Feasibility Study of Online Supercritical Fluid Extraction–Liquid Chromatography–UV Absorbance–Mass Spectrometry for the Determination of Proanthocyanidins in Grape Seeds

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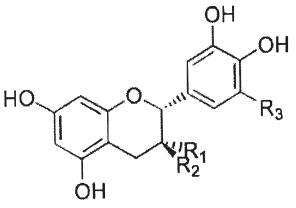
Abstract

Online coupling of supercritical fluid extraction (SFE) with liquid chromatography (LC)–UV absorbance–electrospray ionization (ESI)-mass spectrometry (MS) is evaluated for the determination of proanthocyanidins in grape seeds. The solid-phase intermediate trap is optimized in order to enhance the collection efficiency for the extracted polar components. Pure supercritical CO_2 is used first to remove the oil in the seeds. Then methanol-modified CO_2 is used to remove the polar components (e.g., phenolic compounds). Catechin and epicatechin (90%) are extracted out of the de-oiled after 240 min with 40% methanol as a modifier. Both singly-linked (B-type) and doubly-linked (A-type) procyanidins are identified by LC–ESI-MS, as well as their galloylated derivatives. The hyphenated system combines the extraction, separation, and detection in series. The experimental design minimizes the chance of analyte oxidation, degradation, and contamination. The traditional off-line SFE–LC method is also studied for comparison with the online method. Both advantages and disadvantages are observed for the online mode.

Introduction

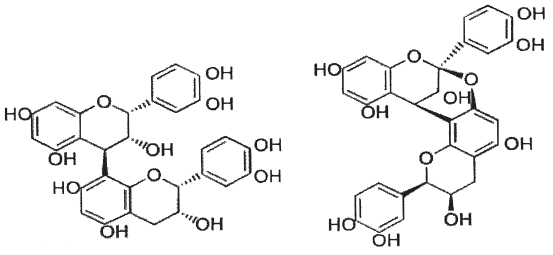
Grapes are one of the most widely consumed fruits in the world. Grapes are rich in polyphenols, a nutritional supplement that possesses antioxidant activity (1). Approximately 30% of total grape polyphenols exist in grape skin, and the other 60–70% are contributed from grape seeds (2). The study of grape seed extracts has been of increasing interests in recent years. Grape seed polyphenols (GSPs) have various physiological effects in vivo, such as protection against x-ray and UV rays (3, 4), anticancer effects (2,5,6), and inhibitory effects against hypercholesterolemia (7).

GSPs have a very complicated molecular composition (8). The main constituents are proanthocyanidins, known as condensed tannin, which include procyanidins and prodelphinidins. Prodelphinidins consist of gallocatechin, epigallocatechin, and their monomeric galloylated derivatives (such as epigallocatechin gallate and gallocatechin gallate, see Figure 1A). Procyanidins, which include monomers and oligomers, are crucial for the therapeutic activity of grape seed extract. The four monomers of procyanidins are catechin (C), epicatechin (EC),



(A) Prodelphindins:
R_1 = H, R_2 = OH, R_3 = OH: gallocatechin (MW = 306 amu)
R_1 = OH, R_2 = H, R_3 = OH: epigallocatechin (MW = 306 amu)
R_1 = H, R_2 = G ester, R_3 = OH: gallocatechin gallate (MW = 458 amu)
R_1 = G ester, R_2 = H , R_3 = OH: epigallocatechin gallate (MW = 458 amu)

(B) Procyanidins:
R_1 = H, R_2 = OH, R_3 = H: C (MW = 290 amu)
R_1 = OH, R_2 = H, R_3 = H: EC (MW = 290 amu)
R_1 = H, R_2 = G ester, R_3 = H: CG (MW = 442 amu)
R_1 = G ester, R_2 = H , R_3 = H: ECG (MW = 442 amu)



(C) B-type dimer (C–C) **(D) A-type dimer (C–C)**

Figure 1. Molecular structures of proanthocyanidins (MW = molecular weight).

