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Sequential Fractionation of Grape Seeds into Oils, Polyphenols, and Procyanidins via a Single System Employing CO_2-Based Fluids

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Pure supercritical CO_2 was used to remove >95% of the oil from the grape seeds. Subcritical CO_2 modified with methanol was used for the extraction of monomeric polyphenols, whereas pure methanol was used for the extraction of polyphenolic dimers/trimers and procyanidins from grape seed. At optimum conditions, 40% methanol-modified CO_2 removed >79% of catechin and epicatechin from the grape seed. This extract was light yellow in color, and no higher molecular weight procyanidins were detected. Extraction of the same sample after removal of the oils and polyphenols, but now under enhanced solvent extraction conditions using methanol as a solvent, provided a dark red solution shown via electrospray ionization HPLC-MS to contain a relatively high concentration of procyanidins. The uniqueness of the study is attested to by the use of CO_2-based fluids and the employment of a single instrumental extraction system.

KEYWORDS: Grape seed; supercritical fluid; extracts; polyphenols; procyanidins

INTRODUCTION

Grape seed extract has become popular in recent years as a nutritional supplement that possesses antioxidant activity. These antioxidants are believed to exhibit antiulcer (*1*), anticarcinogenic (*2*), antimutagenic, and antiviral (*3*) activities. The molecular distribution and total content of antioxidant (e.g., polyphenols) in grape seed extracts principally depend on the raw materials used, but the distribution can be modified by the procedure used to extract them. Furthermore, for a single variety of grape, the phenolic composition depends on whether the extraction is performed on the pulp, skin, or seeds. For example, catechin and procyanidins are found in grape seeds, which contribute to the bitterness and astringency of wines (*4*−*6*).

Common extraction methods for the removal of polyphenols from grape seed use traditional organic solvents such as methanol, ethanol, ethyl acetate, and acetone. It has been shown that water added to the organic phase can improve extraction recovery (*7*). Extraction times vary from a few minutes to several hours (*8*). Different procedures are required to separate polyphenols from procyanidins. Torres et al. (*7*) used cysteamine hydrochloride as a thiolysis reagent to depolymerize the polymeric procyanidins. They used cation exchange to separate the depolymerized mixture into different functional groups. Next, RP-HPLC was used to separate the mixture. Other groups have used different thiolysis reagents, but the procedure to depolymerize the polyphenols in the grape seed extract and analyze them via RP-HPLC is similar (*9*, *10*).

Supercritical fluid extraction (SFE) has significant advantages over more conventional solvent extraction techniques. The absence of light and air, for example, during the extraction can reduce the degradation process that can occur easily with other extraction techniques. Previously, it has been shown that pure CO_2 can be used for the removal of oils from grape seed (*11*). It was later demonstrated (*12*) that removal of phenolic compounds with CO_2 from de-oiled seed required 40% modifier; Palma et al. used different pressures, temperatures, extraction times, modifiers, and modifier concentrations to optimize the extraction efficiency of povlyphenols. It was determined that methanol was a better CO_2 modifier than ethanol. In the study reported here, we have used a single SFE instrument in conjunction with supercritical CO_2 coupled to enhanced solvent extraction (ESE) with methanol for sequential recovery of oils, polyphenols, and extensively polymerized procyanidin compounds from grape seeds. It will be demonstrated that pure CO_2 removes most of the oil from the seeds, whereas methanol-modified CO_2 removes most of the monomeric polyphenol and some dimers, and pure methanol in the ESE mode removes the remaining dimers, trimers, and higher polymeric polyphenols.

EXPERIMENTAL PROCEDURES

Sample Preparation. Chardonnay grape seeds were provided by Synthon, Inc. (Blacksburg, VA). They were cultivated in Washington state and hand-picked during the harvest of 1997. Seeds were crushed in a coffee grinder for 2 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.

