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A Multiresidue Derivatization Gas Chromatographic Assay for Fifteen Phenolic Constituents with Mass Selective Detection

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We have developed a GC/MS method to simultaneously measure the concentrations of 15 biologically active phenolic components of wine: vanillic acid, gentisic acid, *m*- and *p*-coumaric acid, gallic acid, ferulic acid, caffeic acid, *cis*- and *trans*-resveratrol, epicatechin, catechin, morin, quercetin, and *cis*- and *trans*-polydatin. Wine (1 mL) was diluted 1:1 with water to reduce the alcohol content and extracted on a preconditioned C-8 solid-phase extraction cartridge. The phenolic compounds were eluted with ethyl acetate, evaporated to dryness, and derivatized with bis(trimethylsilyl)trifluoroacetamide/pyridine. The TMS derivative of each phenolic compound was analyzed on a GC/MSD coupled to a DB-5HT capillary column using one target and two qualifying ions for each compound in a total run time of 26 min. Resolution and quantitation of all compounds were excellent, with linear calibration curves over a wide range. The lowest detection limit was for gentisic acid (24 μg/L) and highest for quercetin (843 μg/L). The average percent recovery and coefficient of variation (mean precision) ranged from 90.7 to 104.6 (except morin, 72.2%) and 4.0 to 10.2 (except morin, 16.1%, and quercetin, 16.0%) respectively. This method has been applied to solid vitaceous plant materials as well as wine and should be suitable to measure polyphenols in fruit, vegetables, and other foods provided that efficient extraction techniques are employed.

Due to the large number of phenolic compounds of interest and the chemical complexity of the wine matrix, analytical methods to assay the polyphenols present in wine have in the past involved difficult and complicated chromatographic techniques. Historically, the most widely used have required the utilization of high-pressure liquid chromatography (HPLC), specifically with reversed-phase silica-based columns.[1−3]

Depending on the constituents under investigation, different prechromatographic purification treatments of the sample have been necessary, including solvent extraction at different pH ranges,[2] passage through a polyamide column,[4] or fractionation on C_{18} cartridges.[3,5,6] For this reason, rather large initial volumes were required and variable losses occurred due to incomplete extraction or oxidation.

One of the major problems underlying separation of these phenolic compounds is their similarity in chemical characteristics. Many show similar UV spectra with maxima in a narrow range of 280−320 nm. In order to prevent interference, some investigators fractionated the phenolic compounds of wine into neutral and acidic groups before HPLC analysis.

The most recent advances in HPLC technology accomplished by a number of groups[7−10] enabled the quantitation of up to 20 wine phenolics simultaneously, using direct injection of wine on a C_{18} reversed-phase column followed by gradient elution and photodiode array detection. Other than the last-cited paper,[10] none of those published presented analytical data validating their method nor have they been adequately described; neither have they been applied to a sufficiently representative number or range of wines to establish their suitability and robustness for routine use. Most failed to include some of the major compounds of biological interest.

Although not as popular as HPLC, gas chromatographic (GC) techniques with or without mass spectrometric (MS) detection have been employed for the analysis of phenolic compounds in wines. Baranowski and Nagel[11] were among the first to use this approach in which trimethylsilyl (TMS) derivatives of caffeic, ferulic, and *p*-coumaric acid were made after extensive solvent extraction of a large volume of sample (1 L). This method was only used for identification purposes and was not adequately described or validated. Jeandet et al.[12] used a similar approach in which TMS derivatives of resveratrol were made after extensive solvent extraction, and they were able to measure both isomers of this compound.

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Goldberg et al.[13,14] reported direct GC/MS methods to measure *trans*-resveratrol and *cis*-resveratrol which incorporated several novel features: first, and synchronously with Mattivi,[15] they used solid-phase extraction of 1 mL of wine on a C_{18} cartridge followed by elution of the resveratrol isomers with the same volume of ethyl acetate. Only 1 μL of this extract was then injected onto a special heat-resistant column (DB-5) for the final chromatographic separation. Excellent results were obtained by selective ion monitoring (SIM) of the molecular ion at mass 228.

