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Determination of polyphenolic compounds in grape seed extracts using reverse-phase high performance liquid chromatography

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SUMMARY

Oxidative stress is associated with many kinds of chronic diseases. Antioxidants such as polyphenols are compounds that protect cells against the damaging effects of reactive oxygen species. Grape seeds are considered good resources of polyphenols, and grape seed extracts have a very strong antioxidant effect. In the present study, we established a simple gradient reverse-phase high performance liquid chromatography method to determine polyphenol content from three different grape seed resources. An ODS (2), 150 × 3.2 mm column has been employed, and six polyphenols have been determined: gallic acid, protochatechuic acid, (+)-catechin, (-)-epicatechin, procyanidin B2, and epicatechin gallate. Catechin and epicatechin were the main polyphenol compounds in all three extracts. The amount of procyanidin B2 was higher in Extract 1 (from a company of China), while Extract 2 (extracted in our lab) and Extract 3 (from a company of USA) contained higher proportions of epicatechin gallate. For the total polyphenol content, Extract 1 was much higher than that of Extract 2 and 3. The results suggest that the dietary dose of grape seed extracts from different resources should be adjusted according to polyphenol content.

Key words: Grape seed extracts; Polyphenols; Determination method; High performance liquid chromatography

INTRODUCTION

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species such as superoxide, hydrogen peroxide, singlet oxygen, peroxyl radicals, hydroxyl radicals, and peroxynitrite (Ji and Peterson, 2004). Some reactive oxygen species, such as superoxide and hydrogen peroxide, are normally produced in cells as by-products of biochemical reactions or as signaling molecules (Martindale and Holbrook, 2002). When reactive oxygen species-generating reactions are activated excessively, pathological quantities of reactive oxygen species are released to create an imbalance between antioxidants and reactive oxygen species. Oxidative stress has been linked to many diseases such as cardiovascular illnesses, dementias, diabetes, and cancer (Rao and Balachandran, 2002; Ceconi *et al.*, 2003; Hoeldtke *et al.*, 2003; Cejas *et al.*, 2004).

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Polyphenols are a group of naturally occurring plant chemicals with antioxidant potential. Grape seeds are considered good resources of polyphenolic compounds, and extracts of grape seeds are known as powerful antioxidants. The antioxidant capacity of grape seed extracts (GSE) comes from phenolic acid and proanthocyanidins (Kammerer *et al.*, 2003). Scientific studies have shown that proanthocyanidins antioxidant power is 20 times stronger than Vitamin E and 50 times stronger than Vitamin C (Shi *et al.*, 2003). GSE have been used as a botanical supplement material all over the world.

The polyphenol composition of grape seeds is different and complex based on its origin. Because there are many manufacturers of GSE products spread out in different countries, sources of GSE may contain different proportions and amounts of phenolic compounds.

Reverse-phase high performance liquid chromatography (RP-HPLC) is an effective and accurate technique for the analysis of polyphenols. Previous reports on the use of this technique display their advantages. However, some factors can be modified for the improvement. Almost all reported chromatography condition used a column within 250 mm in length (Peng *et al.*, 2001; Pineiro *et al.*, 2004). Though 150 mm analytical column has been employed, the author used three elution phases (Ana *et al.*, 2004). Other HPLC conditions also selected a complex elution phase system (Yang and Chien, 2000; Eduardo *et al.*, 2003). Some other conditions contained an elution program, which consumed more than 85 minutes (Revilla and Ryan, 2000; Kammerer *et al.*, 2004; Yilmaz and Toledo, 2004).

In the present study, we established a simple gradient RP-HPLC method with a 150 × 3.2 mm reverse phase analytical column. This technique has been employed to determine the contents of the 3 different grape seed extracts.

MATERIALS AND METHODS

Reagent and standards

Acetonitrile, methanol and acetic acid (HPLC grade) were purchased from Fisher Scientific (Norcross, USA). Mili Q water was obtained from our lab (Purelab Plus UV/UF type: PL5114, US Filter, Palm Desert, USA). Pure standards of gallic acid (GA, 97% purity), protocatechuic acid (ProA, 99% purity), (+)-catechin (C, 96% purity), (-)-epicatechin (EC, 98% purity), procyanidin B2 (B2, 90% purity), and epicatechin gallate (ECG, 98% purity) were purchased from Fluka (Milwaukee, USA) and Sigma (St. Louis, USA).

GSE samples and preparations

Three GSE samples were used in this study.

