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An LC-MS Method for Analyzing Total Resveratrol in Grape Juice, Cranberry Juice, and in Wine

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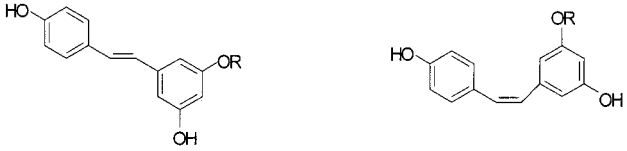
Resveratrol is an antioxidant found in grapes, grape products, and some other botanical sources with antiinflammatory and anticancer properties. In grapes and wine, it occurs both as free resveratrol and piceid, the 3β-glucoside of resveratrol. Here we report a liquid chromatography−mass spectrometry method to analyze total resveratrol (including free resveratrol and resveratrol from piceid) in fruit products and wine. Samples were extracted using methanol, enzymatically hydrolyzed, and analyzed using reversed phase HPLC with positive ion atmospheric pressure chemical ionization (APCI) mass spectrometric detection. Following APCI, the abundance of protonated molecules was recorded using selected ion monitoring (SIM) of *m*/*z* 229. An external standard curve was used for quantitation, which showed a linear range of 0.52−2260 pmol of *trans*-resveratrol injected on-column with a correlation coefficient 0.9999. The coefficient of variance of the response factor over the same concentration range was determined to be 5.8%, and the intra-assay coefficient of variance was determined to be 4.2% ($n = 7$). The limit of quantitation, defined as signal-to-noise 10:1, was determined to be 0.31 pmol injected on-column. The extraction efficiency of the method was determined to be 92%. The stability of resveratrol under different conditions was also examined. For example, resveratrol was stable for up to 5 days at 4 °C in the dark but was not stable at room temperature without protection from light. Resveratrol was detected in grape, cranberry, and wine samples. Concentrations ranged from 1.56 to 1042 nmol/g in Concord grape products, and from 8.63 to 24.84 μmol/L in Italian red wine. The concentrations of resveratrol were silmilar in cranberry and grape juice at 1.07 and 1.56 nmol/g, respectively.

KEYWORDS: Resveratrol; piceid; LC-MS; stability; grape; cranberry; wine

INTRODUCTION

Resveratrol (3, 5, 4′-trihydroxy-stilbene) (**Figure 1**) is a naturally occurring antioxidant found in grapes (*1*), grape products such as wine (*2*), and some other botanical sources, like peanuts (*3*). In grapes, resveratrol occurs both as free resveratrol and piceid (**Figure 1**), which is the 3-β-mono-D-glucoside of resveratrol. In recent years, research on resveratrol has discovered several beneficial biological effects of this compound to human health. These include anticancer activity (*4*), cardioprotection (*5*), antioxidant activity (*6, 7*), inhibition of platelet aggregation (*8*), and antiinflammatory activity (*9*).

The pharmacological activity of resveratrol has stimulated development of analytical methods for its measurement in different matrixes such as plant extracts, wine, serum, and tissue. Most of these methods are based on HPLC with UV absorbance detection (*10, 11*), although fluorometric (*12*) and electrochemical detection (*13, 14*) have been used. Optimization of HPLC parameters is essential to separate *cis*- and *trans*-resveratrol from potential interfering substances since HPLC with UV absorbance detection lacks selectivity. Therefore, HPLC−UV analyses of resveratrol typically require careful method development and validation, and the application of the resulting assay can be time-consuming and low throughput. For example Sato et al. (*10*) reported a gradient HPLC−UV method in which *cis*-resveratrol eluted at 40.5 min with gradient elution, and a minimum of 50 min was required per HPLC analysis. As an alternative to HPLC based assays, capillary electrophoresis has been used for the analysis of resveratrol, and these methods have been reviewed recently (*15*). Also methods using gas chromatography−mass spectrometry (GC-MS) have been reported (*16, 17*). Although GC-MS analysis provides excellent sensitivity and specificity, derivatization of resveratrol is required prior to analysis in order to increase its thermal stability and volatility. Furthermore, the high temperature used at the injector, column, and ion source



trans-Resveratrol R=H
trans-Piceid R=Glc

cis-Resveratrol R=H
cis-Piceid R=Glc

Figure 1. Structures of resveratrol and piceid.