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catechin gallate (CG), and epicatechin gallate (ECG) (Figure 1B). The procyanidin oligomers are composed of C and EC units, linked together through C(4)–C(8) interflavanoid bonds to form a B-type dimer (Figure 1C). The structural variations include an A-type dimer, with the formation of a second interflavanoid bond by C–O–C bonding (Figure 1D). The phenolic molecular distribution and total content of polyphenols in grape seed extracts not only depends on the raw materials but also on the extraction method employed (1). Extraction, identification, and quantitation of polyphenols pose a challenge because the natural matrix has high enzymatic activity, so precautions should be taken to avoid any oxidative, photochemical, and biochemical degradation. Sample preparation is, therefore, a critical step in the analysis of natural polyphenols. Mild extraction conditions are generally believed to be expedient in order to preserve maximum antioxidant capacity.

Supercritical fluid extraction (SFE) with CO_2 affords significant advantages in this regard over conventional solvent extraction techniques. The absence of both air and light during the extraction process can reduce the incidence of degradation, which may easily occur in other extraction techniques. Supercritical CO_2 is an inert extraction medium with a low critical temperature. The analytes, therefore, can be extracted out at very mild conditions (moderate temperature) in which the possibility of thermal degradation is reduced. Unfortunately, the solvating power of pure CO_2 is insufficient for removing polyphenols from grape seed. However, pure supercritical CO_2 can be used for the removal of oils from grape seed. Subsequently, methanol-modified CO_2 can then be used for the extraction of the polar components (e.g., principally the polyphenols) from the deoiled seeds.

Palma and Taylor (9) previously described an off-line extraction method that utilized 35% methanol-modified supercritical fluid CO_2. Three components [i.e., gallic acid (G), C, and EC] were identified and quantitated in the de-oiled grape seed extracts via high-performance liquid chromatography (HPLC)–UV with standards of the three materials. Mass spectrometric (MS) detection was not attempted with these polar extracts.

In a previous study, we reported online coupling of SFE–liquid chromatography (LC)–UV absorbance for the quantitative analysis of nonpolar polynuclear aromatic hydrocarbons (PAHs) in an environmental sample (e.g., soil) (10). In this study, the aim—with the addition of electrospray ionization (ESI)-MS detection—was to evaluate the ability of SFE–LC–UV–ESI-MS for the identification and analysis of proanthocyanidins in similar grape seeds. This study is more challenging because proanthocyanidins are much more polar than PAHs. SFE with a CO_2-based fluid yields extracts that are cleaner than conventional liquid solvent extracts because of lower concentrations of interfering co-extractives in the former. This feature permits the direct introduction of supercritical fluid extracts into an analytical system without a preliminary clean-up step. After the removal of all grape oils via pure CO_2, the polar extracts obtained via methanol-modified CO_2 can be directly transferred to a reversed-phase LC analytical scale packed column for identification and quantitation by both UV and MS detection. Online coupling of SFE and LC provides an alternative technique for the analysis of natural phenolic compounds in trace level quantities. It is less labor intensive and more sensitive than off-line analysis and the opportunity for sample degradation or loss during sample processing is minimized, because all of the

Table I. SFE Conditions

Step 1: De-oil via pure CO_2	
Oven temperature	80°C
Pressure	400 atm
Static time	30 min
Dynamic time	120 min
Flow rate	2.0 mL/min
Modifier	none
Collection temperature	70°C
Trap rinse solvent	10 mL DCM*–methanol
Extract destination	to waste
Step 2: Polyphenol extraction via modified CO_2	
Oven temperature	80°C
Pressure	400 atm
Static time	0 min
Dynamic time	120 min
Flow rate	1.0 mL/min
Modifier	40% methanol
Collection temperature	70°C
Trap rinse solvent to remove CO_2	4 mL water
Trap rinse solvent to remove analytes	LC mobile phase
Extract destination	to LC online analysis

* Dichloromethane.