Extraction. An Isco (Lincoln, NE) 3560 Automated Supercritical Fluid/Enhanced Solvent Extractor with a 10 mL PEEK cell was used

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Table 1. Sequential Extraction Conditions Applied to Grape Seeds

Step 1: SFE Conditions (Pure CO_2)	
oven temp	80 °C
CO_2 pressure	9500 psi
dynamic ext time	60 min
flow rate	2 mL/min
fluid	pure CO_2
sample mass	5 g
collection trap	empty vial 1
trap temp	25 °C
restrictor temp	60 °C
Step 2: SFE Conditions (Methanol-Modified CO_2)	
oven temp	80 °C
CO_2 pressure	9500 psi
dynamic ext time	60 min
flow rate	2 mL/min
fluid	60:40% CO_2/MeOH
sample mass	raffeinate from step 1
collection trap	empty vial 2
trap temp	70 °C
restrictor temp	60 °C
Step 3: ESE Conditions (Pure Methanol)	
oven temp	80 °C
pressure	2000 psi
static ext time	30 min
flush volume	10 mL
solvent	100% MeOH
CO_2 purge	2000 psi for 5 min
sample mass	raffeinate from step 2
collection trap	empty vial 3

for all extractions. Three sequential yet different extraction steps constituting a single method were used for each sample. The first two steps involved the use of CO_2 as an extraction fluid (SFE), whereas the third step used only methanol as an extraction fluid (ESE). **Table 1** shows the extraction conditions for each step. In each extraction, 5 g of crushed grape seeds was used. The extract was analyzed immediately after preparation. For all SF and ES extractions, the total volume of extract in each vial was adjusted to 10 mL prior to analysis.

Analysis. All supercritical fluid chromatographic analyses were performed using a Berger Instrument Inc. (Newark, DE) SFC equipped with autosampler, oven, and diode array UV detector. Separations were perfomed on two stacked Supelcosil diol (Supelco, Bellefonte, PA) columns (2 × 250 mm × 4.6 mm, 5 μm d_p) using supercritical CO_2 modified with methanol containing 0.25% citric acid as the mobile phase. SFC conditions were as follows: 40 °C; liquid flow, 2 mL/min; column back pressure, 125 atm; modifier program, 93:7% CO_2/MeOH, hold for 1 min and then ramp to 83:17% at a rate of 1.7%/min, then ramp to 55:45% at a rate of 4%/min, and hold for 10 min.

HPLC analyses were performed with an Agilent (Wilmington, DE) 1050 quaternary HPLC pump and multiwavelength UV detector. The HPLC was interfaced to a MicroMass (Milford, MA) Platform mass spectrometer equipped with an APCI-ES ionization chamber. The HPLC column output was split 1:10 with only 1 part going into the MS and 9 parts going to the UV detector. Separation of polyphenols via HPLC was performed on an Agilent Eclipse C_{18} column (250 × 4.6 mm, 5 μm d_p). The binary mobile phase consisted of (A) 1% aqueous formic acid and (B) CH_3CN containing 1% formic acid. Separations were performed with a series of linear gradients at a flow rate of 1 mL/min. Elution started with 5% B, which was raised to 25% B in the initial 20 min. B was increased to 35% over the next 4 min, then to 40% over 8 min, and then to 100% B from 32 to 40 min. Data were collected with both UV detector at 280 nm and MS. The MS conditions for analysis in the negative ion mode included a cone voltage of 31 V, a capillary voltage of 3.15 kV, and a source temperature of 120 °C. A scan time of 1.4 s and interscan delay of 0.05 s were used (*m*/*z* 200−700 and 500−1200 amu).

Table 2. Recovery of Oil Using Pure CO_2 from 5 g of Crushed Grape Seeds[a]

extraction	oil wt (g)	recovery (%) (relative to total wt)
1	0.456	9.1
2	0.501	10.0
3	0.480	9.6

[a] Extraction conditions: 9500 psi, 80 °C, 2 mL/min, 60 min dynamic.