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(250−300 °C) might cause partial isomerization or degradation of the sample and result in inaccurate quantitation.

Recently, a method using tandem solid-phase extraction coupled with LC-MS was reported for the determination *trans*-resveratrol in wine (*17*). Here, we report a LC-MS method to determine *cis*- and *trans*-resveratrol (including both free resveratrol and the aglycon released from piceid) in wine, grape products, and cranberry juice. In addition, the stability of resveratrol under different storage conditions was evaluated.

MATERIALS AND METHODS

Materials. β-D-Glucosidase and *trans*-resveratrol were purchased from Sigma Chemical (St. Louis, MO). All organic solvents were HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NY). Cranberry and grape samples were provided by Ocean Spray (Lakeville-Middleboro, MA) as juice, concentrate, or powder. Grape juice was purchased from grocery store and was not from concentrate or powder. Cranberry raw juice was prepared by hydraulic pressing of frozen cranberries which have been combined with rice hulls to facilitate the process. Italian wines were obtained from Marco Maci Winery (San Marco, Italy).

Sample Preparation. A 10 mL aliquot of each wine sample was evaporated under vacuum to remove ethanol, and the residue was reconstituted with 6 mL of deionized water and adjusted to pH 6.0 with 0.1 M NaOH. Each solution was treated with 30 mg β-D-glucosidase (2.4 units/mg) and incubated at 37 °C for 18 h. After enzymatic hydrolysis, the samples were extracted four times with 7 mL of ethyl acetate saturated with water. The ethyl acetate extracts were combined and evaporated to dryness under vacuum. The residue from the ethyl acetate extract was redissolved in 250 μL of methanol/water (6:4; v/v) prior to LC-MS analysis. Powder and juice samples were prepared in a similar manner with the following modifications. Each powder sample (0.1 g) was reconstituted with 1 mL of deionized water using vortex mixing, and then 20 mL of methanol was added. Methanol (20 mL) was also added to 1 g aliquots of each juice sample. After stirring overnight, each extraction mixture was centrifuged at 2280*g* for 5 min, the supernatant was removed and saved, and the solid residue was washed with 10 mL methanol and centrifuged again. Methanol in the combined supernatants was removed using a rotary evaporator, and the residue was redissolved in 10 mL deionized water for enzymatic hydrolysis. Finally, each extract was redissolved in 2 mL of methanol/0.5% formic acid (3:7; v/v) prior to LC-MS analysis. In the analysis of grape powder and juice samples, the composition of the injecting solvent was adjusted to match the starting solvent composition to avoid potential peak distortion that might be caused by stronger injecting solvent. A standard solution of resveratrol was prepared by dissolving a weighed aliquot of *trans*-resveratrol in a minimum volume of methanol and then diluting the sample to a designated volume using MeOH/0.5% formic acid (7:3; v/v). For identification of *cis*-resveratrol, a mixture of *cis*- and *trans*-resveratrol was prepared by irradiating an aliquot of the *trans*-resveratrol stock solution with UV light at 254 nm for 2 h (*10*).

To determine the stability of resveratrol, a stock solution of 0.5 μM resveratrol was prepared in methanol, and a 100 μL aliquot was taken and stored at −80 °C as reference. Additional aliquots were stored in polypropylene Eppendorf tubes under the following conditions: (1) room temperature with exposure to light; (2) room-temperature sealed in the dark; (3) 4 °C in the dark; and (4) −20 °C in the dark. Aliquots were removed from each tube at specific intervals and stored at −80 °C until analysis using LC-MS in a single assay. The stability of resveratrol was determined by the ratio of signal response obtained from each sample to the signal response from the reference stored at −80 °C, which was assumed to be stable.