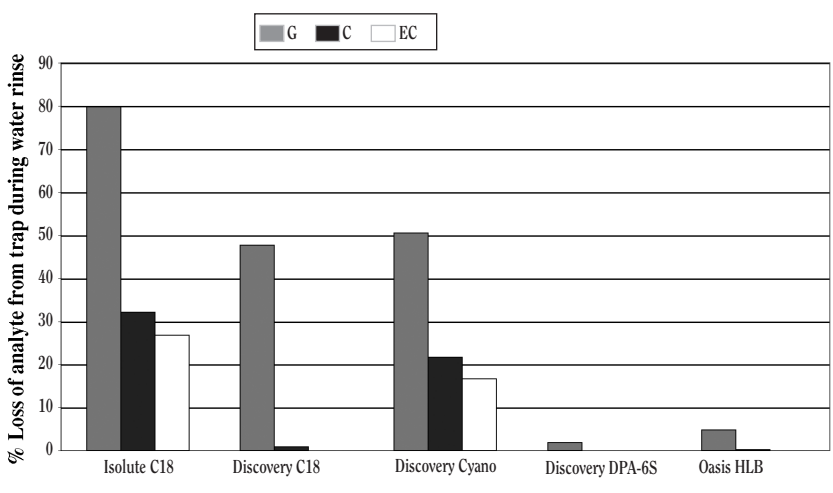


Figure 2. Comparison of loss of analytes from five trapping materials during water rinse. (10 µL of aqueous solution spiked on trap, 4 mL water used to rinse, and each compound has the concentration of 100 µg/mL). Average value of two measurements (*).

extract is directly transferred to the analytical system in an air/light-free environment.

Experimental

Apparatus

An Isco-Suprex (Lincoln, NE) Prepmaster SFE system equipped with solid-phase Accutrap and methanol modifier pump was used for all parts of the study. SFE–supercritical-fluid chromatograpy grade carbon dioxide with 2000 psi helium head pressure was purchased from Air Products and Chemical Inc. (Allentown, PA). Extractions were performed using a 2.5-mL stainless steel extraction vessel (Keystone Scientific, Bellefonte, PA). Previously-ground grape seed (50 mg) was mixed with enough Ottawa sand (Fisher Scientific, Fair Lawn, NJ) to fill the vessel. The SFE variable electronically controlled restrictor was heated to 60°C for all extractions. A 10- × 0.2-cm i.d. stainless steel column filled with Discovery DSC-18 (particle size: ~ 50 µm) (Supelco, Bellefonte, PA) was used as the SFE trap. Other trapping materials were also evaluated, including isolute sorbent C_{18} (particle size: 40–70 µm), Discovery Cyano (~ 50 µm), and DPA-6S (polyamide resin, 50–160 µm), all from Supelco. Oasis HLB (hydrophilic-lipophilic balance materials, ~ 60 µm) from Waters (Milford, MA) was also used. The optimized supercritical extraction procedure is shown in Table I. In the off-line SFE mode, a tandem trap was employed by inserting the outlet tubing of the solid trap into a collection vial half-filled with methanol. A tandem trap arrangement was not possible in the online case. The rinse solvent in off-line SFE was methanol. The extraction vessel was 10 mL, and each time 5 g of crushed seeds were extracted in the off-line experiment.

An Agilent 1050 HPLC system (Wilmington, DE) with programmable multiwavelength UV detector was used to quantitatively analyze the chromatographically separated extracts. A polar embedded alkyl phase Discovery RP Amide C16 column (250 × 4.6 mm, 5-µm particles) (Supelco) was used for the separation. The mobile phase was acetonitrile (ACN) and water (each containing 0.5% formic acid, v/v) with a flow rate of 1.0 mL/min. The chromatographic gradient program began at 5% ACN for 1 min, then linearly increased to 15% at 20 min (~ 0.5% per minute), then linearly increased to 30% at 30 min (1.5% per minute), to 40% at 40 min (1% per minute), and then to 100% ACN at 50 min (6% per minute) and held for 10 min, with a total analysis time of 60 min. The UV detection wavelength was set at 280 nm.

LC–MS was performed (to afford identification information) with an Agilent 1100 HPLC system, which was interfaced to a Finnigan TSQ Quantum MS (ThermoFinnigan, San Jose, CA). In order to operate at low flow rate so that the electrospray (ESI) has high ionization efficiency, the LC column for online SFE–LC–MS study was a small dimension Zorbax Eclipse XDB-C18 column (2.1 × 150 mm, 5 µm) (Wilmington, DE). The mobile phase was ACN and water (each containing 0.5% formic acid, v/v) with a flow rate of 0.2 mL/min. The chromatographic gradient program began at 0% ACN for 3 min, then increased to 25% at 45 min (0.6% per minute), then increased to 40% at the 55 min (1.5% per minute), and to 100% ACN at 60 min (12% per minute). The MS was operated in the negative electrospray mode. ESI conditions were electrospray voltage, 3.0 kV; capillary temperature, 300°C; source collision-induced dissociation, off; scan range, *m/z* 200 to 1500. The experimental details concerning operation of the hyphenated SFE–HPLC interface appear elsewhere.

