Fractional Extraction of Compounds from Grape Seeds by Supercritical Fluid Extraction and Analysis for Antimicrobial and Agrochemical Activities

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White grape seeds were subjected to sequential supercritical fluid extraction. By increasing the polarity of the supercritical fluid using methanol as a modifier of CO_2, it was possible to fractionate the extracted compounds. Two fractions were obtained; the first, which was obtained with pure CO_2, contained mainly fatty acids, aliphatic aldehydes, and sterols. The second fraction, obtained with methanol-modified CO_2, had phenolic compounds, mainly catechin, epicatechin, and gallic acid. The fractions were bioassayed. Antimicrobial activities were checked on human pathogens, and a high degree of activity was obtained with the lipophilic fraction. Agrochemical activities on phytopathogenic fungi and activities on the etiolated wheat coleoptile bioassay were also checked. The more polar fraction was active in the latter bioassay.

Keywords: *Supercritical fluids; seeds; antimicrobial; extraction; grapes*

INTRODUCTION

Grape seed extracts have shown a broad range of pharmacological activities including, among others, antiulcer properties (Saito et al., 1998), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced lipid peroxidation and DNA fragmentation in hepatic and brain tissues (Bagchi et al., 1998). They have also exhibited properties against the oxidation of low-density lipoproteins (Meyer et al., 1997). In most cases, the activity is related to the antioxidant properties of the compounds present in the extracts and is mainly attributed to phenolic compounds. Much of the interest in phenolic compounds from grape seeds stems from their antioxidant properties (Vinson et al., 1995) and their ability to serve as free radical scavengers (Maffei-Facino et al., 1994). To date, the most abundant phenolic compounds isolated from grape seed are catechins and their polymers (Fuleki and da Silva, 1997; Escribano-Bailón et al., 1992).

Supercritical fluid extraction (SFE) allows for the isolation of compounds without interference from air and light, thereby guaranteeing conservation of their antioxidant properties. The literature cites examples concerning the higher antioxidant activity of extracts obtained by SFE compared to that of extracts produced by conventional means (Tipsrisukond et al., 1998). Most likely, this is because the extraction conditions for SFE, when CO_2 is used as the extracting fluid ($P_c = 73$ atm, $T_c = 31$C), are gentler than extraction conditions used for other methods, such as Soxhlet extractions. Thus, the degradation of labile compounds with SFE is avoided (Cheung et al., 1998).

Apart from the presence of phenolic compounds in grape seeds, fatty acids were also detected in supercritical CO_2 extracts (Molero-Gómez et al., 1996; Sovova et al., 1994). The main interest in grape seed oils lies in the level of unsaturated fatty acids, such as linoleic and oleic acids (Rao, 1994; Horrobin and Manku, 1983; Fedele, 1983). In addition, grape seed oil contains high amounts of tannins, in levels 1000-fold higher than in other seed oils (Rao, 1994).

When SFE was applied to extract oils, 100% CO_2 was used as the extracting fluid, without any organic modifier. Thus, lipophilic compounds were extracted. However, to extract phenolic compounds, it was necessary to use an organic modifier to increase the solubility of the compounds in the supercritical fluid (Palma and Taylor, 1998).

In our study, different extraction conditions, that is, pure CO_2 and CO_2/MeOH (5:1) as extracting fluids in supercritical and subcritical conditions, were applied to the same sample to obtain fractionation of the compounds. By using increasing amounts of modifiers, it was possible to first extract the low polar compounds and, later, the more polar compounds, mainly phenolics, from the same sample.

