JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Resveratrol in Raw and Baked Blueberries and Bilberries

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Resveratrol in the fruits of bilberry (*Vaccinium myrtillus* L.), the lowbush "wild" blueberry (*Vaccinium angustifolium* Aiton), the rabbiteye blueberry (*Vaccinium ashei* Reade), and the highbush blueberry (*Vaccinium corymbosum* L.) were measured using a new assay based on high-performance liquid chromatography−tandem mass spectrometry (LC-MS/MS). The LC-MS/MS assay provided lower limits of detection than previous methods for resveratrol measurement, 90 fmol of *trans*-resveratrol injected on-column, and a linear standard curve spanning >3 orders of magnitude. The recoveries of resveratrol from blueberries spiked with 1.8, 3.6, or 36 ng/g were 91.5 ± 4.5, 95.6 ± 6.5, and 88.0 ± 3.6%, respectively. *trans*-Resveratrol but not *cis*-resveratrol was detected in both blueberry and bilberry samples. The highest levels of *trans*-resvertatrol in these specimens were 140.0 ± 29.9 pmol/g in highbush blueberries from Michigan and 71.0 ± 15.0 pmol/g in bilberries from Poland. However, considerable regional variation was observed; highbush blueberries from British Columbia contained no detectable resveratrol. Because blueberries and bilberries are often consumed after cooking, the effect of baking on resveratrol content was investigated. After 18 min of heating at 190 °C, between 17 and 46% of the resveratrol had degraded in the various *Vaccinium* species. Therefore, the resveratrol content of baked or heat-processed blueberries or bilberries should be expected to be lower than in the raw fruit. Although blueberries and bilberries were found to contain resveratrol, the level of this chemoprotective compound in these fruits was <10% that reported for grapes. Furthermore, cooking or heat processing of these berries will contribute to the degradation of resveratrol.

KEYWORDS: Blueberry; bilberry; *Vaccinium* sp.; resveratrol; mass spectrometry; LC-MS/MS

INTRODUCTION

In vivo and in vitro studies indicate that diets rich in botanical antioxidants such as vitamin E, vitamin C, and carotenoids are helpful in preventing chronic diseases such as heart disease and cancer (*1*). As a result, there is growing interest in the biological effects of other dietary botanical compounds including phenolics, flavonols, flavonoids, coumarins, and phytoalexins (*2*). Some potential functions of these compounds include free radical scavenging, antioxidant activity, and the protection or regeneration of other antioxidants. One of these botanical compounds is resveratrol (3,5,4′-trihydroxystilbene), which is a polyphenolic compound occurring in dietary sources such as grapes (*3*), wine (*4*), peanuts (*5*), and cranberries (*6*). In fruits, resveratrol occurs both as free resveratrol and as piceid, which is the 3-β-mono-D-glucoside of resveratrol. Resveratrol in wine might be responsible at least in part for the French paradox, which is the low incidence of heart disease among people in France who consume relatively high amounts of fat and red wine (*7*−*9*).

Blueberries, such as *Vaccinium angustifolium* Aiton, *Vaccinium ashei* Reade, and *Vaccinium corymbosum* L., contain resveratrol, anthocyanins, phenolics, and other antioxidants (*10, 11*). There is considerable variability in the antioxidant capacity of different *Vaccinium* species (*12, 13*). Another potential source of variability in the resveratrol content in fruits such as blueberries is food processing or preparation such as baking, boiling, pasteurization, and irradiation. In this investigation, the resveratrol contents of blueberries and the related bilberry, *Vaccinium myrtillus* L., which were cultivated in several different geographical regions, were investigated before and after baking. We know of no previous studies that have addressed the effect of baking on resveratrol in these fruits.

The pharmacological activity of resveratrol has stimulated the development of analytical methods for its measurement in different matrixes such as plant extracts, wine, serum, and tissue.

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10.1021/jf034150f CCC: $25.00
Published on Web 08/19/2003

Most of these methods are based on HPLC with UV absorbance detection (*14*, *15*), although fluorometric detection (*16*) and electrochemical detection (*17*) have been used to enhance selectivity during the separation and measurement of *cis*- and *trans*-resveratrol. Although the use of gas chromatography−mass spectrometry (GC-MS) has been reported (*18*) and has the potential to improve sensitivity and selectivity, derivatization of resveratrol is required prior to analysis to increase its thermal stability and volatility. Furthermore, the high temperatures used at the injector, column, and ion source (250−300 °C) might cause isomerization or partial degradation of the sample and result in inaccurate quantitation. As a solution to this problem, an LC-MS method was developed for the determination of *cis*- and *trans*-resveratrol (including both free resveratrol and its aglycon released from piceid) in wine, grape products, and cranberry juice (*6*). Here we report a new LC-MS/MS method for the determination of *trans*-resveratrol in fruit products that offers improved selectivity and sensitivity compared to HPLC with mass spectrometric detection alone.