Chemicals and samples

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 ground using a coffee grinder. The grinding was halted for approximately 15 s at periodic intervals to prevent heating of the sample. The

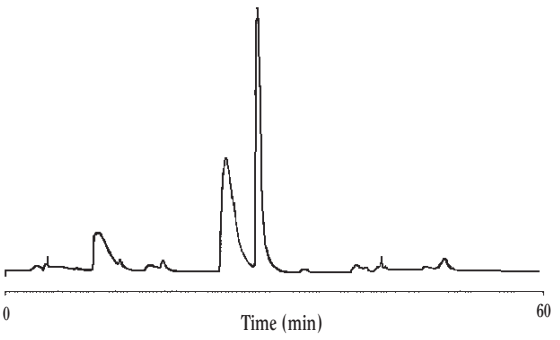


Figure 3. Chromatogram of online SFE–LC, Discovery DPA-6S as the trapping materials. See Table I for SFE conditions. Dynamic extraction, 120 min; LC conditions, Discovery RP Amide C16 column (250 × 4.6 mm, 5 µm); and mobile phase, ACN–water (0.5% formic acid, v/v). Gradient elution: 5% ACN for 1 min, then increased to 15% at 20 min (~ 0.5% per minute), then linearly increased to 30% at 30 min (1.5% per minute), to 40% at 40 min (1% per minute), and then to 100% ACN at 50 min (6% per minute), and held for 10 min. Flow rate, 1.0 mL/min; and UV detection, 280 nm.

Table II. The Influence of Modifier, Extraction Temperature, and Pressure on Extraction Efficiency*

Experiment	CO_2 density (g/mL)	Modifier (methanol) %	Extraction temperature	Extraction pressure (atm)	C extracted (µg)	EC extracted (µg)
1	0.79	30%	50°C	200	11.4	13.6
2	0.93	30%	50°C	400	15.1	16.8
3	0.83	30%	80°C	400	20.2	22.0
4	0.83	40%	80°C	400	36.6	38.7

* Crushed grape seeds (50 mg); dynamic extraction for 120 min; liquid CO_2 flow rate, 1 mL/min.

crushed seeds were stored at room temperature prior to extraction. G, C, EC standards, and formic acid were purchased from Sigma-Aldrich Chemical Co., (Milwaukee, WI). HPLC-grade methanol, acetone, ACN, dichloromethane, and water (Burdick & Jackson, Muskegon, MI) were used.

Results and Discussion

Optimization of extraction recovery

Extract trapping materials

Polyphenols have relatively wide polarities and molecule weights, including very polar compounds such as G, intermediate polar compounds such as C and EC, and less polar compounds that are higher molecular mass oligomers. To achieve quantitative results, the extract trapping system must be highly efficient. In our online SFE–LC design, the trap, (i.e., SPE cartridge) should have the ability, not only to hold the extracted analytes during dynamic extraction and CO_2 decompression, but also to hold the adsorbed analytes during water rinsing of the trap. The latter step is necessary in order to displace residual CO_2 gas that appears in the trap and connection line. Otherwise, the gas may enter the LC and interfere with UV detection.

Five types of SPE products were tested as online SFE trapping materials. Three representative grape seed polyphenolic components (e.g., G, C, and EC) were chosen as probes to evaluate these materials. Each time, 10 µL of an aqueous solution composed of the three standards was spiked into the SPE cartridge. After rinsing the trap with 4 mL of water, the aqueous eluent was analyzed by LC to determine if any of the phenols had been removed from the trap (Figure 2). The cyano phase failed to show good retention for the three highly polar phenolic compounds during the water rinsing step. All three representative components were detected in the rinse water employed for residual CO_2 removal. Similar results were found when Isolute Sorbent C_{18} was used as the trapping material. Discovery DSC-18, which has high carbon loading (18%), offered better retention during water elution. Only G was detected in the rinse water with the latter trap material.