Subsequently we developed a conventional GC/MS method[16] in which solid-phase extraction of both resveratrol isomers was followed by derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA). Both isomers could be measured with a detection limit of 10 μg/L using the target ion of $m/z = 444$ (relative abundance 100%) with ions of $m/z = 445$ (relative abundance 44%) and 446 (relative abundance 19%) as the qualifying ions. The present paper represents an extention of this principle that has permitted the simultaneous assay of up to 15 phenolic compounds in wine and is inherently capable of detecting and measuring many more. Further, the method has also been applied to extracts of solid vitaceous materials such as skins, pips, and stems and should likewise be applicable to extracts of phenolic compounds from any fruit, vegetable, or food source.

EXPERIMENTAL SECTION

Wines. Commercial wines, usually in 750-mL bottles, were opened and analyzed within 24 h. In the event that samples required storage, they were kept at 4 °C in a glass vial filled to completion and protected by foil against sunlight. Analyses were completed within a 3-day period.

Standards, Chemicals, and Solvents. Suppliers, purity, and dilution solvents for all phenolic standards used for this study are tabulated in Table 1. *cis*-Resveratrol was prepared from the trans isomer by UV irradiation.[17] *trans*-Polydatin was isolated from the dried roots of *Polygonum cuspidatum*, and a portion was converted to the cis isomer by UV irradiation.[18]

Instrumentation. A DB-5HT capillary column (J&W Scientific, Folsom, CA, USA; part no. J122-5731) with 5% (v/w) phenyl-substituted methylpolysiloxane nonpolar stationary phase, cross-linked and double bonded to the capillary wall with excellent thermal stability and low bleed levels, was used. The dimensions of the column were 30 m × 0.25 mm i.d., 0.10 μm film thickness, and it was preceded by a 1-m guard column of the same inner diameter connected to the column via a Chromfit glass connector (Chromatographic Specialties Inc., Brockville, ON, Canada).

A Hewlett-Packard (HP) Model GC-5890 was used for the analysis, equipped with a split/splitless injection port, interfaced to a MSD-5970; the GC/MSD was controlled by an HP Vectra 486/50N PC utilizing the MS ChemStation software-G1034C (DOS series) and reporting to a HP laser jet IV printer (Hewlett-Packard, Mississauga, ON, Canada).

Table 1. Source of Phenolic Standards and Dissolving Solvents

compound	supplier	stock std concn (mg/L)	dissolving solvent (v/v)
caffeic acid	Sigma[a]	1013	80% ethyl acetate 20% acetone
(+)-catechin	Sigma	1012	ethyl acetate
m-coumaric acid	Sigma	1076	95% ethyl acetate 5% acetone
p-coumaric acid	Sigma	1013	95% ethyl acetate 5% acetone
(−)-epicatechin	Sigma	1000	20% ethanol 80% acetone
ferulic acid	Sigma	1072	95% ethyl acetate 5% acetone
fisetin	Aldrich[b]	1044	40% acetone 60% ethyl acetate
gallic acid	Sigma	1004	95% ethyl acetate 5% acetone
gentisic acid	Lancaster[c]	1163	ethyl acetate
morin	Sigma	1032	95% ethyl acetate 5% acetone
quercetin	Sigma	1004	methanol
trans-resveratrol	Sigma	4802	absolute ethanol
vanillic acid	Sigma	1032	95% ethyl acetate 5% acetone

[a] Sigma-Aldrich Canada, Ltd, Mississauga, ON, Canada. [b] Aldrich Chemical Co., Inc., Milwaukee, WI. [c] Lancaster Synthesis Inc., Windham, NH.

