Extraction of Natural Complex Phenols and Tannins from Grape Seeds by Using Supercritical Mixtures of Carbon Dioxide and Alcohol

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Proanthocyanidins are supposed to have some therapeutical properties as antioxidants and antineoplasics. Most of the proanthocyanidins, however, are not commercialized since their separation from natural sources is either very expensive or not well-known. In this work, the feasibility of application of mixtures of carbon dioxide and alcohol under supercritical conditions for selective extraction of some phenolic compounds from grape seeds has been studied, among them some low polymerized proanthocyanidins, their main monomer units, (+)-catechin and (−)-epicatechin, and some low molecular weight phenolic compounds, like gallic acid. An analytical-scale supercritical fluid extractor, whose operation was previously optimized, was used to carry out the experiments. A commercial concentrate of complex phenols and tannins from grape seeds was subjected to supercritical extraction in order to find the best operation conditions before directly extracting defatted milled grape seeds. The solvent capacity was found to increase with pressure and with the amount of alcohol used as cosolvent as expected. Such variation in solvent capacity could be used for design of a selective separation process where individual phenolic compounds or groups of them could be obtained. HPLC coupled with two types of detectors, diode array and mass spectrometry, was used for tentative identification and quantification of complex phenols and tannins in the extracts and in the raw materials used for extraction.

Keywords: *Supercritical fluid (SCF) extraction; grape seed; complex phenols and tannins (CPT); proanthocyanidins (PA); HPLC-MS*

INTRODUCTION

Extraction processes that use supercritical fluids (SCF) have been widely studied and used at laboratory, pilot, and industrial scales in the past decades. The food and pharmaceutical industries have rapidly taken advantage of the possibilities of carbon dioxide as a nontoxic, environmentally safe, cheap, and selective extraction solvent (Brunner, 1994; McHugh and Krukonis, 1994). Carbon dioxide has the advantage compared to liquid solvents that its selectivity or solvent power is adjustable and can be set to values ranging from gas to liquidlike. Carbon dioxide has a low critical temperature (304.1 K) and moderate critical pressure (7.28 MPa), thus being an ideal solvent for compounds that may suffer thermal degradation (Palmer and Ting, 1995). Furthermore, CO_2 processing creates a medium without oxygen where oxidation reactions can be avoided, which is of great interest when antioxidant compounds are to be extracted.

Grape seeds are a complex matrix containing approximately 40% fiber, 16% oil, 11% proteins, and 7% complex phenols including tannins (CPT), in addition to sugars, mineral salts, etc. Some research has been published on supercritical extraction of oil from grape seeds (Sovová et al., 1994; Molero et al., 1996) but grape seeds may also constitute an important source of CPT. Under the term CPT, a high variety of natural phenolic compounds are included; among them, proanthocyanidins (PA) form an important subgroup. The fundamental structural unit in the PA subgroup is the phenolic flavan-3-ol (catechin) unit linked principally through the 4- and the 8-positions. CPT are widely found in natural products and their effects on health have been largely studied (Cook and Samman, 1996; Chung et al., 1998). It is generally accepted that CPT are efficient free radical scavengers and could inhibit lipid peroxidation (Rice-Evans et al., 1996; De Freitas et al., 1998). The precise mechanism in vivo is still not completely explained but dietary intake of these natural antioxidants is inversely related to the risk of coronary heart disease and certain forms of cancer (Okuda, 1993; Haslam, 1996; Scott, 1997). CPT possess distinct properties from one another; thus, their isolation is of high interest. However, most of the condensed PA have never been isolated; only very small amounts (around 10^{-3} g) of some oligomeric PA (dimers, trimers) have been isolated so far (Sun et al., 1998).

Tsuda et al. (1995) reported the supercritical carbon dioxide extraction of some polyphenols from tamarind (*Tamarindus indica* L.) seed coat. They found that some flavan-3-ol molecules, such as (−)-epicatechin, were soluble in supercritical CO_2 and their solubility increased when the supercritical CO_2 was modified with small amounts of ethanol. Tsuda et al. (1994) also determined the antioxidant activity of components isolated from tamarind seed and concluded that they can be used for increasing the shelf life of foods by preventing lipids peroxidations and scavenging active

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reactive oxygen species (ROS). Le Floch et al. (1998) reported extraction of some phenolic compounds from a natural matrix using carbon dioxide modified with ethanol and methanol and found high recoveries and selectivity.