MATERIALS AND METHODS

Materials. *trans*-Resveratrol and β-D-glucosidase were purchased from Sigma Chemical Co. (St. Louis, MO). Formic acid (88%) was obtained from J. T. Baker (Phillipsburg, NJ) and was diluted to the desired concentration using deionized distilled water. HPLC grade methanol, water, acetonitrile, and ethyl acetate were purchased from Fisher (Fair Lawn, NJ). Naringenin was purchased from Indofine Chemical (Somerville, NJ). To convert *trans*-resveratrol into *cis*-resveratrol for use as a standard, 0.7 mL of a 0.01 mg/mL *trans*-resveratrol solution in methanol was exposed to UV light ($\lambda = 254$ nm) for 10 min. The resulting solution contained *cis*- and *trans*-resveratrol in a ratio of approximately 2:1.

Blueberry and bilberry fruit samples were obtained from suppliers in various regions in North America and Europe. Lowbush "wild" blueberries (*V. angustifolium* Aiton) were obtained from Nova Scotia and rabbiteye blueberries (*V. ashei* Reade) from Mississippi. Highbush blueberry (*V. corymbosum* L.) fruits were obtained from Michigan and British Columbia, and bilberry (*V. myrtillus* L.) fruits were obtained from Poland. Samples were shipped for analysis frozen on dry ice and were stored at −20 °C until analysis.

Preparation of Standard Solutions and Samples. A 1 mM stock solution of *trans*-resveratrol and a 4 μM stock solution of the internal standard naringenin were prepared in methanol. Appropriate dilutions of the resveratrol stock solutions were also made with methanol. A 50 μL aliquot of each resveratrol standard solution was mixed with a 50 μL aliquot of naringenin and then diluted with 150 μL of acetonitrile and 50 μL of deionized water to make calibration curve standard solutions. Aliquots (10 μL each) of these calibration standards were injected onto the LC-MS/MS for generating calibration curves.

To investigate the effect of baking on the resveratrol content of blueberries and bilberries, berries were heated in an oven at 190 °C for 18 min. This temperature and length of exposure were selected to simulate exposure of these berries to heat during the baking of blueberry or bilberry muffins. The mass of each blueberry and bilberry sample was determined before and after heating to determine losses due to evaporation. Heated and raw blueberries and bilberries were stored at −20 °C under identical conditions until analysis using LC-MS/MS. These heat treatment experiments were carried out three times for each species of blueberry and bilberry.

After thawing, each blueberry or bilberry sample (~1 g) was weighed and then extracted three times in 10 mL of methanol using sonication for 30 min. After each extraction, samples were centrifuged for 5 min at 10000*g* and 4 °C, and the supernatants were removed and saved. The combined supernatants of each sample were evaporated to dryness and reconstituted in 6 mL of deionized distilled water. Each sample was treated with 30 mg of β-D-glucosidase (2.4 units/mg) and incubated at 37 °C for 18 h as described previously (*6*). After enzymatic deconjugation, the samples were extracted three times with 6 mL of ethyl acetate saturated in water. The ethyl acetate extracts were combined and evaporated to dryness under vacuum, and the residues were redissolved in 1.0 mL of methanol. A 25 μL aliquot of the 4 μM naringenin stock solution was added to 100 μL of each sample, and 10 μL aliquots of each sample were injected into the HPLC system for LC-MS/MS analysis. Five raw samples of each species of blueberry and bilberry were analyzed three times each for resveratrol content.

The recovery of resveratrol was investigated by spiking highbush blueberry samples with *trans*-resveratrol at levels of 0.18, 0.36, and 3.6 ng of resveratrol/g of berries. The spiked berries were then processed as described above and then analyzed using LC-MS/MS. These recovery experiments were carried out three times. The recovery was calculated by comparing the differences in *trans*-resveratrol concentration between spiked and unspiked samples to the amount of standards added.

