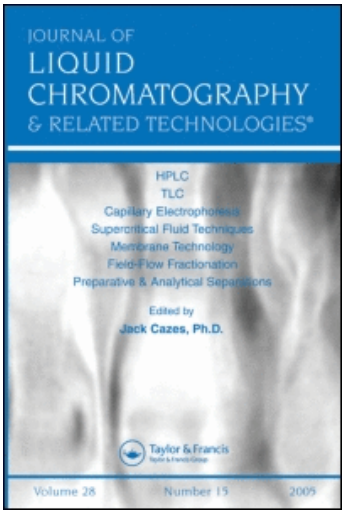

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DETERMINATION OF CATECHINS IN COMMERCIAL GRAPE SEED EXTRACT

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ABSTRACT

An HPLC-UV method with gradient elution for the quantification of catechins [(+)-catechin, (−)-epicatechin and (−)-epicatechin gallate] in grape seed extract was developed. The presence of monomers, dimers, and/or trimers of catechin were assayed with an initial HPLC-APCI-MS analysis, which was followed by isolation, purification, and characterization with spectral (NMR and MS) analyses of the compounds of interest. The results confirmed the presence of above catechin monomers in grape seed extract. Three peaks of interest in HPLC-APCI-MS

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chromatogram were identified to be gallic acid, procyanidin-B_4, and procyanidin-B_2, respectively.

INTRODUCTION

Grape seed extract is a well-known dietary supplement. It exhibits antioxidant and free radical scavenging activity and acts as chemopreventive agents (1–11). As a number of diseases are being related to free radical damage (12), the use of grape seed extracts together with grape products (white and red wine) may help to alleviate a number of conditions, including cardiovascular and inflammatory diseases (13–20).

Chemically, the active antioxidants in grape seed extract have been identified as flavanols (proanthocyanidins, catechols, and gallocatechols). Analysis of the flavanol contents in grape seed extract is therefore important for the standardization of grape seed extract products. In 1999, Palma and Taylor, reported an extraction procedure of polyphenolic compounds from grape seed with near critical carbon dioxide; three main constituents (gallic acid, (+)-catechin, and (−)-epicatechin) were detected (21). However, no information on other catechins was provided. Afterwards, Zhao et al. reported an RP-HPLC separation of grape seed catechins by using acetonitrile and acetate buffer (pH 4.8) in gradient as the mobile phase (19). Unfortunately, no validation in terms of linearity, reproducibility, and recovery was available. In attempting to implement a grape seed extract assay based on the existing chromatographic methods (19,21–23), we experienced difficulties in identifying the potential catechin analogues because of the shortage of reference standards. In this paper, we report a validated HPLC procedure to determine the content of catechins ((+)-catechin a, (−)-epicatechin, and (−)-epicatechin gallate) in a commercial grape seed extract. Meanwhile, an initial HPLC-APCI-MS analysis was carried out for more information on monomers, dimers, and/or trimers of catechin. In order to confirm this information, compounds of interest were isolated and purified from grape seed extracts by means of open column silica gel chromatography, medium pressure silica gel column chromatography, and semi-preparative reverse phase high-performance liquid chromatography, and their structures were characterized by analyzing their NMR and MS spectra.

EXPERIMENTAL

Chemicals and Reagents

(+)-Catechin, (−)-epicatechin, and (−)-epicatechin gallate were purchased from Sigma Chemical Co. (St Louis, MO, USA). HPLC grade acetic acid,

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acetonitrile, and methanol were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Deionized water was generated via an in-home Nano-pure® water system (Barnstead, Newton, MA, USA). Grape seed extract was provided by Dr. K. W. Singletary of University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.

Chromatographic System Conditions

A Waters 2690 HPLC system equipped with a 996 photodiode diode array UV detector was used for solvent delivery and detection, respectively. A Hewlett-Packard column (Zorbax SC-C_{18}, 250×4.6 mm, 5 μm particle size, Part # 880975-902, Hewlett-Packard Co., New Castle, DE, USA) protected by a Waters Delta-Pak™ RP-C_{18} guard column (Waters Technological Ireland, Limited, Wexford, Ireland) was used as the stationary phase. The solvents used for separation were: solvent A, 1% acetic acid; solvent B, acetonitrile containing 1% acetic acid. Solvent gradient conditions were as follows: 0–20 min, 95–87% A; 20–30 min, 87% A, 30–46 min, 87–78% A, 46–55 min, 78−10% A, 55–65 min, 10% A. The column was thermostated at 20°C. The sampling system was kept at 4°C. UV detection was achieved at 277 nm.