The solubility of a solute in a SCF is probably the most important thermophysical property that must be determined and modeled for an efficient design of an extraction process based on supercritical solvents. The most recent literature review on experimental methods and systems investigated, regarding solubilities, is given by Dohrn and Brunner (1995). Some solubility measurements of complex phenols in pure SC−CO_2 have been reported recently. Coutsikos et al. (1995) reported the solubility of some simple phenols, Chen and Tsai (1995) of methoxybenzoic acids, Uchiyama et al. (1997) of flavone and 3-hydroflavone, and Choi et al. (1998) of *p*-coumaric acid isomers.

It is known that the solubility of polar substances in nonpolar SCF, such as CO_2, is very low; however, if a small amount of some polar cosolvent is added, the solubility of polar solutes can be significantly increased (Dobbs et al., 1987). The cosolvent may be selected to interact strongly with the solutes of interest in order to facilitate their extraction. Methanol and ethanol are capable of hydrogen-bonding and dipole−dipole interactions with phenols, which indicates that they would be good carbon dioxide cosolvents for extraction of the phenolic components from grape seeds.

This work is part of a broader project to study the integral recovery of some interesting components in grape seeds. Grape seeds are a byproduct of the wine industry that may be used as a source of natural antioxidants in order to increase its value. The feasibility of application of supercritical fluids for selective extraction of individual CPT, or groups of them, from grape seeds is particularly reported in this paper. Two variables have been used to achieve selectivity: pressure and amount of alcohol used as CO_2 cosolvent. The experimental part consisted first of the determination of the solubility of gallic acid, catechin, and epicatechin with an analytical-scale SCF extractor whose operation was previously optimized to achieve saturation of the solvent. A subsequent extraction carried out over an aliquot mixture of the three components indicated the feasibility of their individual separation in a specifically designed process. Second, a commercial concentrate of CPT from grape seeds (CCPTC) was subjected to supercritical extraction in order to detect the CPT that may be extracted under different operation conditions. Finally supercritical fluid extraction was applied to defatted milled grape seeds (DMGS), obtained from the grape seed oil industry. The different amount of each phenolic compound obtained under different operation conditions indicates the possibility of fractionating the CPT content of grape seeds by using supercritical extraction.

Methods using high performance liquid chromatography (HPLC) and a diode array detector (DAD) have been most often used for identification of CPT. However, the development of advanced technologies, which allow a mass spectrometry detector (MSD) to be directly connected to HPLC, present new interesting possibilities for CPT identification and quantification (Lazarus et al., 1999). Some recent works demonstrate the MS capabilities to analyze CPT in crude plant extracts (Vivas et al., 1996; Saucier et al., 1997; Poon, 1998) and specifically in grape seed extracts (Cheynier et al., 1997). HPLC coupled with DAD and MSD have been used in this work for analysis of all CPT mixtures.

EXPERIMENTAL PROCEDURES

Materials. The standards used in this work for HPLC analyses were syringic acid (>98%), methyl gallate (98%) and gallic acid (98%) from Sigma (St. Louis, MO), (+)-catechin hydrate (98%) and (−)-epicatechin (>97%) from Aldrich (WI), and HPLC-grade (−)-epicatechin-3-*O*-gallate, protocatechuic acid, and protocatechuic aldehyde from Extrasynthèse (Lyon, Genay, France). Ethanol (Merck, > 99.8%) and HPLC-grade methanol (Lab-Scan, Dublin, Ireland) were used as cosolvents. Glass beads 30/60 mesh (Phase separations) were used as support of the raw matter in the SCF extraction cell. Two different qualities of carbon dioxide, one used as supercritical fluid (CO_2−SCF-TP), and another used as refrigerant fluid (99.998%), were supplied by Air Liquide (Spain). Liquid N_2 necessary for MS detection was obtained from Carburos Metálicos (Spain). Milli-Q water was used in all work. HPLC-grade acetonitrile (Lab-Scan, Dublin, Ireland) and puriss formic acid (Fluka, Buchs, Switzerland) were used as HPLC mobile phase after filtration through a 0.45 μm pore size filter. A commercial concentrate of CPT from grape seeds (Solgar laboratories, Leoria, NJ) was used for scanning the CPT that can be dissolved by the SC solvents assayed. Defatted milled grape seeds obtained from the grape seed oil industry were also used for this work.