LC-MS Analysis. LC-MS was carried out using an Agilent (Palo Alto, CA) G1946A LCMSD quadrupole mass spectrometer equipped with a series 1100 HPLC system consisting of a binary pump, automatic solvent degasser, thermostated antosampler and column compartment, and a Hypersil ODS column (5 μm, 100 × 2.1 mm). The solvent system consisted of a 28 min gradient from 25 to 39% methanol with 0.5% formic acid in water as the cosolvent at a flow rate of 250 μL/min. At the end of the gradient, the column was flushed with 95% methanol for 2 min at 500 μL/min to remove strongly retained compounds before re-equilibration with 25% methanol for 5 min at 500 μL/min. The column temperature was 25 °C, and the autosampler was maintained at 4 °C. Positive ion atmospheric pressure chemical ionization (APCI) mass spectrometry was used for the detection and quantification of resveratrol without solvent splitting. The optimum APCI conditions included a nitrogen nebulizer pressure of 40 psi (2.8 bar), a vaporizer temperature of 340 °C, a nitrogen drying gas temperature of 250 °C at 7.5 L/min, a capillary voltage of 4000 V, a corona current of 5.5 μA, and a fragmentor voltage of 70 V. Selected ion monitoring (SIM) was used to record the abundance of the protonated molecule of resveratrol at *m*/*z* 229 with a dwell time of 1.00 s. Quantitation was based on the LC-MS peak area of resveratrol, and a standard curve was run everyday immediately before and again after analysis of samples.

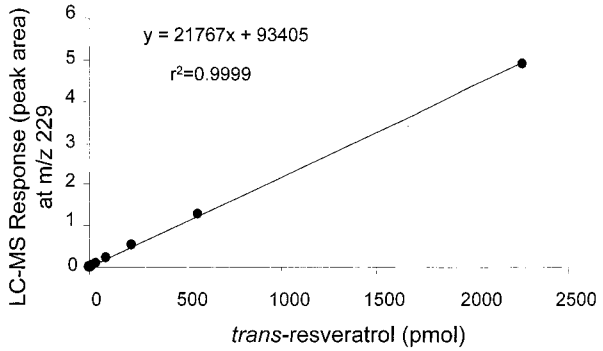

Figure 2. Calibration curve for the LC-MS analysis of *trans*-resveratrol. Positive ion APCI mass spectrometry was used with selected ion monitoring (SIM) for detection. The correlation coefficient was determined to be 0.9999 over the range of 0.52–2260 pmol.

RESULTS AND DISCUSSION

Method Validation. Resveratrol ionizes well in both positive and negative ion APCI and electrospray. In the positive and negative ion APCI spectra of resveratrol, protonated and deprotonated molecules formed the base peak at *m*/*z* 229 and 227, respectively. No peak other than the isotope peak showed abundance over 10%. Containing three phenolic hydroxyl groups, resveratrol is acidic in solution. Therefore, formic acid was added to the HPLC mobile phase to suppress on-column ionic dissociation of resveratrol. The strong acidity of the mobile phase used in this study suppressed negative ion formation and facilitated the formation of protonated molecules. Therefore, positive ion mode was used for all analyses. Since the concentrations of resveratrol in the samples might vary over a wide range (possibly 3 orders of magnitude), APCI was selected over electrospray in order to obtain the broadest possible dynamic range and linearity of response (*18*, *19*).

The recovery of resveratrol using methanol and ethyl acetate extraction was also determined. Grapes were purchased from local supermarket. Peel and seeds were removed, the remaining flesh was homogenized using a blender, and standard solutions of *trans*-resveratrol were spiked into the grape homogenates. The methanol extraction, enzymatic hydrolysis, ethyl acetate extraction, and LC-MS quantitation steps were then carried out for the spiked and unspiked grape homogenates. The recovery was calculated by comparing the differences in resveratrol concentrations between spiked and unspiked samples to the amount of standard added. For this extraction procedure, the recovery was determined to be 92%.