Two novel-type SPE materials, Discovery DPA-6S (polyamide resin, designed for retaining polar compounds with an hydroxyl group), and Oasis HLB (*N*-vinylpyrrolidone and divinylbenzene copolymer, designed for retaining both polar and non polar compounds) were also evaluated. With these two phases, C and EC remained on the trap during water rinsing, but only 1% (w/w) of G was removed. Unfortunately, when online

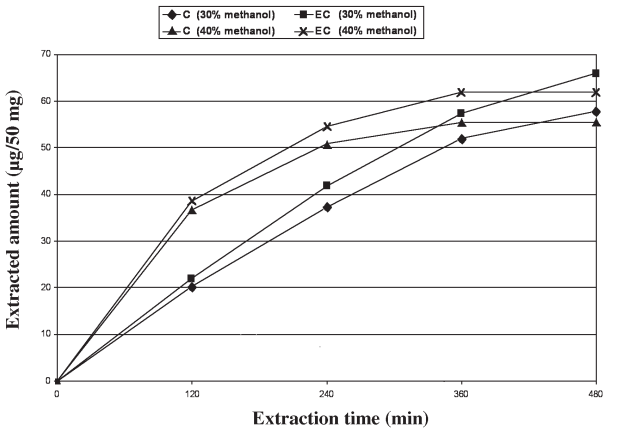
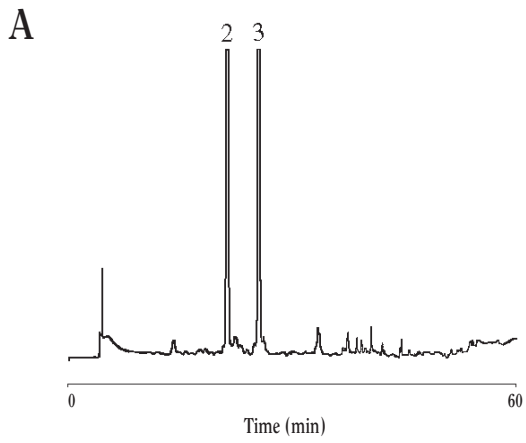


Figure 4. Extraction profile of C and EC at diffferent modifier percentages (see Table I for extraction conditions). Average value of two measurements (*).



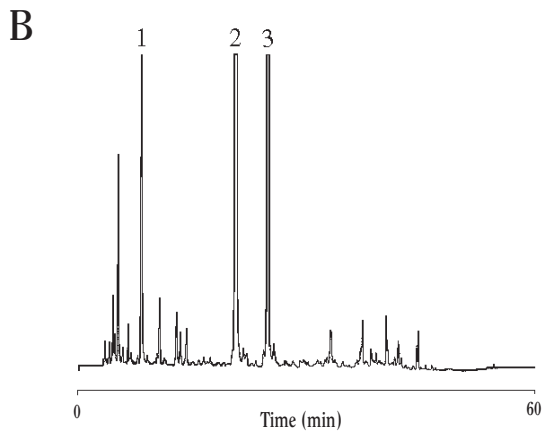


Figure 5. (A) Chromatogram of online SFE–LC extract (see Table I for SFE conditions). Dynamic extraction, 120 min; trapping material, Discovery C_{18}; LC conditions, Discovery RP amide C16 column (250 × 4.6 mm, 5 µm); and mobile phase, ACN–water (0.5% formic acid, v/v). Gradient elution: 5% ACN for 1 min, then increased to 15% at 20 min (~ 0.5% per minute), then linearly increased to 30% at 30 min (1.5% per minute), to 40% at 40 min (1% per minute), and then to 100% ACN at 50 min (6% per minute), and held for 10 min. Flow rate, 1.0 mL/min; and UV detection, 280 nm. (B) Chromatogram of off-line SFE–LC extract. (See Table I for SFE conditions). Dynamic extraction, 120 min; 5 g of sample and 10 mL vessel for extraction; tandem trap, C_{18} solid trap plus methanol liquid trap; and rinse solvent, 4 mL methanol. Collection was concentrated to 1 mL and 10 µL injected for separation (for LC conditions see Figure 5A). For the figure, 1 = G, 2 = C, and 3 = EC.

SFE–LC experiment was performed, the chromatogram showed serious band-broadening (Figure 3). One possible reason for the poor peak shape is that a high percentage of mobile phase water (95%) was initially used in the reversed-phase LC gradient in order to obtain sufficient retention and separation of the three polar phenolic compounds. In other words, both solid phases have strong retention for polar compounds so that when the LC mobile phase with low organic content (5% ACN) enters the trap, the analytes cannot be efficiently eluted from the trap to the LC column. This phenomenon was not observed with the other three phases. As a compromise consideration, Discovery DSC-18 was selected as the trapping material in our research. Therefore, in the online method, we were unable to obtain quantitative results on G and more polar compounds than G because of less retention by the trap during the preliminary water rinse.