In the search for practical applications of extracted compounds, the antimicrobial activity was checked using assorted microorganisms. Gram-positive and Gram-negative bacteria and a human fungal pathogen were used. Activities of extracts as potential agrochemicals

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Table 1. SFE Conditions for Grape Seed Extractions

extraction conditions	fraction A	fraction B
supercrit fluid	CO_2 (100%)	CO_2/MeOH (5:1) (v/v)
solid trap	C18	C18
restrictor temp	55 °C	55 °C
pressure	450 atm	450 atm
extraction temp	35 °C	35 °C
SFE static time	15 min	15 min
SFE dynamic fluid mass	25 g of supercrit fluid	25 g of supercrit fluid
trap rinsing solvent	10 mL of hexane	10 mL of methanol

for phytopathogenic fungi were also examined, using three fungi. Moreover, to ascertain other possible bioactivities, the etiolated wheat coleoptile bioassay was used.

MATERIALS AND METHODS

Samples. White grape seeds were obtained from Synthons Inc. (Blacksburg, VA). They were crushed in a coffee grinder for 2 min, but during this time the grinding was halted for 15 s at periodic intervals to prevent heating of the sample. The samples were stored at room temperature until the extractions were completed. The grape variety was Chardonnay, cultivated in Washington state and hand-picked during the 1997 harvest.

Chemicals. The solid trap used on the supercritical fluid extractor was packed with Upchurch-C18 (0.81 g) from Chrom Tech (Apple Valley, MN). Ottawa Sand Standard (20−30 mesh) and HPLC grade solvents, including hexane, methanol, acetic acid, and water, were obtained from Fisher Scientific (Houston, TX). The phenolic compounds, gallic acid, catechin, and epicatechin, were obtained from Sigma-Aldrich (St. MO) and used as received. Carbon dioxide, with helium headspace, was obtained from Air Products and Chemicals Inc. (Allentown, PA).

Apparatus. A Suprex PrepMaster (Pittsburgh, PA) equipped with an SSI 222D HPLC pump was used for the supercritical fluid extractions. A Hewlett-Packard (Wilmington, DE) GC 5890 Series II gas chromatograph equipped with a Hewlett-Packard MSD 5972 and an HPLC Series 1050 from Hewlett-Packard (Little Falls, DE) equipped with an autosampler, quaternary pump, and a UV−visible multiwavelength detector were used for analyses of the extracts.

Extraction Process. The extractions were conducted in 8 mL vessels. A mixture of 7.5 g of crushed grape seeds (1.5 g) and sand (6.0 g) was used for each extraction. The extraction conditions are presented in Table 1. Fraction B was obtained by re-extracting the sample after fraction A had been obtained.

GC Analysis. The analysis was done with a DB-5MS column (0.25 mm × 30 m, dp = 0.25 μm) from J&W Scientific (Folsom, CA). The injector temperature was 250 °C, and the flow of carrier gas (He) was 1 mL/min. The temperature gradient and the resulting chromatogram for the extract are shown in Figure 1.

HPLC Analysis. The analysis was done using a Luna-C18 column (150 × 2 mm, 5 μm particle size) from Phenomenex (Torrance, CA). The solvents were 2% acetic acid in water (A) and 2% acetic acid in methanol (B). The flow was 0.5 mL/min, and the elution gradient and the resulting chromatogram from the extract are presented in Figure 2.

Bioassays. The microbial bioassay utilized human pathogens: Gram-positive and Gram-negative bacteria [*Bacillus cereus* (+), *Staphylococcus aureus* (+), *Staphylococcus coagulans niger* (+), *Citrobacter freundii* (−), *Escherichia cloacae* (−), *Escherichia coli* (−)] and the fungus *Aspergillus flavus*, the strain used being an aflatoxin producer. The fungal phytopathogens *Botrytis cinerea*, *Cladosporium echinulatum*, and *Penicillum griseofulvum* (as a genus representing the Penicillia) were also used for the microbial bioassay. The Gram-positive and Gram-negative bacteria were grown on nutrient agar and the fungi on potato−dextrose agar. Each organism was heavily seeded onto diagnostic sensitivity test agar (DST) in plastic Petri dishes, to ensure a dense lawn. To these were added 4-mm disks (Whatman No. 3 paper) that had been impregnated with various concentrations of fractions A and B. The fractions were placed on the seeded agar surface. Plates were incubated at 37 °C for 18 h, and then the diameter of the inhibited area was measured. Coleoptile bioassay was performed by germinating wheat (*Triticum aestivum* L. cv. Wakeland) on moist sand at 22 ± 1 °C for 4 days in the dark. Seedlings were removed and prepared under a green safelight at 540 nm. Shoots were fed into a Van der Weij guillotine; the apical 2 mm was cut and discarded, and the next 4 mm of each individual coleoptile was removed for bioassay. Ten 4-mm sections were placed in each test tube with phospate−citrate buffer (pH 5.6) containing 2% sucrose and the fraction to be tested. The test tubes were placed in a roller tube apparatus