Table 3. Relative Recovery (Percent) of Catechin and Epicatechin from De-oiled Grape Seed

modifier/extraction time	catechin SFE	epicatechin SFE	catechin ESE	epicatechin ESE
30% MeOH/60 min	60[a]	59[a]	40[b]	41[b]
30% MeOH/90 min	66	64	34	36
30% MeOH/120 min	75	75	25	25
35% MeOH/60 min	72	73	28	27
40% MeOH/60 min	77	79	23	21

[a] Percent of total catechin or epicatechin recovered via SFE. [b] Percent of total catechin or epicatechin recovered via ESE.

RESULTS AND DISCUSSION

SFE and ESE. In the first step of our extraction, we used 100% CO_2 at 9500 psi and 80 °C to remove grape oil from the seeds. Extraction results using pure CO_2 showed that this type of grape seed has ∼10% oil by weight. It appeared that 60 min of extraction with a flow rate of 2 mL/min of liquid CO_2 was sufficient to remove >95% of the oil from the sample. **Table 2** shows recovery of oil for triplicate extraction of 5 g of crushed grape seed. Increasing the extraction time further did not significantly increase the recovery of oil.

Next, we used methanol-modified CO_2 at 9500 psi and 80 °C to remove polyphenols from the de-oiled grape seeds. **Table 3** shows the recovery of two polyphenols using various percentages of methanol-modified CO_2 and several extraction times. As can be observed, increasing the supercritcal fluid extraction time from 60 to 120 min using 30% methanol-modified CO_2 increased extraction recovery of catechin and epicatechin by 15 and 16%, respectively, of the total. Increasing the modifier concentration from 30 to 40% and keeping the extraction time at 60 min yielded a further increase in recovery of ∼20%. Thus, it was felt to be more economical and faster if

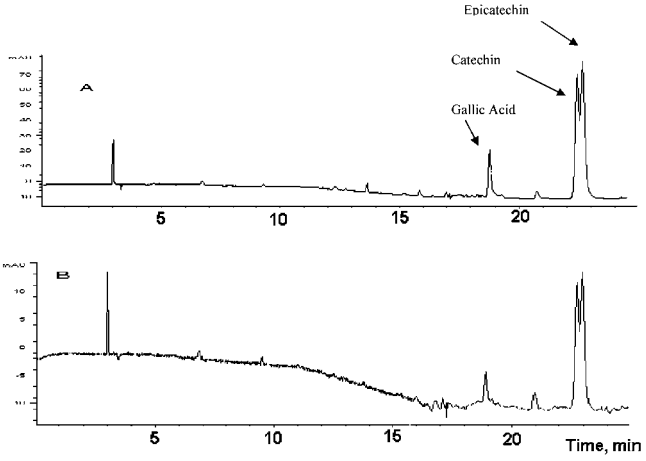

Figure 1. SFC separation of grape seed extract via SFE (**A**) and ESE (**B**): (**A**) methanol-modified CO_2 of de-oiled seeds; (**B**) methanol extract after two-step SFE of seeds. See Experimental Procedures for additional information.