Apparatus and Procedure. Supercritical extraction and solubility experiments were performed on an analytical-scale SCF extractor (Hewlett-Packard, 7680T), whose operation was previously optimized to determine solids solubility through the dynamic analytical method (Murga et al., 1997). The apparatus was equipped with a 7-mL thimble that was used as the solvent-saturation cell. The cell was filled with the raw matter to be extracted or dissolved (≅3 g) distributed in glass beads 30/60 mesh (≅1 g) to improve the contact between solute and solvent and avoid preferential channels when the supercritical solvent passes through the cell. The supercritical solvent used was either pure carbon dioxide or modified with 2, 5, 10, and 15% volume of cosolvent. The carbon dioxide flow was adjusted to the minimum allowed by the SCF extractor (0.5 mL/min) and quantified with a totalizer flow-meter, after recovering the solute at the exit of the extractor. A low flow was used for the solubility and extraction experiments in order to allow the highest residence time of the solvent in the SCF extraction cell, which would allow the saturation of the solvent. A double piston pump (Hewlett-Packard, RSD < 0.3%) was connected to the SCF extractor to have the possibility of pumping the liquid cosolvent up to the extraction pressure. For solute trapping after separation from the solvent, a stainless steel balls packed column was used. The trap was rinsed repeatedly with a solvent (methanol/water, 20/80) in order to quantitatively collect the solute and prepare the sample for analysis.

Sample Analysis. Analysis of all samples was carried out with a HPLC equipped with MSD and DAD (Hewlett-Packard, 1100 series). The HPLC method and the identification and quantification procedure used for CPT mixtures have been described in detail elsewhere (Murga et al., 2000). Some of the main features follow: samples were analyzed on a reverse-phase column

(Spherisorb ODS2, 150 mm × 4.6 mm inside diameter, particle size 5 μm) at constant temperature of 303 K. The mobile phase was a mixture of solvent A (water/formic acid, 95.5/4.5) and solvent B (H_3CN/solvent A, 10/90). Formic acid was added to solvent A to achieve stabilization of the samples through the HPLC column. The flow rate was 0.4 mL/min as the following linear gradient: 100% solvent A was allowed to flow for 2 min; 2.5% solvent B was reached in the following 10 min, 60% B in the next 5 min, and 80% B in the next 63 min. This percentage of solvent B was held 10 min more when the run was finished after a total time of 90 min.

Ionization of the samples for MS detection was achieved at atmospheric pressure through an electrospray interface (API-ES). Negative ionization mode was used (Pérez-Magariño et al., 1999). The API-ES was adjusted as follows: nitrogen was used as nebulizer and drying gas with a total flow of 10 mL/min; the nebulizer pressure was 276 kPa; the temperature of the drier gas was 623 K; the capillary voltage was set at 4000 V and the fragmentor voltage was fixed at 80 V. Flow from HPLC was introduced into the MS without split.

For identification of the different CPT in the samples, a 3-fold information was processed: UV−vis spectra ranging from 220 to 400 nm showed whether the components belong to the CPT family or not, since CPT present characteristic absorption wavelengths and spectra; MS spectra allowed distinguishing monomers from dimers, trimers, etc., and, in some cases, the chemical structure of such components; MS and UV−vis spectra together with relative retention times regarding the standards (Escribano et al., 1992) allowed to tentative identification of most of the components in the samples.

The chromatogram obtained at a wavelength of 280 nm was used for quantification in all cases. Response factors for each of the HPLC standards were obtained by linear regression of known concentrations versus peak areas in the range where the Lambert−Beer law was valid. When standards were not available, the calibration factor obtained for catechin was used.

