of extraction conditions

Since only traces of p-coumaric acid and salicylic acid were extracted using pure CO_2, the addition of methanol as CO_2 modifier was examined. The influence of the methanol percentage in the mixture on the recovery is shown in Fig. 4. All phenolics studied except myricetin were extracted using methanol-modified CO_2. However, in most cases, increased methanol percentages caused the recoveries to decrease. This behaviour is probably caused by a decrease in the trapping efficiency at a flow rate of 2 mL/min and higher modifier percentages. This results in trap flooding by the condensed methanol and analyte losses through blowing droplets of liquid methanol. Despite a trap temperature above the modifier boiling point being used, methanol condensation in the trap was observed for 20% methanol-modified CO_2. A methanol percentage of 5% was selected for further experiments.

Collection and elution conditions

Collection of phenolics on various trap packings was examined. The differences in collection efficiencies using various traps were evaluated through the recoveries obtained under the same extraction conditions. Those recoveries are shown in Table 1. As can be seen, PorapackQ

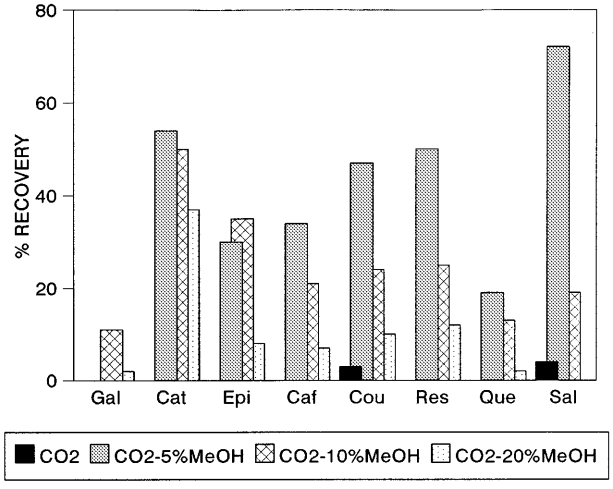

Fig. 4 Influence of the modifier percentage on the SFE recovery of phenolics. Amount added, 20 μg of each phenolic compound. SFE conditions: Pressure, 350×10^5 Pa; extraction temperature, 50°C; flow rate, 2 mL/min; extraction time, 30 min; ODS trap; nozzle and trap temperature during collection, 45 and 70°C, respectively; rinse solvent, acetonitrile; rinse volume, 1.5 mL; nozzle and trap temperature during rinse, 20°C

trapped phenolics better than any of the others tested. A *t*-test for the recoveries obtained with ODS and PorapackQ trap showed that there are significant differences only for p-coumaric acid and t-resveratrol (t values are 2.06, 2.82, 4.63 and 0.70 for caffeic acid, p-coumaric acid, t-resveratrol and salicylic acid, respectively). Critical t value is 2.78 for 4 degrees of freedom and 95% confidence level. More polar packings such as Florisil and Diol provided the least satisfactory results. Since analytes are non-volatile, the ability of packings to favour methanol evaporation or retain the undesirably condensed methanol may have caused their different trapping efficiencies. The results for gallic acid, (+)-catechin, (–)-epicatechin and quercetin were highly irreproducible, and hence were omitted from Table 1.

Elution of extracted phenolics from the PorapackQ trap with acetonitrile was compared with elution with methanol. Recoveries and relative standard deviations (n = 3) of each phenolic compound obtained for methanol and acetonitrile are listed in Table 2. Although methanol gave higher mean recoveries than acetonitrile in most cases, according to t-values only significant differences were observed when acetonitrile was replaced by methanol in the case of salicylic acid. Thus, t values of 0.19, 0, 1.22 and 12.25 were obtained for caffeic acid, p-coumaric acid, t-resveratrol and salicylic acid, respectively (critical t value is 2.78 for 4 degrees of freedom and 95% confidence level). A volume of 1.5 mL of methanol at 1.0 mL/min and 20° C was sufficient to remove all phenolics extracted from the PorapackQ trap; no analytes were found in a subsequent methanol rinse.

Table 1 Effect of trap packing on percent recovery (± s.d., n = 3) of phenolics

Compound	ODS	Diol	CN	PorapakQ	Florisil
Caffeic acid	20 ± 10	16 ± 4	26 ± 6	41 ± 15	N.D.
p-Coumaric acid	43 ± 9	32 ± 2	48 ± 4	66 ± 11	9 ± 3
t-Resveratrol	31 ± 5	26 ± 3	36 ± 1	48 ± 4	37 ± 13
Salicylic acid	36 ± 9	9 ± 15	33 ± 2	40 ± 5	N.D.

