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Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide

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Abstract

A new analytical method using near critical carbon dioxide to extract polyphenolic compounds from white grape seeds has been developed. Carbon dioxide density, organic modifier, percentage of modifier, and extraction temperature were optimized utilizing an experimental design. Gallic acid, catechin, and epicatechin were the main phenolic compounds detected in the HPLC chromatogram of each extract. Recovery and reproducibility of catechin from grape seed was calculated. Under optimized conditions recovery was estimated to be 79% with a RSD equal to 7.3%. Results from the supercritical fluid method were compared with results obtained via liquid–solid extraction using methanol–water. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Grape seed; Polyphenolic compounds; Catechin

1. Introduction

During the past few years it has been demonstrated that phenolic compounds contribute to both the flavor properties of wine and the pharmacological effects of wine. A broad distribution of phenolic compounds appears inside grapes. Furthermore, for a single variety of grape, the phenolic composition depends upon whether the extraction is performed on the pulp, skin, or seed. For example, catechins and polymers of catechins (e.g. procyanidins) are likely to be found in grape seeds [1–3].

Phenols in the grape seed contribute to the total phenolic composition of the grape-derived wine. This comes about due to the several grape maceration steps that must be taken in the processing of grapes to make wines. It is at the maceration process that phenols are extracted from the seed. Therefore, the contribution of phenolic compounds from seeds is increased when long processing times are used. This phenomenon is most notable in the production of red wines [4].

Phenolic compounds such as catechins and procyanidans from seeds have been shown to affect the bitterness and astringency of wines [5–7]. In terms of pharmacological properties, these phenols may act against in vitro oxidation of low density lipoprotein [8]. They have also been suggested to have antiulcer [9], anticarcinogenic [10], antimutagenic [11], and antiviral [12] activities. The high antioxidant power of phenols is generally believed to account for these activities [13,14].

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Common methods for the isolation of phenolic compounds from grape seed use organic solvents such as methanol [2,3,15], ethanol [15,16], and acetone [17]. Extraction recoveries have been shown to improve if mixtures of methanol–water and acetone–water are employed [15]. Various extraction temperatures have been used with times that range from a few minutes to several hours.

Supercritical fluid extraction (SFE) affords big advantages over more conventional extraction techniques. The absence of both light and air during the extraction process can reduce the incidence of degradation reactions that can more easily occur when using other extraction techniques. For example, Tipsrisukond, et al. have reported that supercritical fluid extracts exhibit higher antioxidant power than extracts obtained by classical methods [18]. Furthermore, it is not necessary to concentrate the resulting extract because only a small volume of organic solvent is normally used. SFE has been previously applied to grape seeds for the removal of oils [19]. Pure CO_2 without an organic modifier was used in this extraction. Phenolic compounds were apparently not removed under these conditions. The use of small quantities of organic solvent with supercritical CO_2 (i.e. modifier) should extract phenols from the seed. Since the addition of a modifier to the CO_2 raises the critical temperature, many times near critical conditions rather than supercritical conditions are employed.

In this paper we have focused on phenolic compounds (i.e. polyphenols). In a previous report [20], we described the optimized extraction conditions for removal of eight polyphenols from an inert, spiked matrix using modified CO_2. The optimization study suggested that the most influential variables on phenol recovery were: (a) supercritical fluid CO_2 density, (b) nature of organic modifier, (c) modifier percentage, and (d) the extraction temperature. While this information is valuable, the specific parameters must be re-optimized for grape seed due to differences in the interaction of phenols with the inert matrix and the grape seed matrix. Although the distribution of polyphenols is not homogeneous inside the seed, we nevertheless analyzed the global grape seed composition as opposed to analyzing different parts of the seed. Each extract was kept cold and protected from air and light no more than 24 h prior to chromatographic analysis. Since the age of the raw material can modify its phenolic composition, our goal was to develop a method to extract the phenolic compounds from grape seed, not to quantify phenols in these particular grape seeds.

2. Experimental

2.1. Pre-extraction sample preparation

Grape seeds were provided by Synthon, Inc. (Blacksburg, VA). The variety of grapes was Chardonnay. They were cultivated in Washington State and hand-picked during the harvest of 1997. Seeds were crushed in a coffee grinder for two min, but at 15 s intervals the process was stopped for 15 s to avoid heating of the sample. The crushed seeds were stored at room temperature prior to extraction.