The LC-MS calibration curve of *trans*-resveratrol was linear over more than 3 orders of magnitude. As shown in **Figure 2**,

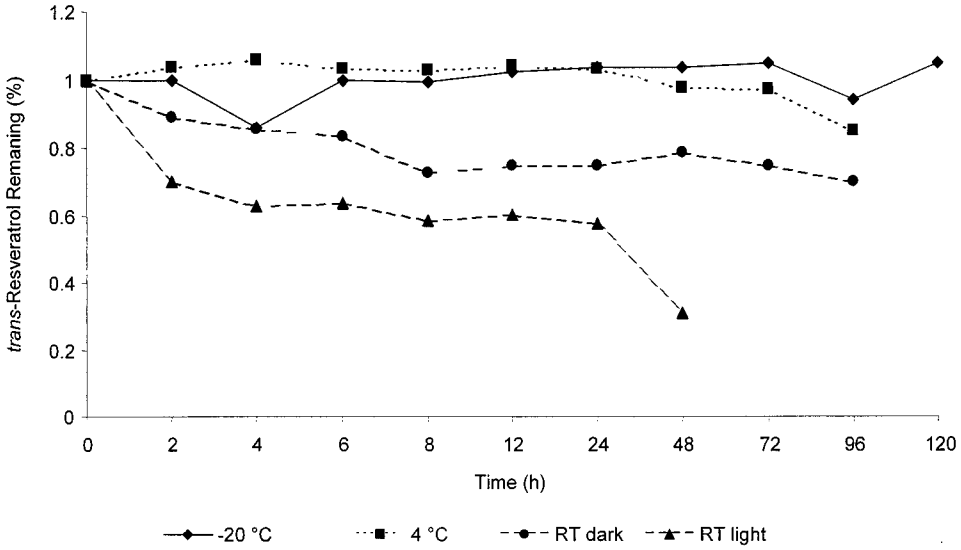

Figure 3. Stability of resveratrol under different storage conditions.

the correlation coefficient of the calibration curve was determined to be 0.9999 over the range of 0.52−2260 pmol of *trans*-resveratrol injected on-column. When response factor was defined as (peak area)/(amount injected), the coefficient of variance of the response factor over the same concentration range was determined to be 5.8%. The limit of quantitation, defined as signal-to-noise of 10:1, was determined to be 0.31 pmol injected on-column with a coefficient of variance of 2.6% ($n = 6$). The intra-assay coefficient of variance was determined to be 4.24% ($n = 7$).

The stability of resveratrol in methanol in the dark was investigated under various storage conditions including room temperature, 4 °C, and −20 °C. Resveratrol stability was also determined at room temperature when exposed to light. As shown in **Figure 3**, resveratrol was stable at −20 °C for up to 5 days and at 4 °C for up to 3 days. However, when stored in methanol at 4 °C for 4 days, the resveratrol concentration decreased to ∼85% of the reference value. At room temperature, a decrease in resveratrol was observed even at 2 h, and the amount was reduced to ∼75% after 8 h. When exposed to light at room temperature, only 70% of resveratrol remained after 4 h of storage, and the level decreased to 30% after 48 h. In conclusion, resveratrol should be protected from light during extraction, storage, and analysis. Standard solutions and extracts containing resveratrol should be stored at ≤20 °C and analyzed within 5 days. Fresh standard solutions should be prepared at least weekly. When large numbers of samples are to be analyzed using automated injection, the autoinjector should be thermostated to ≤ 4 °C, and samples should be analyzed within 2 days.