Amount of each phenolic added, 10 µg. SFE conditions: 5% methanol modified CO_2; pressure, 350×10^5 Pa; extraction temperature, 50° C; flow rate, 2 mL/min; extraction time, 30 min; nozzle and trap temperature during collection, 45° C and 70° C, respectively; nozzle and trap temperature during rinse, 20° C; rinse solvent, acetonitrile; rinse volume, 1.6 ml. N.D., not detected

Table 2 Percent recovery (± s.d., n = 3) for the phenolics under different extraction/collection conditions

Compound	A	B	C
Caffeic acid	71 ± 10	70 ± 3	64 ± 29
p-Coumaric acid	90 ± 7	90 ± 5	97 ± 8
t-Resveratrol	77 ± 4	83 ± 8	95 ± 5
Salicylic acid	59 ± 3	104 ± 6	109 ± 6

Amount of each phenolic added, 8 µg. SFE conditions: 5%-methanol CO_2; pressure, 350×10^5 Pa; flow rate, 2 ml/min; extraction time, 15 min; PorapackQ trap; nozzle and trap temperatures during collection, 45° C and 70° C, respectively; nozzle and trap temperature during rinse, 20° C; rinse volume, 1.5 mL. **A**: Rinse solvent, acetonitrile. Extraction temperature, 50° C. **B**: Rinse solvent, methanol. Extraction temperature, 50° C. **C**: Rinse solvent, methanol. Extraction temperatura, 90° C

Extraction time and flow rate

Extractions were conducted to optimize CO_2 flow rate and extraction time. The effect of these variables is shown in Fig. 5, which lists only the compounds providing reproducible recoveries (s.d. < 10%). A flow rate of 2 mL/min gave the highest recovery in all cases. Increasing flow rate up to a value of 2 mL/min resulted in increased recoveries; above that value recoveries diminished slightly. High flow rates favor analyte losses, particularly when modified carbon dioxide was used. Analyte losses were also apparent from Figure 5b, which shows the influence of

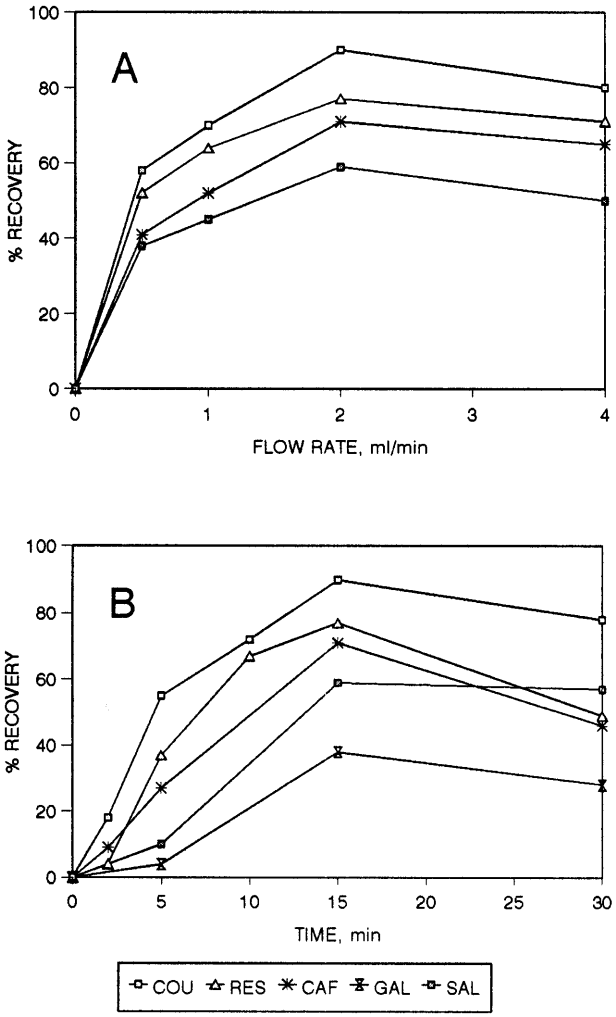

Fig. 5 A, B Effect of fluid flow rate (**A**) and extraction time (**B**) on the percent recovery of the phenolics. Amount of each phenolic added, 10 µg. SFE conditions: 5%-methanol-modified CO_2; pressure, 350×10^5 Pa; extraction temperature, 50° C; PorapackQ trap; rinse solvent, acetonitrile; nozzle and trap temperatures during collection, 45 and 70° C, respectively; nozzle and trap temperatures during rinse, 20° C; rinse volume, 1.5 mL; extraction time, 15 min (**A**) and flow rate 2.0 ml/min (**B**)