2.2. Extraction process

Extractions were conducted in stainless steel vessels (1.0 ml). For the optimization process, seed extractions were performed in duplicate. Approximately 30 mg of crushed grape seeds were used in each extraction. The optimization study varied only four parameters: CO_2 density, modifier type, percent modifier, and extraction temperature. The following conditions were common to every extraction: liquid CO_2 flow-rate=1 ml/min, amount of organic modifier used in the static mode=0.25 ml (added manually over the sample just before sealing the extraction chamber), static extraction time=20 min, mass of CO_2 used during dynamic extraction=20 g, restrictor temperature=50°C, solid-phase trap temperature=35°C, trap rinse solvent=methanol (3 ml) at 0.5 ml/min. The four variables incorporated into our experimental design are shown in Table 1 along with the specific high/low conditions employed.

Due to the high polarity of the catechins, rather high extracting solvent/sample ratios were employed. An internal standard (i.e. 20 μl of β-resorcylic acid, 1840 ppm) was immediately added after the extraction process to the resulting extract. The experiments called for in the experimental design were done in a random order. Liquid–solid extractions (LSE) were performed using 10 ml of

Table 1
Experimental design optimization parameters and results

Experiment	CO_2 density (g/ml)	Organic modifier[a]	Percentage	Extraction temperature (°C)	Area ratio[b]	Area ratio/g of seed
1	0.85	E	10	35	2.7	95.0
2	0.95	E	10	55	4.7	172.5
3	0.85	M	10	55	8.5	282.5
4	0.95	M	10	35	8.1	270.0
5	0.85	E	40	55	3.7	142.5
6	0.95	E	40	35	4.1	155.0
7	0.85	M	40	35	5.3	182.5
8	0.95	M	40	55	7.7	232.5

[a] E: EtOH; M: MeOH.
[b] Total area relative to internal standard area. Each data is the average of two experiments.

methanol–water (4:1) and 240 mg of seed. LSE was carried out in 20 ml vials that were protected from light with aluminum foil for 16 h at room temperature. Sonicated assisted liquid–solid extraction (SALSE) was also carried out in 20 ml vials, but it was performed for a total of 1 h in two 30 min periods separated by 30 min. This procedure allowed for a longer contact time between sample and extracting medium without increasing the sonication time. During the process, the temperature of the water inside the sonicator was kept under 30°C. The extracts were filtered through 50 μm Nylon filters. Internal standard (20 μl) solution was added to the extract after termination of the extract.

2.3. Apparatus

A Suprex Autoprep 44 (Pittsburgh, PA) SFE equipped with a Varian Star SFE modifier pump was used for all supercritical fluid extractions. Analysis of each extract was carried out with a HPLC series 1050 system from Hewlett Packard (Little Falls, DE) equipped with an autosampler, quaternary pump, and an UV–visible multiwavelength detector. An Aquasonic 75HT (VWR, Boston, MA) ultrasonic bath was used for sonicated-assisted liquid extractions.

2.4. Reagents

The solid-phase trap was filled (0.95 g) with Isolute C_{18} (40–70 μm) from IST (Hengoed, UK). Ottawa sand (20–30 mesh) and all HPLC grade solvents were obtained from Fisher Scientific (Houston, TX). The (+)-catechin standard was obtained from Sigma–Aldrich (St. Louis, MO). Solutions of the standard were prepared in methanol. CO_2 with helium headspace from the Air Products and Chemicals, Inc.(Allentown, PA) was the primary extraction fluid.

2.5. Chromatographic and data analysis

A Luna C_{18} column (150×2 mm, dp=5 μm) from Phenomenex (Torrence, CA) was used for all extract assays. The UV wavelength used was fixed at 280 nm. A binary mobile phase of 2% acetic acid in water (A) and 2% acetic acid in methanol (B) in a gradient mode was incorporated. Injection volumes (5 μl) were always identical. Mobile phase flow-rate was 0.5 ml/min. The gradient schedule was: 0–10% B in 20 min at 0.5%/min, hold for 5 min, then 10–30% in 15 min at 1.33% min, then 30–40% B in 5 min at 2%/min, then 40–100% B in 3 min. Minitab Release 10extra (State College, PA) was used to both carry-out the data analysis and ascertain the experimental design.