Preparation of Sample and Standard Solutions

Grape seed extract (5–20 mg) was exactly weighted into a 4-mL sample vial. 10% methanol (2.0 mL) was added to dissolve the sample. The sample solution was vortexed for 1 min and then filtered through 0.2 μm pore size nylon filter media with polypropylene housing (Whatman Inc., Clifton, NJ, USA) before injection (10 μL) into the HPLC system.

Standard stock solutions of (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate were prepared by dissolving 1–20 mg of reference standards, respectively, in 10 mL of 10% methanol. The stock solutions were stored at 4°C and diluted with 10% methanol before use.

Calibration Curves

Calibration samples were prepared before each assay by mixing the appropriate volume of standard solutions with 10% methanol. These samples were analyzed according to the procedure described above. Calibration curves were structured by linear regression of the peak area versus concentration.

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Accuracy, Precision, and Recovery

The accuracy and precision of the method were assessed by within- (n = 3) and between-run (n = 3) validations. Control samples were prepared by spiking corresponding amounts of (+)-catechin and (−)-epicatechin in 10% methanol to provide final concentrations of around 40, 200, and 400 μg/mL, respectively. During each assay run, the control samples were positioned between the samples and calibration samples. By substituting the peak-area into the calibration curve from the same run, the measured concentration could be obtained. The relative errors were obtained by comparing the calculated and theoretical concentrations. Coefficients of variance were calculated by comparing the measured concentrations in the same assay.

Recovery was assessed by spiking certain standard solutions to the samples and preparing recovery samples as described above. During the run, the recovery samples were positioned between calibration samples and samples without spiking standards. By subtracting the amounts of the compounds in the samples without spiking, the remains were compared with the amounts of compounds spiked for the recovery.

HPLC-MS Analysis

For more information on the monomers, dimers, and/or trimers of catechin in grape seed extract, an initial HPLC-MS analysis was carried out on an HP 1100 Series HPLC (Hewlett-Packard, Palo Alto, CA, USA) equipped with an autoinjector, quaternary HPLC pump, and HP ChemStation for data collection and manipulation. The mobile phase was the same as that run in HPLC-UV. The HPLC system was interfaced to a HP 1100 mass selective detector equipped with an APCI ionization chamber.

Conditions for analysis in the negative ion mode included the same mobile phase as described above, a nebulizing pressure of 30 psig, the drying gas temperature at 400°C, and fragmentor at 40 V. Data were collected on an HP Chem Station using selected ion monitoring at m/z 169 [gallic acid-H]$^-$, 273 [catechin (or epicatechin) gallate – galloyl-H]$^-$, 289 [catechin (or epecatechin)-H]$^-$, 441[catechin (or epicatechin) gallate-H]$^-$, 457 [epigallocatechin-H]$^-$, 577 [catechin (or epicatechin) dimer-H]$^-$, 729 [catechin (or epicatechin) dimer-gallate-H]$^-$ and 865 [catechin (or epicatechin) trimer-H] $^-$.

Data collection was stopped at 50 min in order to protect the mass source, because most of polymers of catechin were eluted after 50 min in the current HPLC assay.

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Isolation, Purification, and Characterization of Catechin Analogues

To confirm the information of HPL-APCIC-MS analysis, grape seed extracts (70 g) were subjected to HPLC-UV guided isolation, including Sephadex LH-20 chromatography with methanol as the eluent, silica gel column chromatography, and medium pressure silica gel column chromatography with $CHCl_3$-MeOH-HOAc in gradient as the eluent. The obtained compounds of interest were further purified by using semi-preparative high performance liquid chromatography on a Supelco RP-C_{18} column (Supelco, Co., Bellefonte, PA, USA) with methanol-water-HOAc in gradient as the eluent. The identities of the purified compounds were determined by Co-TLC and Co-HPLC with authentic standards or NMR and MS spectral analyses.

RESULTS AND DISCUSSION

Chromatography

Several combinations of acetonitrile, methanol, and water [with 0.5% formic acid (pH 2.4), 0.1 M acetate buffer (pH 4.8), and 1% acetic acid (pH 2.8)] were evaluated as possible mobile phases with Waters Spherisorb C_{18} and Hewlett-Packard Zorbax SC-C_{18} columns. After optimization of the chromatographic condition, the reverse-phase system using Hewlett-Packard Zorbax SC-C_{18} column and the mobile combination described above, achieved simultaneous separation of three catechins [(+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate] with more than 12 other major peaks within 60 min as shown in Fig 1. Under the described chromatographic conditions, the retention