Liquid Chromatography—Tandem Mass Spectrometry. LC-MS/MS was carried out using a Micromass (Manchester, U.K.) Quattro II triple-quadrupole mass spectrometer equipped with a Waters (Milford, MA) 2690 HPLC system. Aliquots (10 μL) of blueberry or bilberry extracts were injected onto a Waters XTerra MS C_{18} column (2.1 mm × 100 mm, 3.5 μm particle size) at 25 °C. The solvent system consisted of water and acetonitrile (each solvent containing 0.1% formic acid, v/v) with a 30 min linear gradient of 10−80% acetonitrile. The flow rate was 0.2 mL/min. There was a 10 min re-equilibration period with the initial solvent mixture between analyses.

Negative ion electrospray tandem mass spectra were recorded with the electrospray capillary set at 2.5 keV and a source block temperature of 100 °C. Nitrogen was used as the drying and nebulizing gas at flow rates of 8 and 0.8 L/min, respectively. Argon at 1.4×10^{-3} mbar was used as the collision gas for collision-induced dissociation (CID). To identify abundant fragmentation pathways for multiple reaction monitoring (MRM), product ion scanning was carried out using the deprotonated molecules of resveratrol and naringenin at *m*/*z* 227 and 271, respectively, as precursor ions. On the basis of these initial MS/MS experiments, an assay based on LC-MS/MS with MRM was developed using the transitions *m*/*z* 227 → 185 for resveratrol and *m*/*z* 271 → 151 for naringenin, both of which represent favorable fragmentation pathways for these deprotonated molecules. [For the negative ion electrospray MS/MS CID product ion mass spectrum of resveratrol, see Yu et al. (*19*).] Calibration curves were constructed by plotting the LC-MS/MS peak area ratio of *trans*-resveratrol to the internal standard naringenin (at 0.8 μM) against the analyte concentration. No impurities and no *cis*-resveratrol were detected in the *trans*-resveratrol standard.

RESULTS AND DISCUSSION

The linear regression analysis of the standard curves for the LC-MS/MS measurement of *trans*-resveratrol showed a correlation coefficient of $r^2 = 0.999$ over the range from 90 fmol to 100 pmol of *trans*-resveratrol injected on-column (1.6 nM to 10 μM). The limit of quantitation, defined as a signal-to-noise of 10:1, for the determination of *trans*-resveratrol in blueberries and bilberries was 90 fmol injected on-column. For comparison, the limit of quantitation of our previously published LC-MS method was 310 fmol (*6*). Therefore, the new LC-MS/MS method is ~3-fold more sensitive than LC-MS for resveratrol analysis.

As a representative LC-MS/MS chromatogram, the analysis of a raw highbush blueberry extract is shown in **Figure 1**. The peak for *trans*-resveratrol was detected at a retention time of 12.0 min, and the internal standard, naringenin, eluted at 15.0 min. However, no *cis*-resveratrol was detected in this or any of the other blueberry or bilberry samples. The retention time of *cis*-resveratrol formed by UV irradiation of the *trans*-resveratrol standard was 14.3 min. In addition to the HPLC retention times, the *cis*- and *trans*-resveratrol molecules may be distinguished by their different absorption maxima of 280 and 310 nm, respectively (*19*). The recoveries of *trans*-resveratrol from spiked blueberry samples were 91.5 ± 4.5, 95.6 ± 6.5, and 88.0 ±

Table 1. Content of *trans*-Resveratrol in Various Raw and Baked Blueberries and Bilberries

blueberry or bilberry specimen	*trans*-resveratrol[a] (pmol/g of sample)	*trans*-resveratrol[b] (pmol/g of sample) adjusted for evaporation during heating
raw highbush Michigan blueberry	140.0 ± 29.9	
heated highbush Michigan blueberry	98.3 ± 20.8	75.7 ± 16.0
raw highbush British Columbia blueberry	not detected	
heated highbush British Columbia blueberry	not detected	not detected
raw rabbiteye Mississippi blueberry	not detected	
heated rabbiteye Mississippi blueberry	not detected	not detected
raw lowbush "wild" Nova Scotia blueberry	56.2 ± 15.7	
heated lowbush "wild" Nova Scotia blueberry	49.3 ± 13.5	41.6 ± 11.4
raw Polish bilberry	71.0 ± 15.0	
heated Polish bilberry	89.8 ± 15.1	58.9 ± 9.9

[a] Mean of five samples measured three times each ± SD. [b] Mean of three samples measured three times each ± SD.