Ultrahigh-purity helium with in-line Supelpure moisture trap and hydrocarbon trap (Supelco Canada, Mississauga, ON, Canada) was used as carrier gas. The carrier gas-line pressure was set at 60 psi, column head pressure at 8 psi; the septum purge was at 2.4 mL/min.

GC temperature information: injector, 280 °C; detector (transfer line), 320 °C; oven equilibration time, 1.0 min; initial temperature, 80 °C; initial time, 1.0 min; oven temperature program (total run time, 25.8 min).

level	rate (°C/min)	final temp (°C)	final time (min)
1	20.0	250	1.0
2	6.0	300	2.0
3	20.0	320	4.0
Total run time:	25.8 min.		

GC injector information: injection source, autoinjector; sample washes, 3; sample pumps, 3; sample volume injected, 1 μL; solvent A (acetone) washes, 4; solvent B (pyridine)washes, 4; injection port, splitless with double gooseneck glass insert and gold-plated injector seal and a Viton O-ring for high temperatures (HP).

MS information: aquisition mode, SIM; solvent delay, 7.80 min; electron multiplier voltage (EMV), 1400; EMV offset, 200; resulting EMV, 1600; tune, customized tune with perfluorotributylamine (PFTBA) tuning standard utilizing ions 219, 414, and 502 amu.

Extraction and Derivatization Procedure. Sep Pak C_8 cartridges (Waters Canada Ltd., Mississauga, ON, Canada) were preconditioned with 3 mL of ethyl acetate, 3 mL of 60% (v/v) ethanol, and 3 mL followed by 2 mL of deionized water. Wine samples were diluted with an equal volume of deionized water to bring the alcohol level to approximately 6% (v/v), and exactly 1

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mL of diluted sample was injected onto the preconditioned Sep-Pak and allowed to drain by gravity flow (3−5 min). A gentle flow of nitrogen was then introduced over the sample with simultaneous gradual suction on a vacuum manifold (−100 kPa) for 45 min (Millipore Canada, Mississauga, ON, Canada).

The phenolic compounds were extracted by eluting the dry Sep-Pak with 3 mL of ethyl acetate. The eluate was collected in a centrifuge tube previously spiked with fisetin as internal standard at 1.0 mg/L. The extract was then evaporated to dryness on a nitrogen evaporator (Meyer Organomation Associates Inc., S. Berlin, MA). To ensure complete removal of water, 0.5 mL of methylene chloride was added, and the resultant mixture was vortexed and evaporated to dryness (azeotropic removal of water). Extracts were further dryed in an oven at 70 °C for 15 min and derivatized by incubating with 1.0 mL of 1:1 BSTFA/pyridine (v/v) using vigorous vortexing and incubating at 70 °C for 30 min.

RESULTS

Identification of Phenolic Compound Characteristic Ions. Individual phenol stock standards were prepared as described in Table 1 and further diluted to individual working standards of approximately 10 mg/L. Each was dried and derivatized following the procedure as described above. A 1 μL sample of each derivatized extract was injected separately on to the GC/MSD with the instrument on full-scan mode, from 50 to 800 amu. This allowed the establishment of the retention time and the characteristic TMS derivative spectrum of each phenolic compound. A target ion and two qualifying ions were chosen per compound on the basis of their abundance, reproducibility, freedom from interference, and specificity to the compound. The molecular ion (M^+) was preferred when found in appreciable abundance (Table 2). The phenolic compounds were divided into seven groups of ions (Table 3), each group containing the ions of one, two, or three compounds. The dwell time was set at 100 ms/ion.

Chromatographic Resolution. A composite dry standard of all substances tested, after derivatization and analysis, showed excellent resolution between all compounds of interest. Myricetin, rutin, and isoquercitrin displayed very poor sensitivity even at concentrations as high as 20 mg/L. A method blank showed very low background noise (5000 abundance units for phenolic acids and 2500 abundance units for the remaining segment).