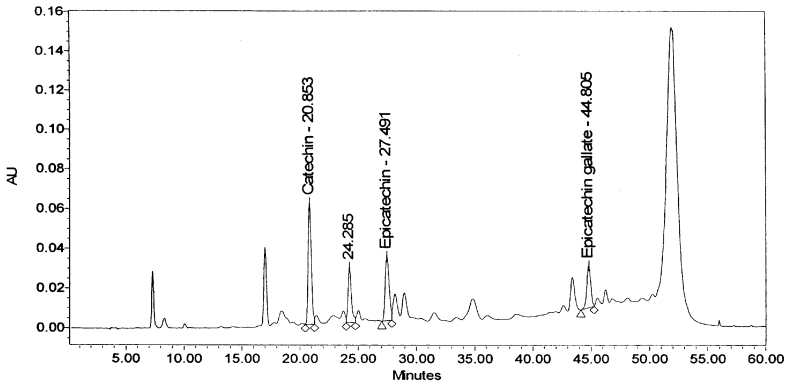

Figure 1. Typical HPLC-UV chromatogram of grape seed extract.

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times for (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate were 20.85, 27.49, and 44.81 min, respectively. Peak tailing effects, probably due to the interaction of polyhydroxyl groups of catechins with the stationary phase were prevented by adding acetic acid to the mobile phase.

It has been reported that catechins are unstable in alkaline media and decompose oxidatively (24). However, they are relatively stable under acidic and neutral conditions. In the current study, 10% methanol was used to prepare the sample and the sampling system temperature was kept at 4°C.

Calibration

Calibration graphs were linear in the concentration range 4 to 430 μg/mL for (+)-catechin and (−)-epicatechin, and 8 to 80 μg/mL for (−)-epicatechin gallate, with regression coefficients (r^2) of 0.999998, 0.99998, and 0.9996, respectively. These concentration ranges are high enough to determine catechins in grape seed extract commercial samples.

Accuracy, Precision, and Recovery

The accuracy and precision of the method were validated on the basis of within and between run assays, for which the results were shown in Table 1. At three different concentrations, the coefficients of variance of within run were found to be less than 0.14 and 0.36% for (+)-catechin and (−)-epicatechin, respectively, and the mean relative errors were less than 0.144 and 1.13% for (+)-catechin and (−)-epicatechin, respectively. The accuracy and precision for between run were also satisfactory. The coefficients of variance were less than 0.61 and 1.71% for (+)-catechin and (−)-epicatechin, and the mean relative errors were less than 0.6 and 1.19% for (+)-catechin and (−)-epicatechin, respectively.

The average recoveries for (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate were 100.18, 100.28, and 100.04%, respectively. Based on the analysis of the recovery data, the coefficients of variation were found to be 3.94, 4.30, and 5.77% ($n = 3$), respectively.

Sample Analysis

Three batches of grape seed extract samples were analyzed according to the method described above. The content [% w/w (STD%), $n = 3$] of (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate was determined to be 4.48 (2.22), 3.08 (1.79), and 0.63% (3.48%), respectively.

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Table 1. Precision and Accuracy Validation of Catechins

		Within-Run			Between-Run		
Group	Spiked Concentration (μg/mL)	Measured Concentration (Mean ±SD, n = 3) (μg/mL)	Coefficient of Variance (%)	Relative Error (Mean) (%)	Measured Concentration (Mean ±SD, n = 3) (μg/mL)	Coefficient of Variance (%)	Relative Error (Mean) (%)
QC-1	(+)-Catechin, 42.78	42.46±0.06	0.14	−0.65	42.52±0.222	0.52	0.6
QC-1	(−)-Epicatechin, 41.88	42.07±0.15	0.36	0.45	42.38±0.57	1.34	1.19
QC-2	(+)-Catechin, 213.9	214.2±0.1	0.05	0.14	213.06±1.30	0.61	0.39
QC-2	(−)-Epicatechin, 209.4	211.8±0.1	0.05	1.13	210.5±1.66	0.79	0.5
QC-3	(+)-Catechin, 427.8	431.67±0.62	0.14	0.91	433.55±3.26	0.75	1.34
QC-3	(−)-Epicatechin, 418.8	426.04±0.58	0.14	1.71	427.46±2.44	0.57	2.05

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Information on Catechin Dimer and/or Trimers

Besides HPLC-UV, HPLC thermospray mass spectrometry (HPLC-TSP-MS) (25), HPLC atmospheric pressure ionization mass spectrometry (HPLC-APCI-MS) (26) with selective ion monitoring (SIM), and MALDI-TOF-MS (26,27) have been employed to the analysis of grape seed extract. In the current study, an HPLC-APCI-MS analysis with SIM was carried out for the information on catechin analogues in a commercial grape seed extract. Although the current HPLC-APCI-MS method might remain to be further modified in terms of sensitivity, the information obtained was of considerable interest. A typical HPLC-APCI-MS chromatogram is shown in Fig 2 and the identity information for peaks of interest is summarized in Table 2. (+)-Catechin, (−)-epicatechin and (−)-epicatechin gallate could be easily identified based on the assignment of their SIM signals and the comparison of their retention times in HPLC-UV and HPLC-APCI-MS, which was further confirmed by Co-HPLC of isolated compounds with reference standards.