Extract 1 was obtained from Shanghai Pharmaceutical Company, China. Extraction method supplied by the manufacturer was as follows. First, dried grape seed powder was extracted with 95% ethanol for 4 hours. Then they spun down the grape seed powder and removed the solvent under vacuum. Afterwards, the residue was solved in water and loaded on a resin column. The column was eluated with water, discarding the water eluents. Then, the column was eluated with 95% ethanol. Finally, the ethanol eluents were collected, dried under vacuum, and lyophilized.

Extract 2 was extracted in our lab and the preparation process was as follows. Red global grape were purchased from a supermarket in Chicago, USA. Second, the grape seeds were collected, washed with water, lyophilized and ground to fine powder. Afterwards, 20 g of grape seed powder was extracted with 400 ml 70% methanol (degassed with Helium) for 1 hour in room temperature. After filtering with cotton, the residue was extracted with 70% methanol for 2 more times. Extracted solutions were mixed and filtered with Watman 1 filter paper. Solvent of filtrate was evaporated under vacuum with Buchi

rotary evaporator (Buchi, Switzerland) and lyophilized with Virtis BenchTop 2K freeze drier (Virtis, Gardiner, USA).

Extract 3 was obtained from a dietary supplement company in Iowa, USA. Information of extraction method was not available. All GSE samples were stored in -20℃.

Chromatographic conditions

We used a LC-10AD *vp* liquid chromatographic system (Shimadzu, Kyoto, Japan) equipped with a SCL-10A *vp* system controller, two 410 LC pumps, a SIL-10A*i* auto injector and sample cooler, a CTO-10A *vp* column oven, a DGU-3A degasser, a SPD-M10A diode array detector, and automated software system (Shimadzu class-*vp* chromatography laboratory software). The separations were carried out on a Prodigy ODS (2) C18 column (150 × 3.2 mm, 5 μm, Phenomenex, Torrance, CA). The precolumn was a C18 column (30 × 3.2 mm, Phenomenex, Torrance, CA). The diode array detector was set to an acquisition range of 200-370 nm at a spectral acquisition rate of 1.25 scans/s.

For the mobile phase, we used eluent A (0.5% acetic acid in water and acetonitrile [50:50, v/v)] and eluent B [2% (v/v) acetic acid in water]. The mixing of the isocratic and gradient solvent elution system were as follows: 90% B (0.01 min), 85% B (5 min), 75% B (20 min), 45% B (50 min), 0% B (51 min), 0% B (56 min), 90% B (58 min), and 90% B (60 min). The flow rate of the mobile phase was 0.6 ml/min and the injection volume was 20 μl. The detection wavelength was set to 280 nm. All solutions were filtered through a 0.2-μm hydrophilic polypropylene membrane (Pall Gelman Laboratory, Ann Arbor, USA) before use. Separation was accomplished at the temperature of 25℃.

RESULTS

Table 1 gives retention times and maximum absorbance wavelengths for the six standards. The compound index number in this table corresponds to their respective peaks in the chromatograms. Most polyphenol standards have maximum absorption near 280 nm and we select 280 nm as the assay wavelength. Fig. 1 shows the chromatogram of six polyphenol standards.

To determine the linearity for phenolic compounds in HPLC coupled with UV vision detection, six standard mixtures: (+)-catechin, (-)-epicatechin, procyanidin B2, epicatechin gallate (1, 5, 20, 100, 200 μg/ml), gallic acid (0.15, 0.75, 3, 15, 30 μg/ml), and protocatechuic acid (0.25, 1.25, 5, 25, 50 μg/ml) were examined under the previously stated conditions. The calibration curves were established using peak area and its related amount of polyphenols. The results obtained by using this method, including regression equations, linear range and correlation coefficiency, were summarized in Table 2. The response regression equation was $Y = bX + a$, where Y was the peak area, X was the sample amount (ng), and b and a were numerical coefficients. The calibration curves for the six polyphenols were linear for peak area versus sample amount over two level ranges. The detection limits were evaluated on the basis of a three times signal-to-noise ratio. The observed detection limits of all the selected standards were less than 1 μg/ml. For all linearity curves, the correlation coefficients were no less than 0.9994 (Table 2).

Six polyphenols in the extract samples were identified by comparing the retention times and UV spectrum of authentic polyphnolic standards obtained from the mixed standards chromatograms. The extract samples from different resources were determined by HPLC. A typical extract chromatogram was shown in Fig. 2. Evidently, there are more compound peaks in the chromatograms from extracts than in those from mixed standards. Nonetheless, the unknown chromatogram peaks did not affect our determination of the selected

Table 1. Index number, retention time and maximum absorption of standards in HPLC assay

Compound	Number	Retention time (min)	Maximum absorption (nm)
Gallic acid	GA	5.2 - 5.4	270.2
Protocatechuic acid	ProA	9.4 - 9.8	257.9, 292.8
Catechin	C	15.6 - 16.2	278.0
Procyanidin B2	B2	19.1 - 19.7	277.8
Epicatechin	EC	22.8 - 23.4	277.6
Epicatechin gallate	ECG	34.5 - 35.2	275.0