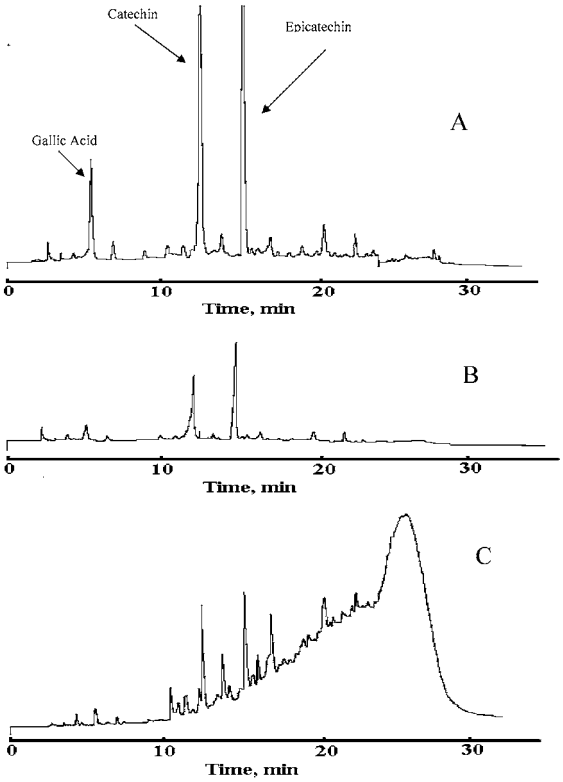

Figure 2. HPLC separation of SF extract of de-oiled grape seed and ES extract of de-oiled/MeOH–CO_2 SFE of grape seed: (**A**) MeOH-modified CO_2 extract, first time; (**B**) MeOH-modified CO_2 extract, second time; (**C**) pressurized MeOH extract. See Experimental Procedures for additional information.

modifier percentage was increased from 30 to 40% rather than increasing the extraction time.

After extraction of grape seed with initially 100% CO_2 and then 40% methanol-modified CO_2, raffeinate in the extraction cartridge was further extracted with 100% methanol using ESE conditions, thus yielding additional catechin/epicatechin (**Table 1**, step 3). Alternatively, the remaining polyphenols (≤20%) could be extracted using either longer SF extraction times or re-extraction of the raffeinate using the same SFE procedure (40% methanol-modified CO_2 for 60 min). Analysis of the resulting extract in both cases showed that more catechin and epicatechin (~10%) could be extracted from the grape seed. Our results showed that the calculated concentrations of catechin and epicatechin in de-oiled grape seeds after being extracted under optimum SFE conditions for 60 min were 3.4 and 4.9 mg/g, respectively. It is important to note here that the color of the supercritical extract after 60 min using 40% methanol wherein all polyphenols could be removed was light yellow. The color of the enhanced solvent extract after 30 min of static extraction and 10 min of dynamic extraction (10 mL solvent) using 100% methanol, on the other hand, was dark red. For the static ESE period, the cartridge with 5 g of crushed grape seed was filled with methanol. It appeared that 30 min of static extraction time was sufficient for complete extraction. Lowering the static extraction time (e.g., 20 min) caused incomplete extraction, and an additional extraction step was required. A dynamic extraction volume of 10 mL was chosen on the basis of the dead volume of the extraction cell, which was determined to be 3−4 mL of methanol. For this reason, 10 mL of solvent was thought to be enough to flush the cell at least three times after the static extraction period.

SFC-UV Analysis. Figure 1 shows the SF chromatogram of the methanol-modified CO_2 extract (**A**) and the 100% methanol ES extract (**B**). Catechin and epicatechin were identified on the basis of the retention time of eluted standards (*13*). It is believed that the peak with a retention at 18.4 min is gallic acid on the basis of the retention time of an eluted gallic acid standard. It is important to note here that after several injections of the ES extract (e.g., previously SF extracted grape seeds), the inlet pressure of the column increased. The pressure could be lowered by periodically changing the guard column frit. It is believed that most of the high molecular weight polymeric polyphenols from the ES extract of grape seeds were precipitated at the head of the guard column after injection into the supercritical mobile phase and, thus, were not subjected to chromatography.

HPLC-UV Analysis. Figure 2A shows the HPLC of the de-oiled grape seed extract using 40% methanol-modified CO_2 for 60 min, whereas **Figure 2B** shows the separation of the same grape seeds after being extracted for a second time under the same SFE conditions (40% methanol-modified CO_2 for 60 min). As can be observed, longer times extracted more catechin and epicatechin from the seeds. **Figure 2C** shows the ES extract of