RESULTS AND DISCUSSION

Solubility of Gallic Acid, (+)-Catechin, and (−)-Epicatechin in SC Mixtures of CO_2 and Ethanol. Solubility is one of the thermophysical properties necessary for extraction process design. Therefore, some solubility measurements were carried out in the first place. Since most of the CPT contained in grape seeds are either not commercially available or much too expensive, one of the low molecular weight phenols, gallic acid, and two proanthocyanidin monomers, (+)-catechin and (−)-epicatechin, were selected. Solubility was determined by carrying out several experiments, in which the total amount of supercritical fluid allowed to flow through the cell—in which pure solute was placed—was changed, varying then the amount of solute extracted. A plot of the total amount of extracted solute versus the total amount of supercritical fluid used to dissolve it gives a linear correlation, whose slope is a measurement of the solubility at the temperature and pressure at which the operation was carried out (Sovová, 1994).

The solutes studied are unstable at high temperatures because their reactivity increases and they may polymerize or oxidize. To avoid such reactivity, temperature was kept at 313 K in all cases. The solvents used were pure CO_2 and CO_2 modified with different amounts of ethanol. The minimum pressure used was 20 MPa, well above the critical locus of ethanol + carbon dioxide mixture (Pöhler and Kiran, 1997). Some experiments carried out at 20 and 30 MPa showed that pure CO_2 dissolves gallic acid (0.005 and 0.013 mg of gallic acid per kg of CO_2, respectively), while it did not dissolve a measurable amount of (+)-catechin or (−)-epicatechin. To increase the solubility of these solutes, ethanol was used as a cosolvent of CO_2. The results obtained are reported in Table 1. It may be observed that the solubility of all solutes increases with the amount of cosolvent, being the largest for gallic acid, then for (+)-catechin, and last for (−)-epicatechin. Solubility was expected to be higher for catechin than for epicatechin since, although they are isomers, catechin presents a lower melting point (Krukonis and Kurnik, 1985; McHugh and Krukonis, 1994; Chen and Tsai, 1995). Differences of solubility of the three components are fairly large when using 10% ethanol as cosolvent, suggesting the possibility of separation of the mixture to obtain their individual components.

Table 1. Solubility (mg of Solute/kg of SC Solvent) of Three Natural Phenols, in CO_2 Modified with Ethanol, at 20 MPa and 313 K

	% ethanol (v/v)		
	2	5	10
solubility of gallic acid	0.11	0.55	7.48
solubility of (+)-catechin	0.06	0.47	4.80
solubility of (−)-epicatechin	0.05	0.45	2.23

To corroborate such separation perspectives, a mixture of identical weight fraction of the three components, gallic acid, (+)-catechin, and (−)-epicatechin, was subjected to extraction with CO_2 + 10% ethanol at 20 MPa and 313 K for 2 h. The composition of the extract was 0.85 weight fraction of gallic acid, 0.14 of (+)-catechin, and 0.01 of (−)-epicatechin. That is, the extract contained a very low amount of epicatechin while it had a high gallic acid content, which agrees with the previous solubility results although solute−solute interactions might vary the individual solubility in multicomponent systems. This experiment leads to the conclusion that selective extraction of the individual components could be obtained when using SC mixtures of CO_2 and ethanol as extracting solvent.

SCF Extraction of CPT from a Commercial Grape Seed Concentrate (CCPTC). Grape seeds are a very complex natural matrix. Therefore, the study of fractionation of the phenolic fraction of grape seeds by using SCF technology was begun with a simpler matrix, i.e., a commercial grape seed CPT concentrate (CCPTC), whose composition in the components of interest for this work is reported in Table 2.