3. Results and discussion

A fractional factorial experimental design that covers only half of the possible experiments was employed. This kind of design had previously yielded valuable insight for the extraction of polyphenols spiked on an inert matrix [19]. Since a ‘‘real world,’’ non-spiked matrix was to be dealt with in this study, we suspected stronger extraction conditions might be

required. In other words, higher CO_2 density and higher extraction temperature (0.95 g/ml, 55°C) were checked. We dared not go any higher in temperature because some phenols are somewhat thermally unstable in the presence of air. For example, several weeks are needed to degrade compounds like catechin at 55°C; whereas, only a few days are required if the temperature is higher. While ethyl acetate was found to be a good modifier in our inert matrix study, we chose not to use it due to the fact that it is a good solvent for grape seed oils and it would be expected that seed oils would co-elute with the phenolic compounds from the solid trap. Therefore, to get cleaner extracts, methanol and ethanol were used at 10% and 40% (v/v). The higher percentage is considerable greater than was found to be necessary to extract polyphenols from an inert matrix. Since we used a relatively low solid-phase trap temperature, we suspected that there would be some modifier condensation on the trap during the extraction which could lead to some analyte loss. Therefore, all of the condensed modifier collected during the dynamic step was combined with the rinse solvent for analysis.

The target value in the experimental design was the area of the combined chromatographic peaks derived from the extract. In other words, the eight highest peaks in the typical extract chromatogram were chosen (noted by * on the chromatogram shown in Fig. 1), and their areas were added to obtain a total area. Due to the fact that different amounts of sample were taken for various experiments and the final extract volume varied, the total area was divided by the amount of each sample and by the area of the internal standard (β-resorcylic acid) used. In this way, the total phenol area per gram of sample and per unit area of internal standard was obtained. The extraction conditions and results are shown in Table 1. From the literature, catechin and epicatechin appear as the more important non-flavonoid phenolic compounds in grape seeds consequently they are the only ones identified in this work.

A graphical analysis of the data from the experimental design was made. Fig. 2 reveals that the most important variable was the identity of the organic modifier. For example, approximately twice as much polyphenol was removed with methanol as opposed to ethanol. CO_2 density, percentage of modifier, and extraction temperature exhibited smaller influences. The average area ratio per gram of seeds using 0.95 g/ml was 13% higher than when using 0.85 g/ml. Methods with 10% modifier produced 12% more extract than methods with 40% modifier. Using 55°C as the extraction temperature, the area ratio per gram was increased 14% versus the methods that used 35°C. In summary, the best conditions with the described SFE instrumentation were 0.95 g/ml, 10% methanol, and 55°C. An analysis of interactions among variables gave no significant interactions.

It is interesting to compare the optimum conditions used previously for the spiked inert matrix

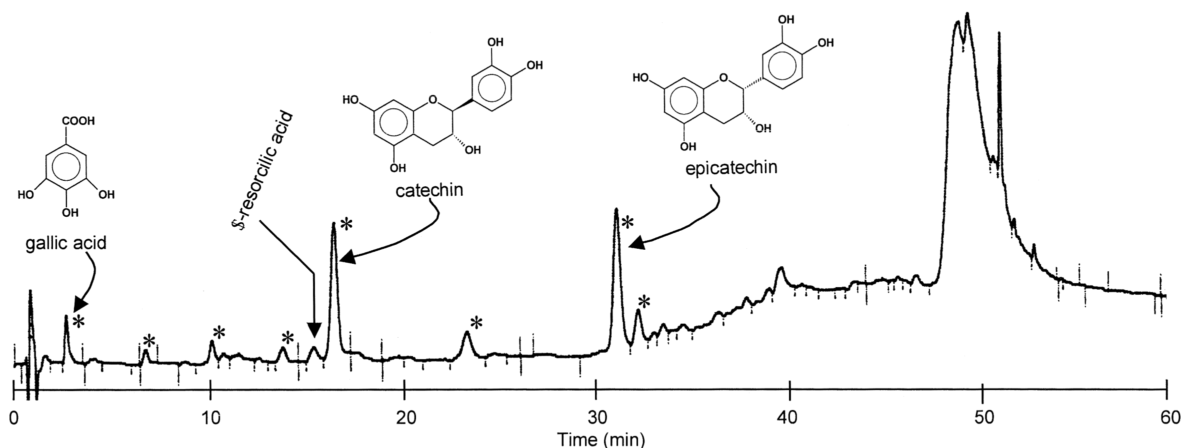


Fig. 1. Chromatogram of extract from experiment three.