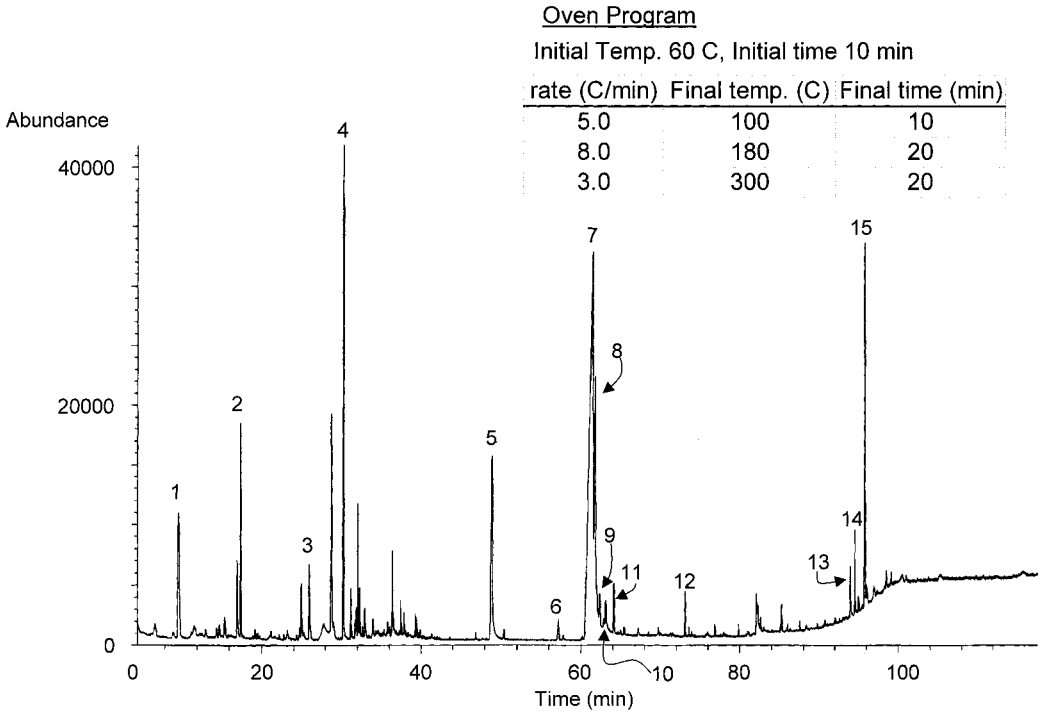

Figure 1. Resulting chromatogram and oven program for GC analysis of fraction A. Assignments of peaks are given in Table 2.