Composite standards of all compounds at three concentration levels, spiked into red wine, extracted, and derivatized, displayed excellent resolution, sharp peaks, and good sensitivity. Examples of total ion chromatograms (TIC) of unadulterated Cabernet Sauvignon and Merlot wines are presented in Figure 1. Although some wine extracts showed minor interferences, the software can apply adequate corrections during intergration, which can also be done manually for sharper definition of the true peaks. The chromatogram baseline was very stable, and no column bleed was noticeable at any time even after >500 injections.

Detection of the compounds of interest was based on the retention time (±0.05 min), the presence of both qualifier ions, and the predetermined ratio between the target ion and each qualifier (±25% tolerance limit) (Table 2). A composite spiked extract (standard) was injected after every five samples. Fisetin was used as an internal standard and therefore spiked in all extracts and standards at a concentration of 1.00 mg/L. The response of fisetin was not used to correct results but rather to monitor unusual instrument fluctuation, most likely due to matrix and most often in compounds found in the epicatechin-to-quercetin window. Such samples were then re-injected. Compounds over-ranging the instrument or whose concentration fell outside the linearity range were diluted and analyzed against a standard with the same dilution factor.

Table 2. Selective Ion Monitoring of a Target and Two Qualifier Ions for Each Phenolic Compound

compound	RT (min)	target ion[a] (*m*/*z*)	qualifier ions (*m*/*z*)	
fisetin	17.90	471.00	399.0 (55)	559.8 (150)[b]
vanillic acid	8.29	297.35	253.0 (58)	312.4 (67)
gentisic acid	8.39	355.4	356.5 (87)	357.4 (40)
m-coumaric acid	8.87	249.0	293.0 (184)	308.0 (178)
p-coumaric acid	9.27	249.0	293.0 (184)	308.0 (178)
gallic acid	9.40	282.0	443.6 (36)	460.0 (55)
ferulic acid	10.07	338.4	323.4 (57)	293.3 (34)
caffeic acid	10.33	396.5	381.5 (25)	307.4 (12)
cis-resveratrol	11.83	444.7	445.6 (41)	446.7 (18)
trans-resveratrol	14.24	444.7	445.6 (41)	446.7 (18)
(−)-epicatechin	15.66	369.5	355.5 (105)	368.5 (233)
(+)-catechin	15.89	369.5	355.5 (87)	368.5 (300)
morin	16.47	648.0	649.0 (57)	560.0 (10)
quercetin	18.70	648.0	649.0 (61)	559.8 (14)
cis-polydatin	20.40	361.0	444.0 (107)	372.0 (59)
trans-polydatin	23.93	361.0	444.0 (66)	372.0 (43)

[a] Target ion was taken to be 100%. [b] Number in parentheses represents the target ion:qualifier ion ratio expressed in percent.

Table 3. GC/MSD Selective Ion Monitoring Parameters[a]

group	group start time (min)	ions in group (amu)
1	8.00	253.0, 297.4, 312.4, 355.4, 356.5, 357.4
2	8.70	293.0, 249.0, 308.0, 282.0, 443.6, 460.0
3	9.80	338.4, 323.4, 293.3, 396.5, 381.5, 307.4, 268.0
4	11.20	444.7, 445.6, 446.7
5	14.80	368.5, 355.5, 369.5
6	16.20	648.0, 649.0, 560.0, 471.0, 399.0
7	19.10	361.0, 444.0, 372.0

[a] Dwell time, 100 ms/ion.

Method Development. In developing this method, a number of variables were manipulated to improve chromatographic resolution, detection, recovery, precision, and analysis time.