Previously, we verified that there is no difference between the ionization efficiency of 13-*cis*- and all-*trans*-retinoic acid, which differ only by the geometry on one carbon−carbon double bond (*20*). Since the only difference between the structures of *trans*- and *cis*-resveratrol is the geometry of the carbon−carbon double bond, in this study we assumed that ionization efficiency of *cis*- and *trans*-resveratrol are the same for quantitation of *cis*-resveratrol. Since only *trans*-resveratrol was available as a standard, *cis*-resveratrol was prepared by irradiating a *trans*-resveratrol methanolic solution with UV light at 254 nm according to a published method (*10*). Identification of *cis*-resveratrol in samples was carried out by comparison of the HPLC retention time, UV absorbance at 306 nm, and positive ion APCI mass spectrometric response with the *cis*-resveratrol standard prepared in-house. *trans*-Resveratrol eluted first from

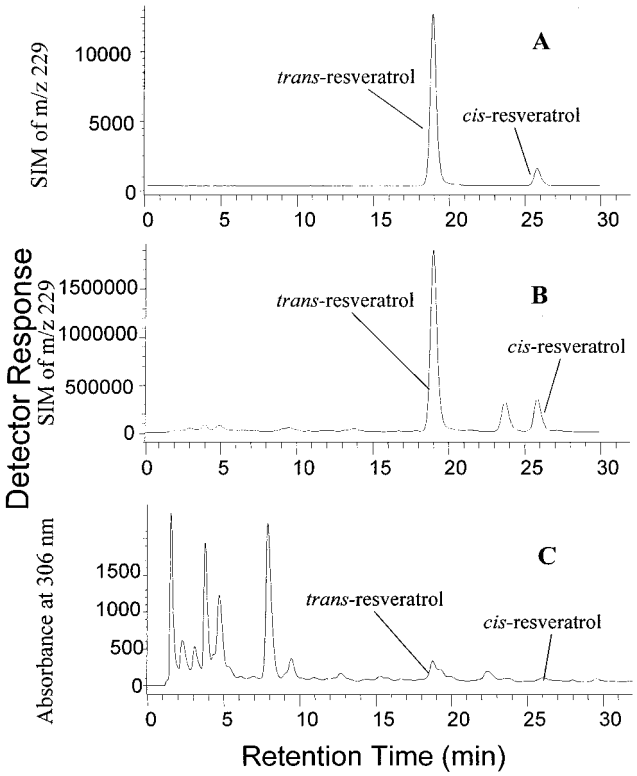

Figure 4. HPLC chromatograms of standard resveratrol and an extract of grape powder. (A) LC-MS chromatogram with SIM of *m*/*z* 229 showing detection of protonated molecules of standard *cis*- and *trans*-resveratrol. (B) LC-MS chromatogram with SIM of *m*/*z* 229 for a grape powder extract. (C) HPLC–UV chromatogram recorded during the grape powder extract analysis shown in panel B indicating lower selectivity and lower sensitivity compared to APCI mass spectrometric detection.

the C_{18} HPLC column with a retention time of 19 min followed by *cis*-resveratrol at 26 min. For example, **Figure 4** shows the LC-MS analysis of a mixture of *cis*- and *trans*-resveratrol standards and the analysis of a grape powder sample. Although three peaks were detected in the selected ion chromatogram of *m*/*z* 229 of the grape powder extracts (**Figure 4B**), the peaks corresponding to *cis*- and *trans*-resveratrol were identified by comparison to the standard mixture (**Figure 4A**). The identity of the third peak eluting at 23.5 min was not determined. However, in a separate analysis, the product ions of *cis*- and *trans*-resveratrol as well as those of the unknown peak were