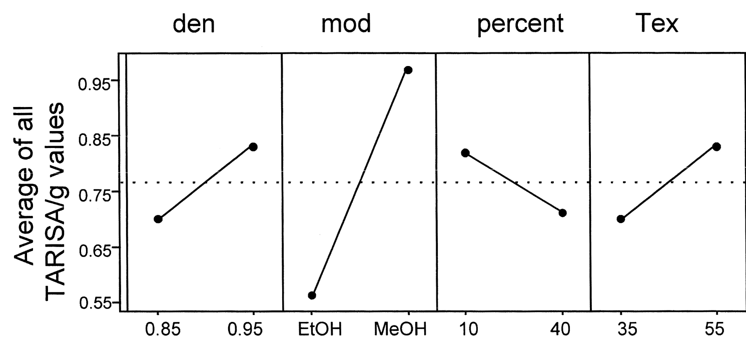


Fig. 2. Main effects plot of variables on the average of TARISA per gram of seed.

versus the non spiked grape seed matrix for the extraction of polyphenols. Higher CO_2 density (0.95 versus 0.85 g/ml) and higher temperature (55°C versus 35°C) were required for the seed matrix probably to overcome analyte–matrix interactions that were not present in the spike inert matrix. The reason for a lower percentage of modifier with the grape seed (10% versus 30%) can be explained by the change in modifier from ethyl acetate to methanol which is a more polar modifier. The higher CO_2 density coupled with a more polar modifier called for less amount of modifier.

We were interested to measure the reproducibility of the developed method. Two modifications in the procedure, however, we made prior to the study. Re-extraction of the seed raffeinate revealed small chromatographic peaks in the resulting HPLC assay. This observation suggested that our optimized method had not yielded an exhaustive extraction. For this reason, the amount of CO_2 used in the dynamic extraction step was increased. A second modification was to include an additional dynamic extraction step with pure CO_2 (e.g. 5 g of CO_2) whose purpose was to ensure that any remaining organic modifier would be removed completely from the extraction vessel. It was felt that polyphenols would favorably partition into the organic modifier as opposed to the CO_2 if modifier remained in the vessel with the seeds.

Five extractions were performed on approximately 30 mg samples. Catechin was selected as the target analyte. It was chosen because of its high sensitivity to degradation processes. We reasoned that it should be one of the polyphenols with a high variability in extraction recovery. Catechin was identified by retention factor and by the addition of an authentic standard of catechin to the extract. The chromatographic peak area of catechin was measured and divided first by the chromatographic peak area of the internal standard and then by the amount of seed sample. Results (i.e. area ratio/gram of seed) for each of the five extractions are shown in Table 2. A relative standard deviation of 7.3% was found.

Table 2
Repeatability of the extraction method

Extraction	Area of catechin	Area of I.S.	g of sample	Area of catechin/ area of I.S. per gram of sample
I	1651	397	0.0310	134.2
II	1168	323	0.0276	131.0
III	1388	351	0.0321	123.2
IV	1244	238	0.0356	146.8
V	1438	335	0.0346	124.1
			Mean	131.8
			SD	9.6
			RSD	7.3