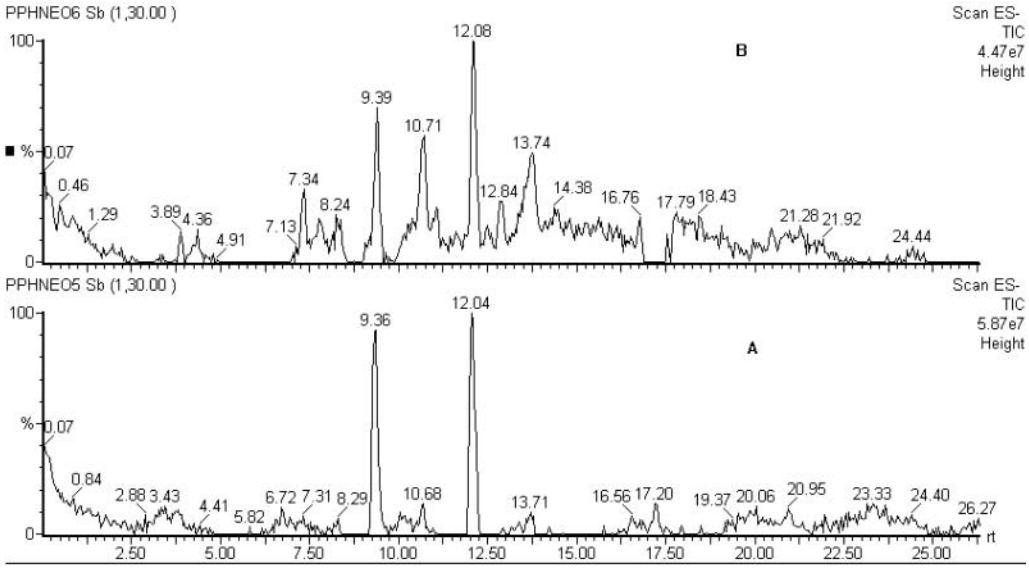

Figure 3. LC-MS (TIC) for separation of SF extract (**A**) of de-oiled grape seed and ES extract (**B**) of de-oiled/MeOH/CO_2 SFE grape seed.

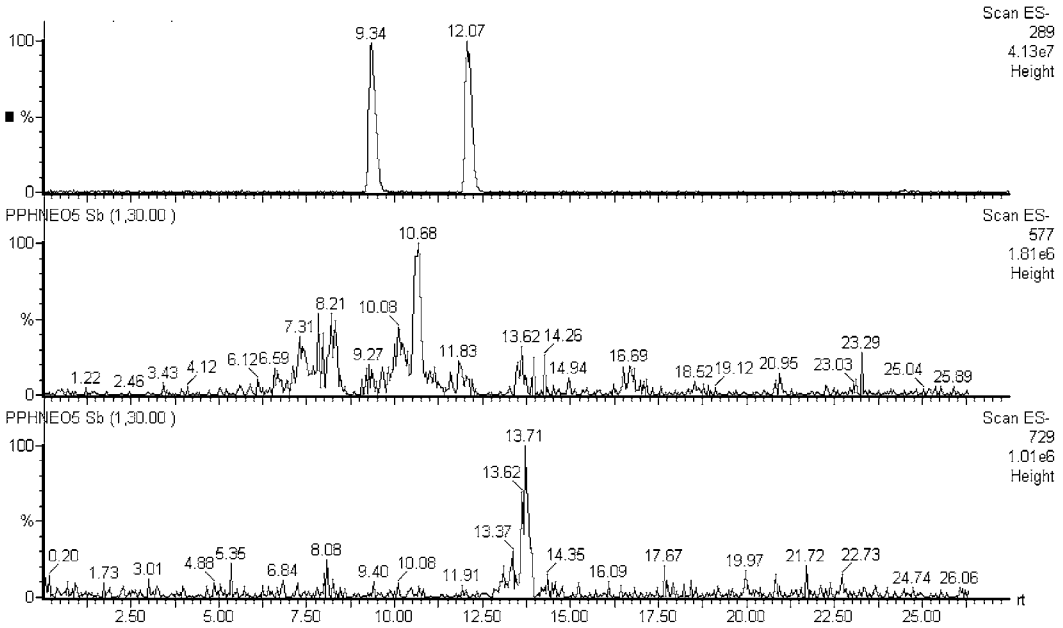

Figure 4. Extracted ions (729, 577, 289 amu) from LC-MS separation of SF extract (MeOH/CO_2) of de-oiled grape seed.