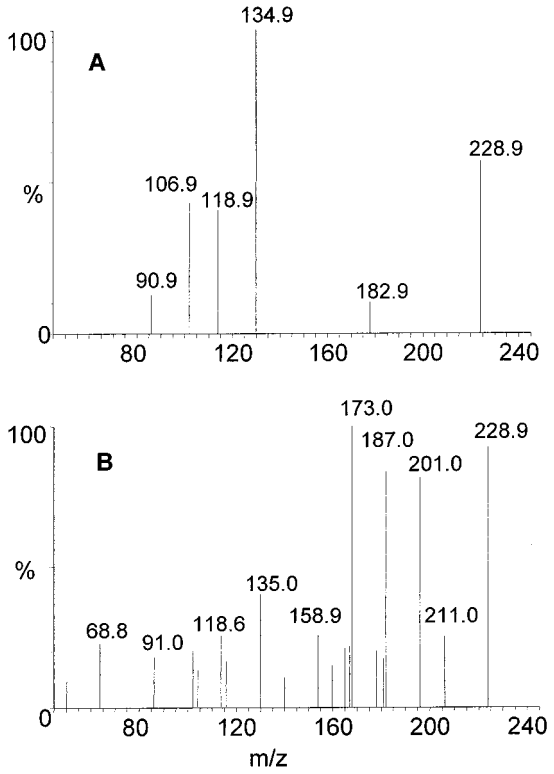

Figure 5. MS-MS spectra of the ion of *m/z* 229 eluting at 22.5 min in the LC-MS-MS analysis of a grape concentrate extract. (A) *trans*-Resveratrol; (B) unidentified peak at 22.5 min.

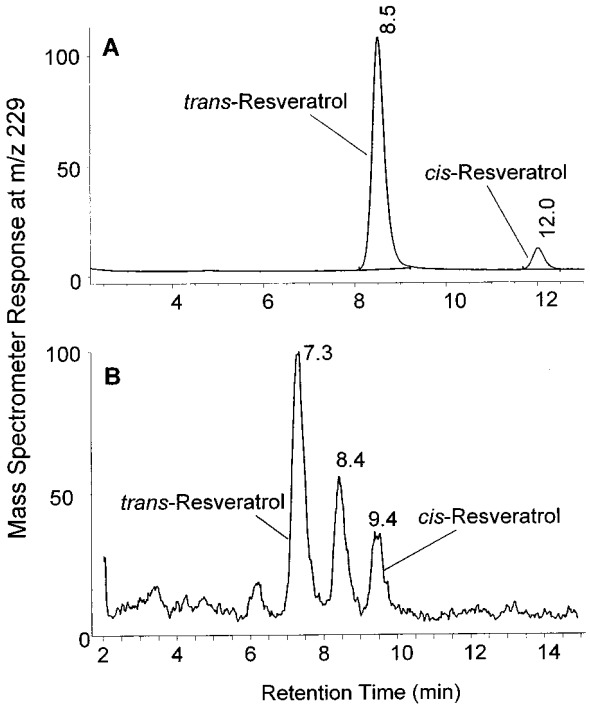

Figure 6. Separation of *cis*- and *trans*-resveratrol using the modified HPLC method. (A) Baseline separation of *cis*- and *trans*-resveratrol standards was obtained. (B) Analysis of a grape concentrate extract showing resolution of *cis*- and *trans*-resveratrol from the unknown peak.

scanned using LC-MS-MS. As shown in **Figure 5**, the fragmentation of this unknown peak (**Figure 5B**) showed no similarity to that of *trans*-resveratrol (**Figure 5A**). The fragmentation of *cis*-resveratrol (not shown) is identical to that of *trans*-resveratrol. Therefore, this unknown peak is unrelated to resveratrol and was not quantified in this study. Since most measurements of resveratrol are being carried out using HPLC with absorbance detection, the UV chromatogram at 306 nm recorded during the LC−UV-MS analysis is shown for comparison in **Figure 4C**. Multiple peaks including many that were more abundant than resveratrol were detected in the UV absorbance chromatogram. The *cis*-resveratrol peak was barely detectable, and there was a peak coeluting with *trans*-resveratrol, which would interfere with its quantification. In summary, LC-MS is more selective and much more sensitive than HPLC with UV absorbance detection for the quantitative analysis of *cis*- and *trans*-resveratrol.

Although the retention time of *cis*-resveratrol was 26 min with the HPLC solvent system used for the analyses reported here, additional method development was carried out to increase the throughput of this separation. Using the same mobile phase and gradient of 35 to 55% solvent B over 20 min, *cis*- and *trans*-resveratrol were resolved with baseline separation in 12 min (**Figure 6**A). By applying the modified method, the throughput of this assay could be doubled without altering the mobile phase composition or mass spectrometer parameters. In this modified HPLC method, the unknown peak in the grape samples was still resolved from *cis*- and *trans*-resveratrol (**Figure 6B**), ensuring accurate determination of *cis*- and *trans*-resveratrol. The HPLC retention times of *cis*- and *trans*-resveratrol in **Figure 6B** are different from those in **Figure 6A** because a different HPLC system (Waters 2690A) was used. However, all relevant species are separated using either chromatograph, illustrating the robustness of the modified method.