Table 2 also reports the results obtained when the CCPTC was subjected to extraction with supercritical mixtures of CO_2 with 2, 5, 10, and 15% (v/v) ethanol or methanol at 20 and 30 MPa for 3 h and a flow of SC−CO_2 of 0.5 mL/min. When a SC mixture of CO_2 and 2% (v/v) ethanol at 20 MPa was used as solvent, only some low molecular weight phenols, such as gallic acid, protocatechuic acid, and protocatechuic aldehyde, were extracted. When using 5% ethanol, three more CPT were extracted, i.e., syringic acid, (+)-catechin, and (−)-epicatechin, and when using 10% ethanol, epicatechin gallate was additionally extracted. Larger amounts of the same components were recovered at 30 MPa, as expected, since the solvent capacity of a SCF increases with pressure. The main feature of the extract obtained

Table 2. CPT Composition of the Commercial Grape Seed CPT Concentrate (mg of Component/g of CCPTC) and Amount of the Individual CPT Extracted by SCF Extraction at 313 K and 30 or 20 MPa

				(mg of component extracted/g of CCPTC)·$(10^2)^c$													
				SCF = CO_2 + ethanol (% v/v)[a]								SCF = CO_2 + methanol					
				P = 20 MPa				P = 30 MPa				P = 20 MPa				P = 30 MPa			
R_t, min	name of the compound	MW	CCPTC mg/g	2% v/v	5% v/v	10% v/v	15% v/v	2% v/v	5% v/v	10% v/v	15% v/v	2% v/v	5% v/v	10% v/v	15% v/v	2% v/v	5% v/v	10% v/v	15% v/v
11.2	gallic acid	170	4.659	0.02	0.24	23.86	65.96	0.03	0.28	31.69	96.70	NQ	0.54	28.82	30.62	NQ	6.85	43.38	99.73
20.8	protocatechuic acid	154	0.065	0.02	0.12	0.56	1.07	0.02	0.16	0.61	1.50		0.19	1.21	1.69		0.43	1.14	1.73
23.6	monogalloyl glucose[b]	332	0.063				0.39				0.77			0.09	0.31		0.02	0.40	2.14
25.2	protocatechualdehyde	138	0.007	0.11	0.21	0.35	0.47	0.18	0.26	0.37	0.64	NQ	0.13	0.36	0.54	0.05	0.12	0.39	0.70
32.2	methyl gallate	184	0.604										0.96	7.72	9.16	0.02	2.63	7.51	10.50
33.0	dimeric PA, B3[b]	578	0.173								0.25								0.06
34.5	dimeric PA, B1[b]	578	1.918				0.35				1.37							0.02	0.44
36.2	(+)-catechin	290	4.255		0.05	1.41	9.00		0.06	2.24	13.91			3.12	3.24		0.19	6.33	22.63
46.7	dimeric PA, B2[b]	578	2.300				0.39				0.80							0.02	0.40
50.9	syringic acid	198	0.188		0.29	1.77	2.62	0.08	0.32	6.96	8.48			0.26	0.40	0.03	0.05	0.10	0.17
52.4	(−)-epicatechin	290	3.853		0.03	0.41	6.10		0.04	0.86	6.49			1.59	1.73		0.01	3.28	11.66
61.6	dimeric galloyl PA[b]	730	0.937				0.40				0.89								0.14
84.7	epicatechin gallate	442	0.629			0.06	0.71			0.10	0.86							0.22	1.14

[a] SCF were mixtures of CO_2 with 2, 5, 10, and 15% volume of cosolvent. MW = molecular weight of the individual components; R_t = retention time in HPLC elution; NQ = detected but not quantifiable compound. [b] Tentative compound. [c] (10^2) means that values of (mg of component extracted/g of CCPTC) are to be multiplied by 10^{-2}.

when using a mixture of CO_2 and 15% (v/v) ethanol was that some proanthocyanic dimers (B1, B2, B3) and a dimeric galloyl PA were obtained in the extract at 30 MPa. High yields (>90%) of some low molecular weight phenols, such as protocatechuic aldehyde, were extracted under these conditions.

When methanol was used as CO_2 cosolvent, the results were qualitatively similar to the case of ethanol although some differences may be observed: When 2% methanol at 20 MPa was used, gallic acid and protocatechuic aldehyde could be detected in the extract but could not be quantified. No dimers or other compounds with higher molecular weight than PA monomers were recovered at 20 MPa, even when 15% methanol was used. Methyl gallate was found when using methanol as cosolvent while it was not found when using ethanol.