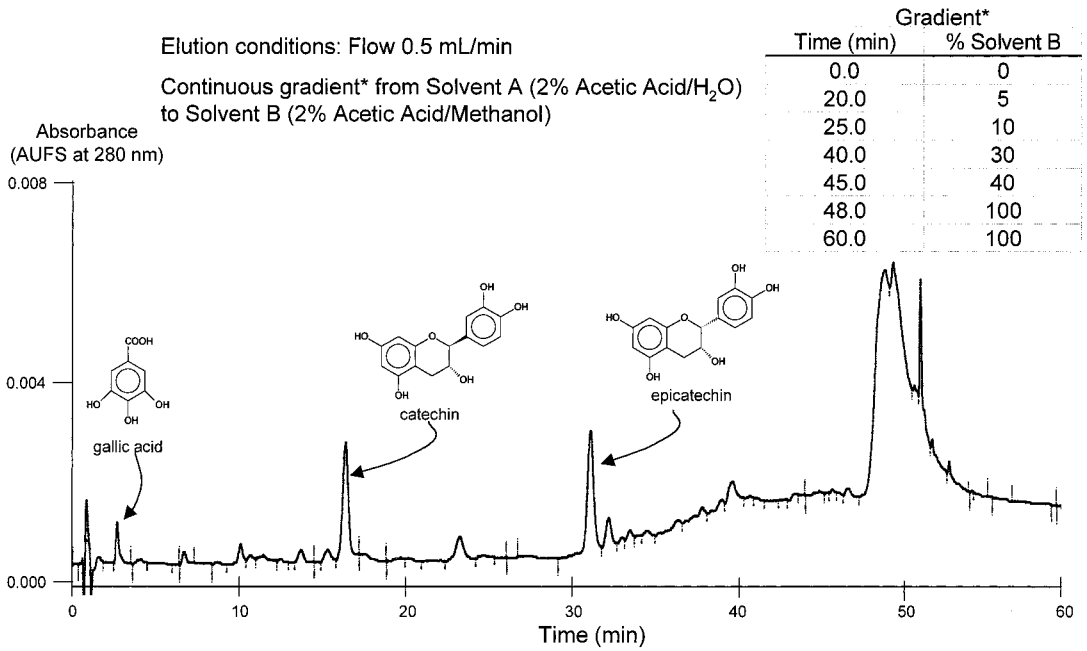

Figure 2. Resulting chromatogram and gradient elution schedule for an LC analysis of fraction B.

Table 2. Assignments for Peaks in Fraction A Analyzed by GC/MS

peak	RT (min)	compound
1	9.7	2-*trans*-heptenal
2	17.4	nonanal
3	26.0	2-*trans*-decenal
4	30.3	2-*trans*-4-cis-decadienal
5	49.0	palmitic acid
6	57.2	linoleic acid methyl ester
7	61.6	linoleic acid
8	61.9	oleic acid
9	62.4	linoleic acid ethyl ester
10	63.1	stearic acid
11	64.1	palmitic acid butyl ester
12	73.1	stearic acid butyl ester
13	94.0	campesterol
14	94.5	stigmasterol
15	95.8	β-sitosterol

for 20 h at 22 °C. The coleoptiles were then taken out and blotted dry, and their magnified (×3) images were measured. All data were statistically analyzed.

RESULTS AND DISCUSSION

Eight extractions were performed on 1.5 g of sample. The average yield was 159.3 mg (10.6%) for the first fraction (fraction A) and 118.4 mg (7.9%) for the second fraction (fraction B). The lowest polarity fraction (A) was a yellow oil after drying under a stream of nitrogen. It was analyzed by GC/MS, and the chromatogram is shown (Figure 1). The Wiley library was used to identify the extracted compounds, and those compounds that we were able to identify are listed in Table 2. All assignments had a correlation level >90% against the library standard.

There were three different kinds of compounds isolated in the oil fraction, that is, aliphatic aldehydes, fatty acids and their derivatives, and sterols. These results agree with the data from the literature, employing both SFE (Molero-Gómez et al., 1996) and conventional liquid extractions (Rao, 1994; Elshami et al., 1992). Some fatty acid derivatives were also found, such as methylated, ethylated, and butylated fatty acids.

The first eluants in gas chromatography were the aliphatic aldehydes: 2-*trans*-heptanal, nonanal, 2-*trans*-decenal, 2-*trans*-4-*cis*-decadienal (Figure 1). These compounds play an important role in the aroma of some oils (Kiritsakis, 1998; Shimoda et al., 1997). It has been proved that nonanal increases in proportion to oxidation of the lipid fraction (Raghavan et al., 1994; Morales et al., 1997).