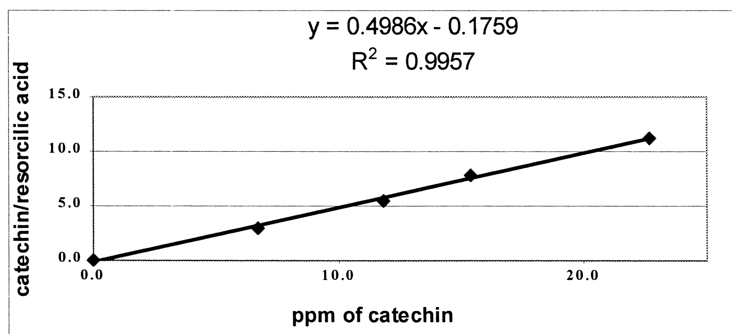
Parenthetic to these extractions, a calibration curve was constructed using four different concentrations (1343, 2366, 3078, and 4537 ppm) of catechin. The solutions were prepared by mixing 50 μl of standard dissolved in methanol with 20 μl of internal standard diluted to 10 ml with methanol. The entire process was done in duplicate and the averaged results are tabulated and graphed in Fig. 3. The *y*-axis of the graph represents the ratio of catechin chromatographic peak area and internal standard chromatographic peak area. It was of interest to compare the efficiency of the SFE method with the conventional extraction method using organic solvent. Several methods for extracting catechins from grapes seed are available in the literature [2,3,15–17]. The most general methodology involves the use of aqueous methanol for 16–24 h at room temperature. SALSE has been found to be useful for various kinds of solid matrices [21,22], among them cereal seeds [23,24]. Therefore a sonicated assisted extraction was attempted. For one hour, a seed/aqueous methanol mixture was sonicated. The time used for SALSE matched the time used for SFE. The results are shown in Table 3 expressed as milligrams of catechin extract per 100 grams of seed. Three sets of data

Table 3
Comparison of recoveries for three extraction methods (mg of catechin per 100 g of seeds)

	LSE[a]	SALSE[b]	SFE[c]
Extraction 1	62.8	63.2	79.0
Extraction 2	68.3	65.1	77.1
Extraction 3	67.2	64.5	72.5
Extraction 4	64.5	58.9	86.4
Extraction 5	65.3	63.4	73.0
mean	65.6[d]	63.0[d]	77.6
RSD	3.3	3.9	7.3
relative recovery to SFE	84.5	81.2	100.0

[a] LSE: Liquid Solvent Extraction.
[b] SALSE: Sonicated Assisted Liquid Solvent Extraction.
[c] SFE: Suprecritical Fluid Extraction.
[d] Statistically lower than recovery obtained by SFE (*t*-test, 95% confidence level).

catechin (ppm)	average of area of catechin	average of area of IS	ratio area catechin/ area IS
0.0	0.0	0.0	0.00
6.7	170.5	57.0	2.99
11.8	328.0	61.0	5.38
15.4	444.0	56.5	7.86
22.7	639.5	57.5	11.12



* Each point represents the average of duplicated data

Fig. 3. Calibration curve for standards of catechin.

are shown: LSE, SALSE and SFE. The LSE and SALSE results are statistically the same while, SFE produced higher recoveries (e.g. 16–20% greater) than LSE or SALSE. The differences in catechin recovery from grape seeds may be due to insufficient solvating power of the aqueous methanol or due to degradation processes during the time of extraction. Degradation processes are less likely during SFE because the sample is always under a CO_2 atmosphere and is protected from light. Reproducibility, however, with LSE or SALSE was better than SFE. One advantage of SFE over LSE is that full automation is feasible by coupling SFE with a chromatographic assay.

It was of interest to determine the extent to which one could extract catechin that has been spiked onto grape seed. The spike solutions were the same four solutions which were used to build the calibration mentioned earlier. Along with a pre-weighed amount of seed, 50 μl of each solution was individually placed in the extraction vessel prior to the addition of organic modifier. Since the calibration solutions were made up in methanol, only 200 μl of additional modifier was added to each extraction vessel. Extractions were then performed on each spiked seed sample. This experiment was desirable because we could determine the effects of other co-extractives in the ''real world'' sample on the extraction of catechin by SFE. The results are shown in Table 4. The estimated recovery was 79% relative to the respective spiked amount. Therefore estimates of catechin in the seeds should be corrected by this factor. This means that the mass of catechin in grape seed is probably close to 98 mg/g of seed rather than 70 mg/g of seed as previously shown by SFE.

In conclusion SFE using methanol-modified CO_2 yielded higher catechin and other polyphenol recoveries from grape seed than liquid–solid extraction. Although SFE gave a lower reproducibility, it was fast and the extracts are more protected from degradation processes.

Table 4
Recovery of spiked catechin from grape seeds using different levels of standard addition

Catechin added (mg)	Catechin extracted (mg)	Recovery (%)[a]
67.2	53.9	80.2
118.3	99.2	83.8
153.9	108.8	70.7
226.9	187.0	82.4

[a] Average recovery=79.3%.

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