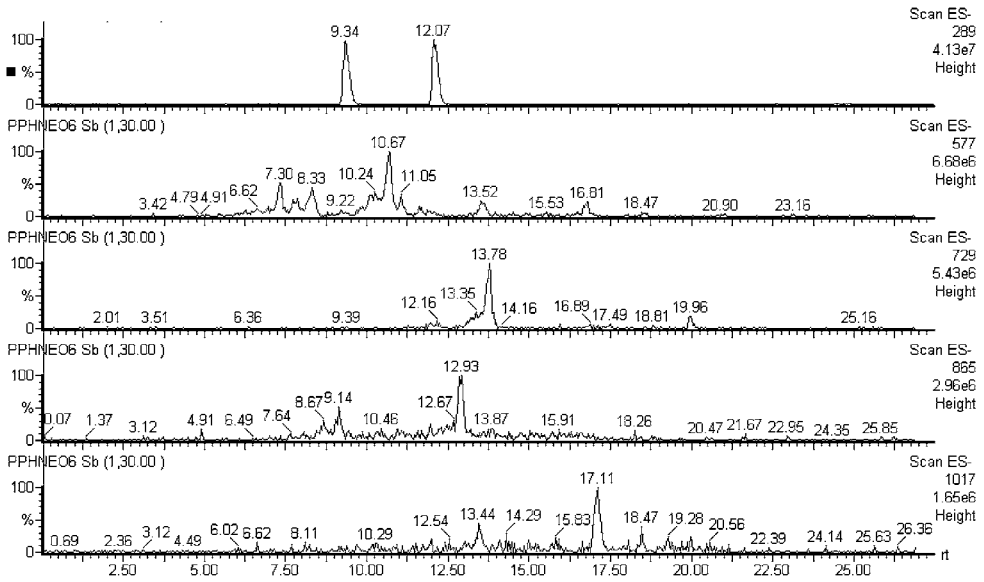

Figure 5. Extracted ions (1017, 865, 729, 577, 289 amu) from LC-MS separation of ES extract of de-oiled grape seed after extraction with methanol-modified CO_2.

de-oiled grape seeds after being extracted for 120 min using 40% methanol-modified CO_2. Some catechin and epicatechin remained in the seeds after SFE. Peaks with t_R of 5.17, 11.69, and 13.17 min were again identified as gallic acid, catechin, and epicatechin using a standard mixture. The HPLC trace of the ESE sample also showed elution of what we believe to be high molecular weight polymeric polyphenols as a broad hump. It is believed that in the HPLC case, polymeric components dissolved in the HPLC mobile phase and were eluted from the column, whereas in the SFC case, they were precipitated at the head of the guard column.

HPLC-MS Analysis. To confirm our extraction results, reversed phase liquid chromatography coupled with on-line mass spectrometry using an atmospheric pressure ionization electrospray chamber (LC-MS) was applied. For this purpose both SF and ES extracts, which were earlier separated by HPLC-UV, were separated again and detected by MS for peak indentification. **Figure 3** shows the LC-MS total ion current chromatogram (TIC) for both SF (**A**) and ES (**B**) extracts in the negative ion mode. The identified components in the SF extract showed mainly the presence of catechin and epicatechin (*m*/*z* 289, $[M - H]^{-1}$) with t_R of 9.36 and 12.04 min, respectively (**Figure 3A**). It is important to note here that the MS was started 3 min after injection of each extract into the HPLC system. The minor peaks in **Figure 3A** were identified via extracted ions (**Figure 4**) as singly linked procyanidin dimers (*m*/*z* 577 $[M - H]^{-1}$ with t_R of 7.3, 8.2, and 10.7 min) and galloylated procyanidin dimers (*m*/*z* 729 $[M - H]^{-1}$ with t_R of 13.6 and 13.7 min) (*14*). No other peaks could be detected or identified in this extract.