Sterols have been previously isolated by SFE from aqueous samples (Jayasinghe et al., 1998), and they have been determined before in seed oils (Yildiz et al., 1998; Tsaknis 1998) and in grape juice (Ng and Hupe, 1998). Campesterol, stigmasterol, and sitosterol have been isolated previously from grape seed oil by organic solvent extraction (Elshami et al., 1992). Therefore, the composition of our fraction A via SFE is similar to the composition of grape seed oil as previously described in the literature by organic solvent.

Due to the high polarity of compounds present in fraction B, it was analyzed by LC, and the resulting chromatogram is shown in Figure 2. The two main compounds in this fraction were catechin and epicatechin. Gallic acid is also present in a significant amount, as well. These compounds were identified by their retention times against standard samples.

On the basis of the chromatographic conditions employed (i.e., column, elution, and detection properties), the other minor compounds are, most probably, phenolic compounds.

The literature states that the main low molecular weight phenolic compounds present in white variety grape seed are catechin and epicatechin (Fuleki and da Silva, 1997; Kallithraka et al., 1995). It was reported that the concentration ratio between these two compounds depends on the grape variety, the viticulture practices, and weather conditions.

Therefore, the two extracted fractions obtained by SFE have the main characteristics previously described for both oils and phenolic compounds conventionally isolated from grape seed. In our study, we isolated these two different sets of compounds from the same starting grape seed material in consecutive extractions, using

Table 3. Effects of Fractions A and B, Catechin, and Epicatechin against Human Pathogen Microorganisms

	μg/disk[a]											
	fraction A			fraction B			catechin			epicatechin		
organism	1500	1000	500	1500	1000	500	1500	1000	500	1500	1000	500
Bacillus cereus (+)	−	−	−	−	−	−	−	−	−	−	−	−
Staphylococcus aureus (+)	+	−	−	+	+	−	−	−	−	+	+	−
Staphylococcus coagulans niger (+)	++	++	++	+	+	−	−	−	−	−	−	−
Citrobacter freundii (−)	++	+	−	+	+	−	−	−	−	+	−	−
Echerichia cloacae (−)	++	++	+	+	+	+	−	−	−	+	+	+
Echerichia coli (−)	+	+	−	+	+	−	+	+	+	+	+	−
Aspergillus flavus	−	−	−	−	−	−	−	−	−	−	−	−

[a] 4-mm disk impregnated with selected concentration of fraction A or B. ++, high inhibition (diameter > 15 mm); +, inhibition (15 mm > diameter > 10 mm); −, no inhibition (diameter < 10 mm).

Table 4. Effects of Fractions A and B, Catechin, and Epicatechin against Crop Plant Fungi

	μg/disk[a]											
	fraction A			fraction B			catechin			epicatechin		
organism	1500	1000	500	1500	1000	500	1500	1000	500	1500	1000	500
Botrytis cinerea	−	−	−	−	−	−	−	−	−	−	−	−
Cladosporium echinulatum	+	+	−	−	−	−	−	−	−	−	−	−
Penicillum griseofulvum	−	−	−	−	−	−	−	−	−	−	−	−

[a] 4-mm disk impregnated with selected concentration of fraction A or B. ++, high inhibition (diameter > 15 mm); +, inhibition (15 mm > diameter > 10 mm); −, no inhibition (diameter < 10 mm).

slightly different extraction conditions. Because the main part of fraction B was composed of catechins, bioassays were performed on pure samples of catechin and epicatechin as well. The amount of pure compounds assayed was half of the amount assayed from the extract.