Table 3 Effect of the addition of water to the sample on phenolic recovery (% Recovery ± s.d., n = 3)

Compound	Dry sample	H_2O addition to sample
Caffeic acid	34 ± 8	13 ± 3
p-Coumaric acid	47 ± 4	24 ± 5
t-Resveratrol	50 ± 2	16 ± 5
Salicylic acid	72 ± 14	24 ± 2

Amount of each phenolic added, 20 µg. SFE conditions: 5% methanol CO_2; pressure, 350×10^5 Pa; extraction temperature, 50° C; flow rate, 2 mL/min; extraction time, 30 min; ODS trap; rinse solvent, acetonitrile; nozzle and trap temperature during collection, 45° C and 70° C, respectively; nozzle and trap temperature during rinse, 20° C; rinse volume, 1.5 mL

extraction time on SFE recovery. Recoveries increased by increasing extraction time up to 15 min. A longer extraction time (30 min) resulted in decreased recoveries. This was because a longer exposure of the extracted analytes on the PorapackQ trap to a high flow of the gaseous carbon dioxide-methanol mixture favoured losses dramatically.

Effect of water on SFE recovery of phenolics

Since water is present in many of the samples containing the phenolic compounds studied (e.g. wine and solid parts of the grape), the influence of water in recovery must be evaluated in order to establish the potential of SFE to recover these compounds directely, without sample drying.

The spiked diatomaceous earth (0.6 g) was soaked with 0.5 mL of distilled water in order to examine the effect of sample moisture. As can be seen from Table 3, recoveries from the wet sample were reduced to less than half compared to those of the dry sample. Extraction of these polar compounds in supercritical methanol-modified carbon dioxide is hindered as a result of partitioning in the aqueous phase. Compounds providing highly fluctuating recoveries have been removed from Table 3.

Extraction temperature

As can be seen from Table 2, increasing temperature from 50° C to 90° C had no significant effect on the recovery from the spiked support. t values of 1.32, 2.26 and 1.02 were obtained for p-coumaric acid, t-resveratrol and salicylic acid (critical t value is 2.78 for 4 degrees of freedom and 95% confidence level). In the case of caffeic acid (significantly different variances), t value (0.36) was also smaller than the critical *t* value (4.30, for 2 degrees of freedom and 95% confidence level). The extraction of spiked phenols seemed to be controlled by solubility, and temperature has two different effects on it; thus, increasing extraction temperature decreases solvating power of the supercritical fluid but increases analyte volatility. However, a very different behavior can be expected in real samples involving a diffusion and/or desorption process, as extraction rate is primarily controlled by analyte/matrix interaction rather than by CO_2 solubility. Furthermore, the amounts of the more hydroxylated phenolics (e.g. gallic acid, catechins and quercetin) extracted at 90° C were not reproducible at all.

Recovery and functional groups

The low-molecular-weight phenolics containing 2–3 polar groups (carboxyl or hydroxyl functions) such as t-resveratrol, p-coumaric acid and salicylic acid can be quantitatively recovered under optimal conditions (mean percent recoveries ≥ 95%). It can be concluded that phenolic acids (C_6-C_1), hydroxycinnamic acids (C_6-C_3) and stilbenes (C_6-C_2-C_6) containing 1, 1 and 3 hydroxy groups, respectively, can be extracted using supercritical 5%-methanol carbon dioxide. Increasing the number of hydroxy groups results in a more difficult extraction. For instance, the recovery of gallic acid, containing two additional OH-groups compared to salicylic acid, was only around 40%. Also, the replacement of an hydrogen atom with a hydroxyl group in hydroxycinnamic acid negatively affected the recovery, which decreased to approximately 30%. Extraction of flavonoids posed a greater difficulty, (+)-catechin, (–)-epicatechin and quercetin containing five hydroxylic groups were barely extracted and myricetin, a flavonol containing six hydroxylic groups, could not be recovered.

Conclusions

The influence of variables on the supercritical fluid extraction of a selection of phenols has been studied systematically. The study has an orientative value for phenols extracted from wine and winery by-products but it is necessary to indicate that it has been developed with spiked diatomaceous earth, so conclusions are refered to minimal extraction conditions free of matrix complications.

The present study revealed that only some of the phenolics studied (e.g. p-coumaric acid, t-resveratrol and salicylic acid) could be successfully extracted by SFE and the use of methanol as modifier was essential in order to extract them. Neither methanol percentages higher than 5%, flow rates above 2 mL/min nor extraction times longer than 15 min are recommended since they produced analyte losses.

The presence of water negatively affected the recovery of these compounds, as a result of their undesirable partitioning.

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