In the same way, the peak with retention time of 6.59 min in HPLC-APCI-MS chromatogram was confirmed to be gallic acid by Co-TLC and Co-HPLC of isolated gallic acid with a reference standard. The peaks with retention times of 15.85 and 23.51 min could be ascribed to dimers of catechin due to the appearance of significant ions at m/z 577 [catechin (or epicatechin) dimer–H]$^-$ and 289 [catechin (or epicatechin)–H]$^-$.

After isolation, purification, and spectral (NMR and MS) analyses, the identity of these two peaks was determined to be procyanidin-B_4 [MS (m/z): 577

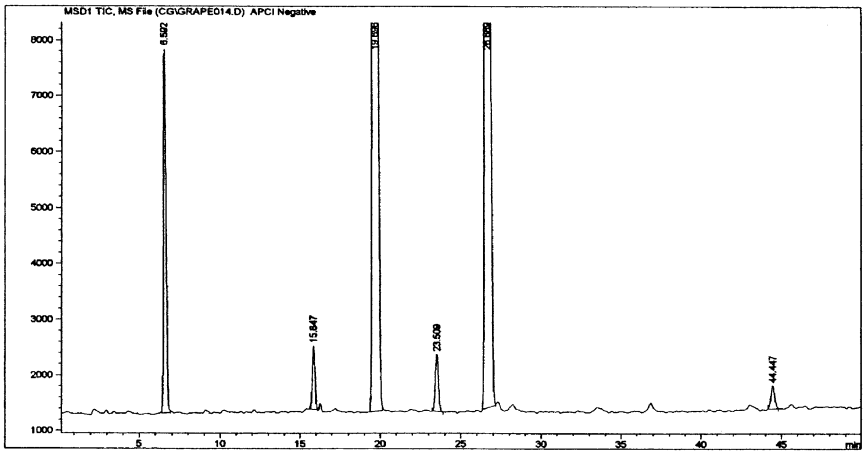

Figure 2. Typical HPLC-APCI-MS chromatogram of grape seed extract.

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Table 2. LC/MS Data of Grape Seed Extract

Retention Time (min)	Ions m/z	Compound Identified
6.59	169	Gallic acid
15.85	577, 289	Procyanidin-B_4
19.70	289	(+)-Catechin
23.51	577, 289	Procyanidin-B_2
26.69	289	(−)-Epicatechin
44.45	441, 289	(−)-Epicatechin gallate

$(M\text{-}H)^-$; ^{13}C NMR (DMSO-d_6) δppm: 30.6, 36.9, 68.2, 72.8, 76.8, 81.7, 95.5, 95.7, 96.2, 97.0, 100.8, 101.5, 107.2, 115.1 (×2), 115.9 (×2), 119.3 (×2), 132.5 (×2), 145.1 (×2), 145.4 (×2), 153.6, 155.2 (×2), 157.3, 157.9, 158.3] and procyanidin-B_2 [MS (m/z): 577 $(M\text{-}H)^-$; ^{13}C NMR (DMSO-d_6) δppm: 29.6, 37.0, 66.9, 73.4, 77.0, 79.6, 96.1, 96.4, 97.3, 100.5, 101.4, 107.3, 115.2 (×2), 115.9 (×2), 119.3 (×2), 132.0, 132.5, 145.5 (×2), 145.7 (×2), 154.5, 156.3 (×2), 157.7, 158.2 (×2)], respectively (28,29). The peak with retention time of 28.15 min in HPLC-UV chromatogram was tentatively ascribed to be epigallocatechin gallate in HPLC-UV, based on the comparison of retention time with authentic standard. But the HPLC-MS assay results did not support the presence of the compound because the characteristic ions at m/z 305 $[\text{epigallocatechin-H}]^-$ and 457 $[\text{epigallocatechin gallate-H}]^-$ for epigallocatechin gallate were not observed in the current HPLC-MS analysis.

CONCLUSION

The current gradient method developed for the determination of catechins is suitable for all grape seed extracts. Work to extend this method to include dimers and trimers of catechin is underway.

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