As can be seen in Table 3, the highest activities exhibited by fraction A were against *S. coagulans niger*, *E. cloacae*, *C. freundii*, and *E. coli*. These bacteria were susceptible at the concentration assayed, even at the lowest concentration, so it would be of interest to determine the compound responsible for this bioactivity. On *S. aureus* there was only moderate activity at the highest concentration. *B. cereus* and the fungus *A. flavus* were resistant to fraction A at all concentrations assayed. In the literature, there are reports concerning the modulation properties of sterols, such as are present in fraction A, over the bioactivities of other compounds (Wangspa and Takemoto, 1998), so it may be possible that sterols produced the effects shown by fraction A.

Activities on human pathogens by fraction B were lower that those exhibited by fraction A. Only at the highest concentration was there some moderate activity. However, the activity profile was more homogeneous than in the case of fraction A (i.e., the activity was almost the same on all bacteria, with the exception of *B. cereus*, which was resistant at all of the concentrations assayed). The activity demonstrated by catechin was nil for all bacteria with the exception of *E. coli*, which exhibited moderate activity. For epicatechin, the resulting activity profile was similar to the profile obtained by fraction B, so it is possible that the antibacterial activity of fraction B is mainly due to epicatechin. There were no effects with fractions on fungal phytopathogens (Table 4), with the exception of moderate activity of fraction A on *C. echinulatum*.

The etiolated wheat coleoptile bioassay has been used as a method to isolate and purify biologically active natural products. (Cutler et al., 1991). This bioassay covers a broad range of activities, not only plant growth regulatory properties but also other biological activities, including antimicrobial, immunosuppressant, and pharmaceutical properties.

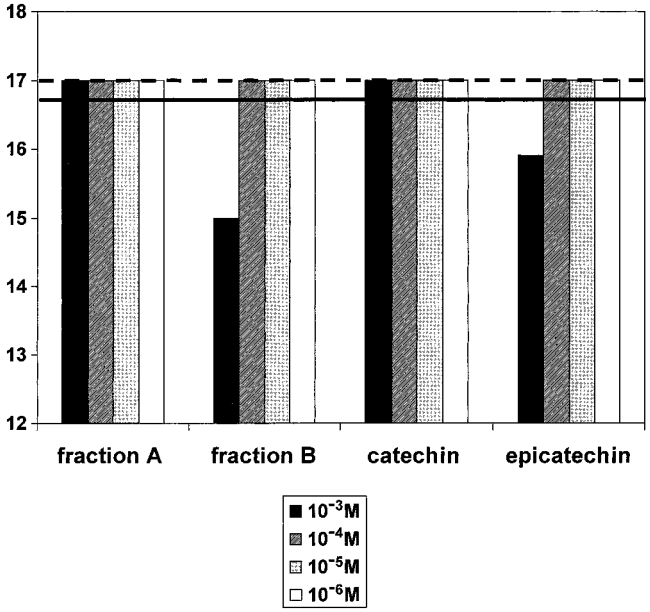

Figure 3. Effects of isolated fractions and pure compounds on etiolated coleoptiles (*T. aestium* L. cv. Wakeland): (dotted line) control; (below solid line) significant inhibition ($P < 0.01$). The initial coleoptile length (×3) was 12 mm.

In this bioassay, fraction B showed moderate activity by inhibiting the growth of wheat (i.e., 40% at the higher concentration tested). Catechin was not active, whereas epicatechin showed a 20% of inhibitory effect at 10^{-3} M (Figure 3). This implies that there are other active compounds in fraction B, different from epicatechin.

It can be concluded that by SFE it is possible to fractionate compounds from grape seeds. The results of the bioassays mean that there are active compounds in fractions extracted by SFE. We do not suggest to winemakers how to process grape seeds because the process is not fully developed for industrial applications. Our study has dealt with the composition and bioactivity of extracts via SFE. More work should be done to determine the compounds responsible for activity in fraction A against bacteria and the responsible compounds of

fraction B in the etiolated wheat coleoptile bioassay. The gentle conditions used and the absence of air and light during the SFE process guarantee the conservation of bioactivities and antioxidant power of extracted compounds because degradation processes are avoided.

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