Table 2. The regression equations and the linear range of polyphenols

Compound	Regression Equations $Y = bX + a$	Correlation Coefficiency R^2	Linear Range (μg/ml)
Gallic acid	$Y = 490X + 1938$	0.9999	0.2 - 30
Protocatechuic acid	$Y = 277.6X - 438$	0.9998	0.3 - 50
Catechin	$Y = 130.2X - 1051$	0.9994	1.2 - 240
Procyanidin B2	$Y = 122.0X - 1513$	0.9999	1.0 - 200
Epicatechin	$Y = 136.3X - 1428$	0.9999	1.0 - 196
Epicatechin gallate	$Y = 163.1X - 1497$	0.9998	1.1 - 216

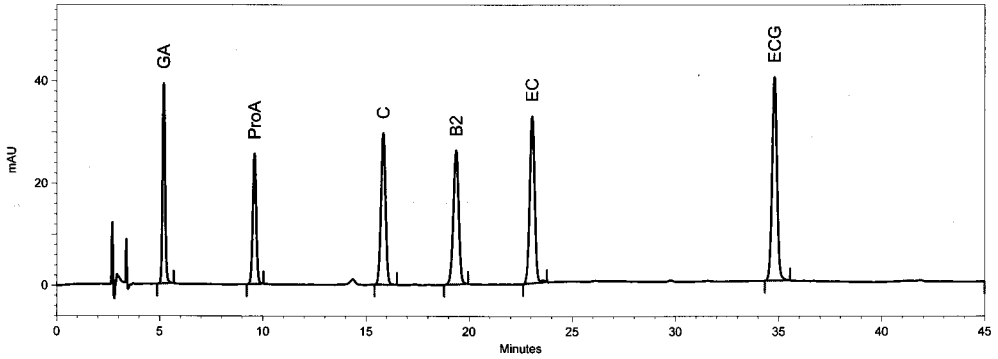

Fig. 1. HPLC-UV (280 nm) chromatogram of polyphenolic standards: GA, gallic acid; ProA, protocatechuic acid; C, (+)-catechin; EC, (-)-epicatechin; B2, procyanidin B2; ECG, epicatechin gallate.

polyphenols because of good resolutions in our experiments. The assay results shown in Table 3 demonstrate that catechin and epicatechin were the two main polyphenols in all the three extracts. This analytical data was similar with other former reports (Auger *et al.*, 2004; Mehdi and Larry, 2004; Yilmaz and Toledo, 2004). The accumulative content of selected compounds of Extract 1 was 5-6 times higher than that of Extract 2 and 3. Fig. 3 showed that the content of polyphenolic compounds was much different from the three extracts.

Table 3. Polyphenols content in GSE samples from different resources (mg/g, n = 3)

Compounds	GSE samples from different resources		
	Extract 1	Extract 2	Extract 3
Gallic acid	9.96 ± 0.46	0.83 ± 0.05	0.85 ± 0.04
Protocatechuic acid	0.17 ± 0.03	0	0.25 ± 0.01
Catechin	41.03 ± 2.54	4.65 ± 0.23	8.39 ± 0.47
Procyanidin B2	55.15 ± 3.66	2.52 ± 0.13	5.20 ± 0.26
Epicatechin	76.41 ± 3.72	11.04 ± 0.52	9.48 ± 0.63
Epicatechin gallate	11.66 ± 0.64	8.22 ± 0.42	11.92 ± 0.79
Total	194.39 ± 11.05	27.27 ± 1.33	36.09 ± 0.43

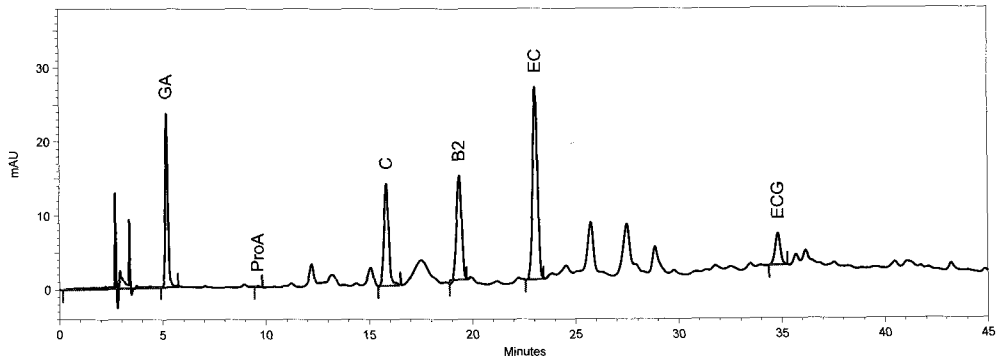

Fig. 2. Typical chromatogram of grape seed extracts (Extract 1).