Online extraction conditions

Previously, it has been shown that pure CO_2 can be successfully used for the removal of oils from grape seed (9). For our online design, it was essential that all of the oils in the first step be removed. If any oil was left in the seed and extracted by methanol-modified CO_2 during the second step, it may enter the LC column with the extracted polyphenols and plug or damage the LC column. During the de-oil step, pure CO_2 (80°C, 400 atm) was employed at 2 mL/min for 120 min in order to fully remove any oil. A mixture of dichloromethane–methanol (1:1, v/v, room temperature) was used to elute the extracted oil from the solid phase C_{18} trap to waste.

To obtain the highest extraction efficiency of the polyphenols, modifier percentage, extraction temperature, and CO_2 pressure were evaluated. The combined amounts of extracted C and EC were used as the criteria to evaluate extraction efficiency at different conditions. Higher extraction pressure (400 atm compared with 200 atm) and higher extraction temperature (80°C compared with 50°C) seemed more favorable for the extraction of C and EC from the grape seed (see Table II). For these polar phenolic compounds, the percentage of modifier had a significant effect on extraction efficiency. The polarity of the extraction medium increases when more polar modifier is added. A former study has demonstrated that methanol is a better CO_2 modifier than ethanol to extract polyphenols (9). Thus, different percentages (v/v) of methanol as modifier were investigated. When methanol was increased from 30% to 40%, a nearly 80% increase in recovery (C 20.2 to 36.6 µg, EC 22.0 to 38.7 µg.) was observed. Extraction time was another important parameter evaluated. Under optimized conditions (400 atm, 80°C and 40% modifier), 360 min (triple extraction, 120 min each) were utilized to obtain an exhaustive extraction (Figure 4). Such a long extraction time is probably dictated by the characteristics of the sample. With natural products, analytes have much stronger interactions with matrices, compared with the interaction with an inert support. When 30% modifier was used, a longer extraction time was necessary in order to achieve an exhaustive extraction. The overall results from the two methods (30% and 40% modifier) are comparable. The total extractable C in a 50 mg de-oiled sample was 55.5 µg (1.1 mg per gram seed). The total extractable EC in the same 50 mg sample was 62.0 µg (1.2 mg per gram seed).

Comparison of online and off-line SFE–LC

The UV trace of online SFE–LC is shown in Figure 5A. Compared with the LC chromatogram via off-line SFE–LC (Figure 5B), the online approach provided less peak quantity and with

Table III. Comparison of Online and Off-line Methods for Deoiled Seeds

	Online SFE–LC	Off-line SFE–LC
Sample amount	50 mg	5 g
Extraction time	De-oil step: 120 min extraction 360 min (triple extraction, 120 min each)	De-oil step: 120 min extraction 360 min (triple extraction, 120 min each)
Extract concentration time	None	30 min concentrate from 4 to 1 mL
Separation time	60 min	60 min
Total time usage for each assey	540 min	570 min
Quantitative results	Only possible for intermediate polar extracts	Possible for intermediate polar to polar extracts

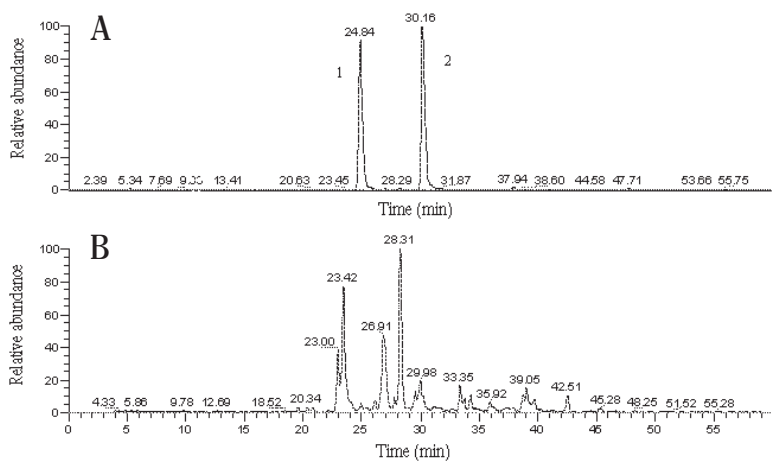


Figure 6. Extracted ions (288–289, 576–578 amu) from online SFE–LC–MS of deoiled grape seeds. For the figure, (A) 1 = C, 2 = EC; and (B) singly-linked (B-type) dimers.