Table 1. Concentrations of Resveratrol in Italian Wines

wine description	*trans*-resveratrol (μM)	*cis*-resveratrol (μM)	total resveratrol (μM)
1. red blend	10.93	9.40	20.33
2. primitivo	9.57	11.13	20.70
3. negroamaro	8.92	15.92	24.84
4. negroamaro + malvesia nero	13.87	10.05	23.92
5. negroamaro	6.79	10.57	17.36
6. negroamaro	7.61	8.77	16.38
7. red blend, vino novello	3.14	5.49	8.63

Analysis of Grape and Cranberry Products. Seven different Italian red wines were analyzed for concentrations of total *cis*- and *trans*-resveratrol. The results are summarized in **Table 1**. Concentrations of *cis*-resveratrol varied from 1.26 to 3.65 mg/L (5.49−15.92 μM), and concentrations of *trans*-resveratrol were in the range 0.72−3.18 mg/L (3.14−13.87 μM). A previous analysis of resveratrol in red wine using HPLC with UV absorbance detection reported similar results of 0.15-3.59 and 0.30−2.95 mg/L for *trans*- and *cis*-resveratrol, respectively (*21*).

In the present study, eight different grape and cranberry preparations were analyzed for resveratrol content, and the results are summarized in **Table 2**. A significant finding of our analyses is that the resveratrol content of cranberry juice is comparable to that of grape juice. To the best of our knowledge, the presence of resveratrol in cranberry juice has not been published previously. This indicates that cranberry juice serves as another dietary source of resveratrol. Another important finding is that powdered preparations and concentrates of grape juice, which are used commercially for preparing juice products, still contain significant levels of resveratrol. In some of these processed grape samples, the relative proportions of *cis*- and *trans*-resveratrol were almost equal and similar to those in red wine. Since resveratrol occurs primarily as the *trans* isomer in grapes (*22*), isomerization probably occurred during processing

Table 2. Resveratrol in Grape and Cranberry Samples

sample	*trans*-resveratrol (nmol/g)	*cis*-resveratrol (nmol/g)	total resveratrol (nmol/g)
1. Concord grape powder	1185.00	216.92	1401.92
2. Concord grape powder	108.63	46.16	154.79
3. Concord grape powder	69.96	16.20	86.16
4. Concord grape powder	2.35	2.69	5.04
5. Concord grape concentrate	4.89	3.05	7.94
6. Concord grape extract	1.91	1.87	3.78
7. cranberry raw juice	0.93	0.14	1.07
8. Concord grape juice	1.13	0.43	1.56

or storage. Since the four grape powder samples were processed differently by the manufacturer, it was expected that levels of resveratrol would be different in each.

CONCLUSIONS

An LC-MS method for the quantitative analysis of *cis*- and *trans*-resveratrol has been developed, validated, and applied to the analysis of wine, grape, and cranberry products. The method provides excellent sensitivity with a limit of quantitation of 0.31 pmol and linearity of over at least 3 orders of magnitude. The extraction efficiency of the sample preparation method was determined to be 92%. The stability of *trans*-resveratrol in methanol was determined and the results shows that *trans*-resveratrol solutions are stable at −20 °C for up to 5 days, or at 4 °C for up to 2 days when protected from exposure to light. Resveratrol was detected in cranberry juice at a concentration similar to grape juice, which shows that cranberry may serve as an alternative dietary source for the antioxidant resveratrol. Grape and cranberry juices contain primarily *trans*-resveratrol, whereas processed juice products contain higher proportions of *cis*-resveratrol. The sensitivity and specificity of this LC-MS method would be suitable for investigations of the pharmacological significance of these different resveratrol isomers.

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