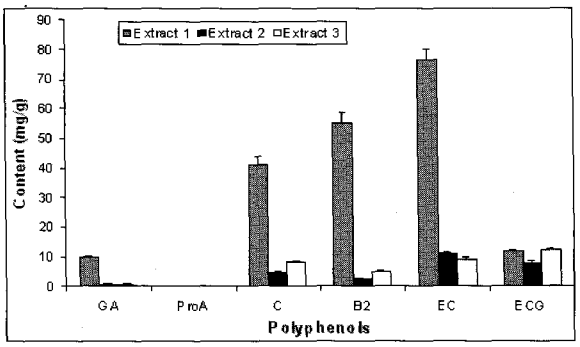

Fig. 3. Polyphenols content in different grape seed extracts.

The ratio of each individual polyphenols is shown in Fig. 4, with procyanidin B2 and epicatechin gallate marked on the pie chart. From this figure, we found that the ratios of polyphenols were similar between Extracts 2 and 3, while the proportion of Extract 1 differed.

DISCUSSION

Based on the HPLC procedures for polyphenols assay described previously, reversed-phase systems use long analytical C-18 column (250 mm in length) and higher flow rate of mobile phase. Although this condition can increase the separation factors among different compounds, it can make the column press higher, prolong the retention time of phenolic compounds, and generate more waste eluents. In the present study, we selected a 150 × 3.2 mm ODS (2) column, a simpler gradient elution program. The polyphenols were separated in the column with lower mobile phase flow rate (0.6 ml/min) and shorter assay time (60 min), while generating a small quantity of waste solution in each single assay.

The total contents of polyphenols were different

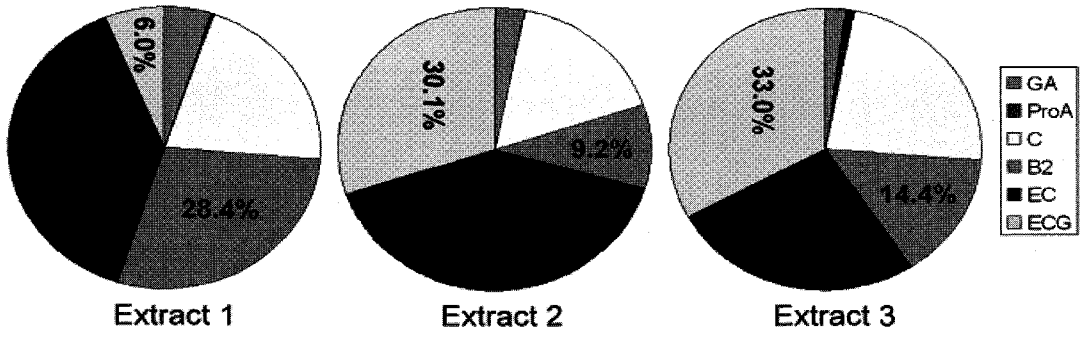

Fig. 4. Polyphenolic compounds proportions in different resources of grape seed extracts.

among different GSE samples. Fig. 3 show that the ratios of polyphenols were different among Extract 1, 2, and 3, which shows that different sources of GSE contain different amounts of polyphenolic compounds. The quantitative analysis method we developed may have a value for GSE manufacturer.

Grape seeds contain a complex phenolic composition. The scavenger capacity for free radicals was different among variety of polyphenols in grape seeds. Catechin and epicatechin are considered radical scavengers. Moreover, gallic acid esterification has been found increased in the scavenger capacity while procyanidin B2 has been shown to be the most effective compound in trapping oxygen free radicals (Silva *et al.*, 1991). In this study, six representative compounds were selected (two phenolic acids and four proanthocyanidins), and a simpler RP-HPLC analysis method was established to separate the polyphenolic compounds. From the assay data, we found that GSE from different origins contain a unique composition of polyphenols. In comparing the extracts, GSE Extract 1 had the highest total polyphenol concentration and higher proportion of procyanidin B2 (28.4%). Although the total contents of polyphenolic compounds were lower in Extract 2 and 3, the proportion of epicatechin gallate were 30.1% for Extract 2 and 33.0% for Extract 3, which are much higher than that of Extract 1 6.0% (Fig. 4.). In conclusion, our data has shown that the total polyphenols and the proportion of individual polyphenolic constituents are different among the three tested extracts. Whether their pharmacologic effects differ in these three extracts remained to be investigated in future studies.

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