a slight increase in band-broadening. The online design introduced extra column dead volume (e.g., trapping cartridge and connection tubing) to the LC system. In addition, some polar compounds were undetectable in the online mode because of: (*a*) less trapping efficiency when a high percentage of methanol was used as the modifier and (*b*) some loss of trapped extract was introduced during the water rinse step. These disadvantages can be avoided in the off-line mode because (*i*) a tandem solid–liquid trap was utilized and (*ii*) residual trapped CO_2 can be removed during the trap rinse/recovery step. Table III compares the two methods concerning time usage, sample amount, and quantitative results. The online method needed less time in that no extract processing step was needed, and all of the extracts were directly transferred to the LC for analysis. Also it required less sample (50 mg) to be extracted. Although with the off-line method, in order to achieve similar sensitivity as the online method, 100 times more sample (5 g: 50 mg) was needed to be extracted, and the extracts had to be concentrated from 4 to 1 mL. Finally, only 10 µL of the 1 mL could be injected off-line to the LC for analysis. However, in the off-line method, there is no sacrifice of LC separation efficiency because of extra column dead volume, and most of the extracts are quantitatable. The extractable C and EC amounts via the off-line method were 1.7 and 1.9 mg/g. The increase in extractables via the off-line method was probably attributable to the use of a tandem trap. A single solid-phase trap (as in online mode) may not be efficient enough to retain all the extracts when a high percentage of methanol is used as modifier. Adding a liquid trap also assisted in retaining the analytes that might have eluted from the solid trap.

Online SFE–LC–MS study

Off-line SFE–LC–MS was performed in a previous study (1). C, EC, singly-linked procyanidin dimers, and galloylated procyanidin dimers were identified in the supercritical CO_2 methanol extracts. Here, MS was coupled with online SFE–LC to provide specific extract compound information. Mass spectral data showed mainly the presence of C and EC

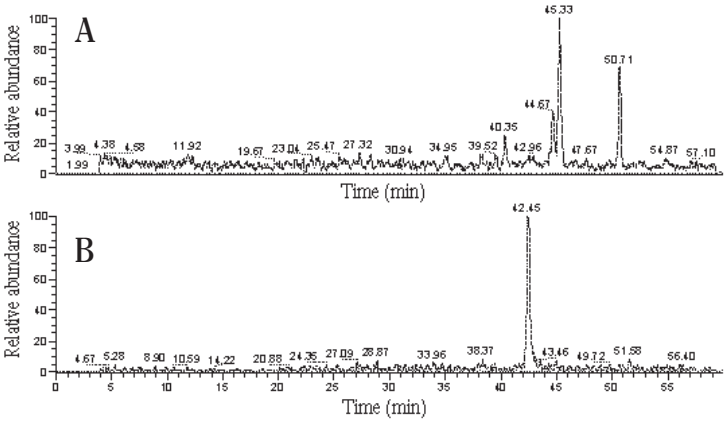


Figure 7. Extracted ions (574–576, 860.8–861.5 amu) from online SFE–LC–MS of de-oiled grape seeds. For the figure, (A) doubly-linked (A-type) dimers and (B) doubly-linked (A-type) trimer.

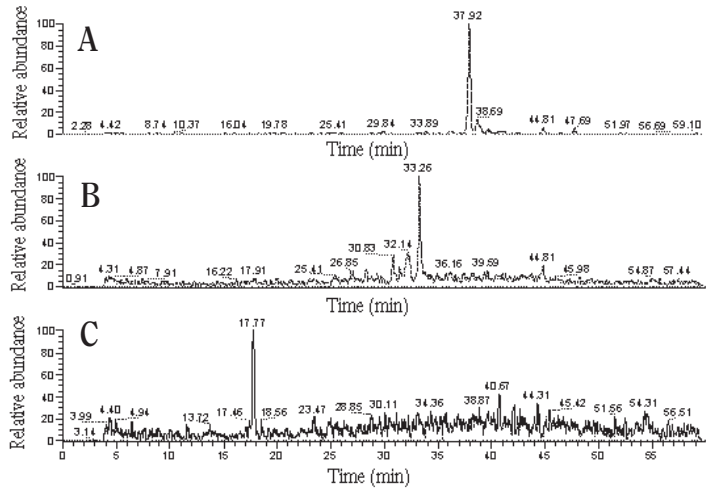


Figure 8. Extracted ions (440–442, 728–730, and 1016–1018 amu) from online SFE–LC–MS of de-oiled grape seeds. For the figure, (A) C gallate; (B) dimer gallate, and (C) trimer gallate.