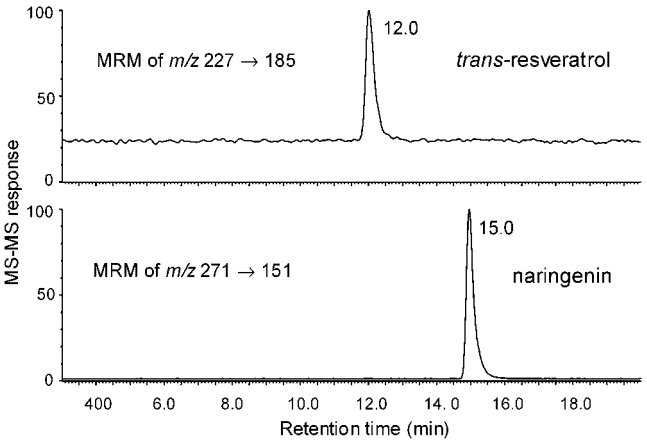

Figure 1. LC-MS/MS chromatograms of resveratrol and naringenin (internal standard) in a raw highbush Michigan blueberry extract. Following reversed phase HPLC separation, MS/MS was carried out using negative ion electrospray, collision-induced dissociation, and multiple reaction monitoring (MRM).

3.6% [± standard deviation (SD), $N = 3$] for 0.18, 0.36, and 3.6 ng of resveratrol/g of blueberry, respectively. This level of extraction efficiency was sufficient for quantitative analyses.

The results of the LC-MS/MS quantitative analyses of resveratrol in raw and heated blueberry and bilberry samples are shown in **Table 1**. The resveratrol content of blueberries ranged from as high as 140 pmol/g of raw highbush blueberries from Michigan to below the limit of detection in highbush blueberries from British Columbia or rabbiteye blueberries from Mississippi. Raw lowbush wild blueberries and bilberries contained intermediate resveratrol levels of 56.2 and 71.0 pmol/g, respectively. In addition, considerable regional variation in the resveratrol content of the blueberries was observed in the two samples of highbush blueberries.

For comparison, the resveratrol concentrations in grapes have been reported to range from 1.56 to 1042 nmol/g (*6*), which is ~10−7500-fold higher than the highest concentration of resveratrol detected in blueberries or bilberries. Although the concentrations of resveratrol in blueberries and bilberries are relatively low compared to the levels reported in grape and cranberry juices (*6*), these analyses indicate that blueberries and bilberries may still serve as another dietary source of resveratrol.

The levels of resveratrol that were measured in each type of raw and heated blueberry and bilberry are summarized in **Table 1**. During heating, the berries lost between 14 and 34% of their mass due to evaporation. Therefore, **Table 1** lists both the actual measured amounts of resveratrol in the heated samples as well as the adjusted values that take into account evaporation during heating. As shown by the adjusted data in **Table 1**, heating blueberries for 18 min at 190 °C resulted in the destruction of between 17 and 46% of the resveratrol (for Polish bilberries and highbush Michigan blueberries, respectively). The variations in stability of resveratrol in these different species of berries were probably a matrix effect. To the best of our knowledge, there are no other reports describing the resveratrol content of baked or heat-processed fruit.

In conclusion, a new LC-MS/MS quantitative assay was developed and applied to the analysis of *trans*-resveratrol in blueberry and bilberry samples. The extraction efficiency of the method was excellent (≥88%), and the linearity of both assays showed a correlation coefficient of 0.999 over >3 orders of magnitude. In addition, the LC-MS/MS assay showed no evidence of interference from coeluting substances. *trans*-Resveratrol was detected in both blueberries and bilberries but at much lower levels than has been reported in grapes and cranberries. Because blueberries and bilberries are often baked or heat-processed before consumption, a significant finding of this study is that resveratrol in these fruits will be at least partially degraded by heating.

ACKNOWLEDGMENT

Blueberries and bilberries were generously provided by Wilhemina Kalt and Jane McDonald of the Agriculture and Agri-Food Canada Research Centre, John Braswell of Mississippi State University, Brian Castor of the Driscoll Strawberry Association, and David Trinca of MBG Marketing. We thank Carol Gaines of Westco-Bakemark for providing a commercial baking protocol for blueberry muffins.

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Received for review February 14, 2003. Revised manuscript received July 24, 2003. Accepted July 28, 2003. Funding for these studies was provided in part by Grants R24 CA83124 from the National Cancer Institute and P50 AT00155 provided jointly by the National Center for Complementary and Alternative Medicine (NCCAM), the Office of Dietary Supplements (ODS), the Office for Research on Women's Health (ORWH), and the National Institute of General Medicine (NIGMS) of the National Institutes of Health (NIH). The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of the NCCAM, NCI, ODS, ORWH, NIGMS, or NIH.

JF034150F