These results suggest that a selective extraction might be performed over a complex CPT concentrate to fractionate the mixture by varying pressure and/or amount and type of cosolvent. Mixtures of SC−CO_2 and a low percentage of cosolvent would extract some of the low molecular weight phenols. A progressive increase in pressure and percentage of cosolvent would allow extraction of increasing molecular weight phenols. These variables conveniently changed in a global SCF extraction process might be the key for isolation of some of the CPT found in grape seed.

A decision on the cosolvent to be used, methanol or ethanol, should be made on the basis of toxicity, solvent cost, and final use of the extract obtained.

SCF Extraction of CPT from Defatted Milled Grape Seeds (DMGS). Some SCF extractions were carried out over DMGS in order to study the possibility to selectively recover their CPT. Experimental conditions were similar to the ones used with the CCPTC, and methanol was used as cosolvent. The results obtained for SCF extraction when using 5%, 10%, and 15% (v/v) methanol, together with the CPT composition of DMGS are reported in Table 3. When 2% (v/v) of methanol was added, no quantifiable amount of any solute was obtained in the extract. By using larger amounts of methanol, up to 15%, only four low molecular weight phenols, (+)-catechin, and (−)-epicatechin were obtained in the extract. The amount of extracted component decreases in all cases compared to extraction of the commercial CPT concentrate. Dimeric PA and other CPT with higher molecular weight were not found in the extracts of DMGS, which was probably due to their lower concentration in the DMGS and to the interactions between the matrix and the single compounds (Björklund et al., 1998). In a natural matrix, the distribution of the solutes in the solid substrate and the interactions among them have a high influence on the course of the extraction.

Table 3. CPT Composition of DMGS (mg of Component/g of DMGS) and Amount of the Individual CPT Extracted by SC Extraction at 313 K and 20 or 30 MPa[a]

			(mg of component extracted/g of DMGS)·$(10^2)^c$					
			P = 20 MPa			P = 30 MPa		
R_t, min	compound name	DMGS mg/g	5% v/v	10% v/v	15% v/v	5% v/v	10% v/v	15% v/v
11.1	gallic acid	0.034	0.05	1.03	2.43	0.21	1.11	2.71
20.5	protocatechuic acid	0.015	0.12	0.48	0.75	0.12	0.55	0.77
23.8	monogalloyl glucose[b]	0.002		0.02	0.04		0.03	0.08
25.4	protocatechualdehyde	0.003	0.20	0.21	0.22	0.21	0.21	0.24
36.6	(+)-catechin	0.058		0.02	0.21		0.03	0.33
52.8	(−)-epicatechin	0.038			0.05		0.03	0.05

[a] SCF were mixtures of CO_2 with 5, 10, and 15% volume of methanol. MW = molecular weight of the individual components; R_t = retention time in HPLC elution. [b] Tentative compound. [c] (10^2) means that values of (mg of component extracted/g of DMGS) are to be multiplied by 10^{-2}.

Conclusion. Classical solvent extraction of the phenolic fraction of grape seed is very well-known and several solvents have been assayed that dissolve the whole phenolic fraction. SC−CO_2 may not be competitive with classical extraction if the whole phenolic fraction is to be extracted; however, CO_2 has the advantage compared to liquid solvents that its solvent power is adjustable by varying pressure and by adding cosolvents, among other variables. The study carried out in this work shows that by adjusting these two variables, the phenolic content of grape seeds may be fractionated. A sequential SCF extraction process could be designed to achieve such fractionation. The results are consistent with the notion that molecular weight might be the dominant factor in the SCF extractions (Van Alsten and Eckert, 1997).

ABBREVIATIONS USED

API-ES, atmospheric pressure ionization electrospray interface; CCPTC, commercial concentrate of complex

phenols and tannins; CPT, complex phenols and tannins; DAD, diode array detector; DMGS, defatted milled grape seeds; HPLC, high-performance liquid chromatography; MS, mass spectrometer; MSD, mass spectrometry detector; MW, molecular weight; NQ, not quantifiable; PA, proanthocyanidins; ROS, reactive oxygen species; R_t, retention time; SC, supercritical; SCF, supercritical fluid; UV−vis, ultraviolet−visible.

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