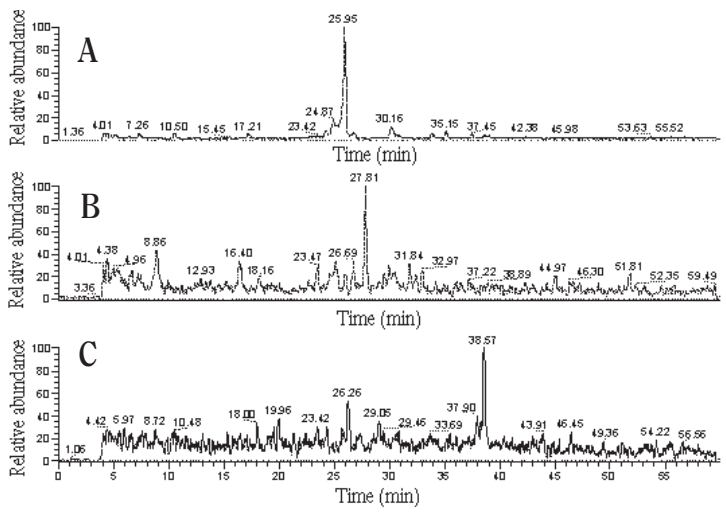


Figure 9. Extracted ions (304–306, 456–458, and 608–610 amu) from online SFE–LC–MS of de-oiled grape seeds. For the figure, (A) gallocatechin, (B) gallocatechin gallate, and (C) gallocatechin dimer.

(Figure 6A). Several singly linked dimers (B-type) were also observed with *m/z* 577 $[M–H]^{-1}$ (Figure 6B), but no higher oligomer was detected. The doubly-linked procyanidins (A-type) are produced via the formation of a second interflavanoid bond (C–O oxidative coupling). Because of the complexity of the structure, A-type procyanidins are not as frequently encountered in nature compared with B-type procyanidins (11). However, several A-type dimers (Figure 7A) and one trimer (Figure 7B) were found in the grape seed supercritical fluid extracts with *m/z* 575 $[M–H]^{-1}$ and *m/z* 861 $[M–H]^{-1}$. The galloylated procyanidins were found at *m/z* 441, 729, and 1017 $[M–H]^{-1}$, corresponding to CG (monomer, Figure 8A), dimer gallate (Figure 8B), and trimer gallate (Figure 8C), respectively. No higher oligomer gallates and higher gallate derivatives (e.g., oligomer digallate and oligomer trigallate) were detected. In addition, ions were observed in the mass spectral trace of grape seed SF extracts, which suggested the presence of prodelphinidins and their galloylated derivatives (Figure 9). Deprotonated molecular ion *m/z* 305 corresponded to gallocatechin (Figure 9A), and *m/z* 457 and 609 $[M–H]^{-1}$ were related to gallocatechin gallate (Figure 9B) and gallocatechin dimer (Figure 9C). No higher oligomer was found.

Conclusion

This study explored the feasibility of extraction and analysis of proanthocyanidins in grape seeds via online SFE–HPLC–UV–MS. The hyphenated design avoided possible analyte oxidation, degradation, or contamination that may occur in the extract-processing step in a traditional off-line method. C and EC were successfully quantitated after optimizing the solid-phase trap. Mass spectral data confirmed the existence of four types of proanthocyanidins (e.g., singly- and doubly-linked procyanidins, galloylated procyanidins, and prodelphinidins). Only low-molecular oligomers (up to trimer) were found in the supercritical CO_2 extracts. Compared with the off-line SFE–LC approach, much less sample was necessary in the online extraction method, since all the extracted compounds can be directly transferred to the LC. Also, no extract processing/concentration step was needed in the online method. However, in the online mode, some highly polar compounds were lost during the collection step (lower trapping efficiency of single solid trap when high percentage modifier was used) and during the water rinsing step (less retention of polar compounds on C_{18} trap).

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