Next, HPLC-MS of the ES extract after SFE of the seeds was performed (**Figure 3B**). Results showed the presence of monomers as was observed in the HPLC-UV and SFC-UV (*m*/*z* 289) with t_R of 9.39 and 12.08 min. Various numbers of both singly linked procyanidin and galloylated procyanidin dimers were detected [*m*/*z* 577 with t_R of 7.30, 8.33, 10.67, 13.52, and 16.81 min and *m*/*z* 13.78 (**Figure 5**)]. The concentration of detected dimers in the SF extract was $\sim^1/_{10}$ of the dimer concentration in the ES extract. Also, procyanidin and galloy-

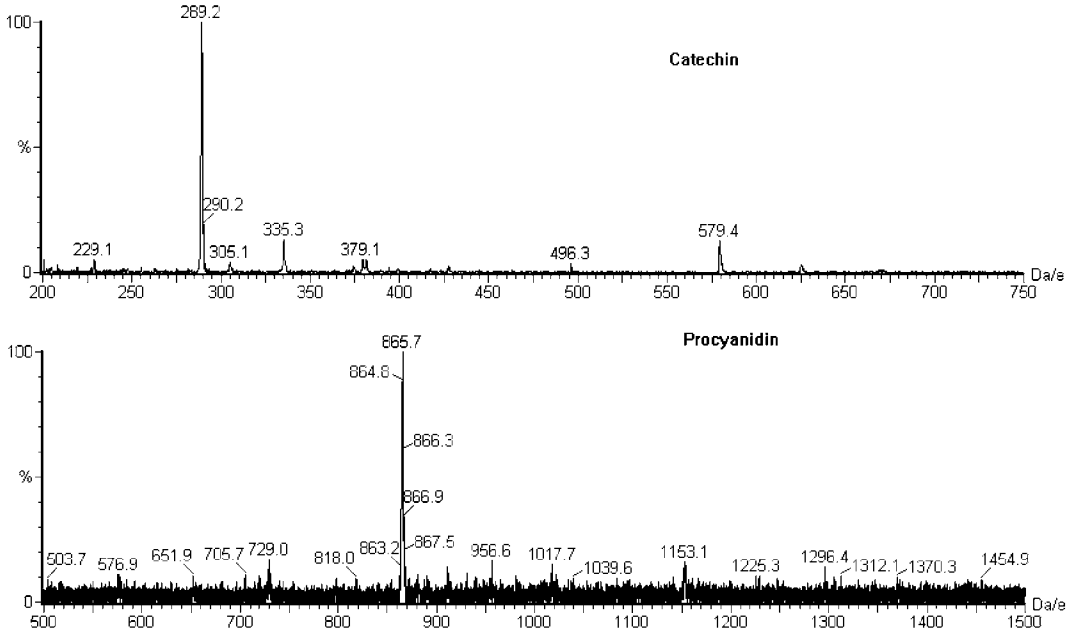

Figure 6. Mass spectrum of eluted peak component (t_R = 12.93 min) in extracted ion (865 amu) chromatogram (**Figure 5**) and mass spectrum of eluted peak component (t_R = 9.34 min) in extracted ion (289 amu) chromatogram (**Figure 5**).

lated procyanidin trimers were detected in the ES extract [*m*/*z* 865 $[M - H]^{-1}$, t_R = 12.93 min and *m*/*z* 1017 $[M - H]^{-1}$, t_R = 17.11 min (**Figure 5**]. Support for the assignment of procyanidin trimer is found in **Figure 6** where the mass spectrum of the trimer and catechin are recorded. We were not able to detect molecules larger than trimers.

In summary, it was demonstrated that with a single instrument both SFE and ESE can be used sequentially in one method. Also, it was determined that the SF extract is much cleaner than the ES extract. Results showed that the SF extract contained only monomers and a few dimers, whereas the ES extract contained mostly dimers, trimers, and higher molecular weight polyphenols. HPLC and SFC results indicated that SFE using 40% methanol-modified CO_2 extracted up to 80% of the monomeric polyphenols that were present in the seed.

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