Instrument Parameters. The injector and detector temperatures were based on previous experience with the analysis of resveratrol in wine and juices.[16] The GC oven temperature program was designed to elute all 15 phenolic compounds at a fast rate without jeopardizing resolution from interferences, giving sharp peaks, flat baseline, and good sensitivity. It was deemed necessary to introduce a 4-min baking period at the end of each run to ensure elimination of ghost peaks and low signal-to-noise ratio. A double

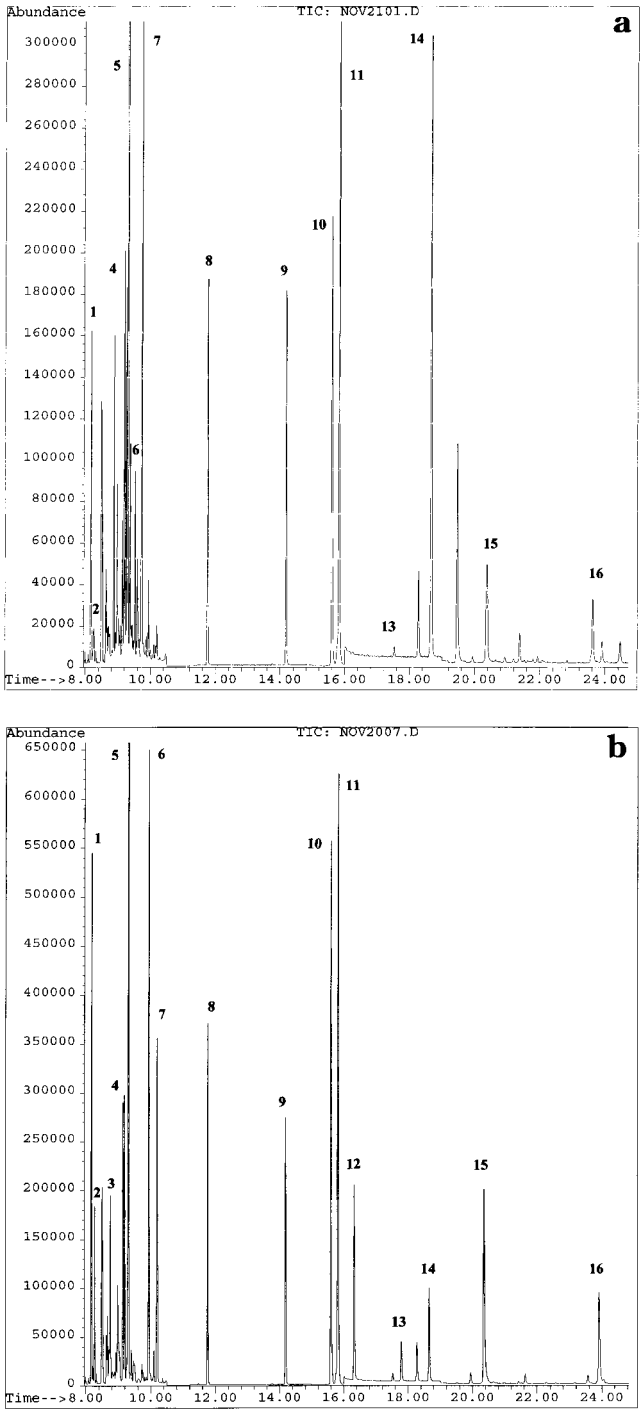

Figure 1. (a) TIC of a 1994 Cabernet Sauvignon wine extract in SIM identified by peak number and concentration (mg/L). Key: (1) vanillic acid (0.40); (2) gentisic acid (0.12); (4) *p*-coumaric acid (0.25); (5) gallic acid (0.21); (6) ferulic acid (0.10); (7) caffeic acid (0.44); (8) *cis*-resveratrol (0.45); (9) *trans*-resveratrol (0.37); (10) epicatechin (7.40); (11) catechin (12.1); (13) fisetin (1.00); (14) quercetin (5.35); (15) *cis*-polydatin (0.15); (16) *trans*-polydatin (0.22). Morin and *m*-coumaric acid were not detected in this wine. (b) TIC of a 1994 Merlot wine extract in SIM. Key: (1) vanillic acid (1.44); (2) gentisic acid (1.02); (3) *m*-coumaric acid (0.10); (4) *p*-coumaric acid (0.42); (5) gallic acid (0.51); (6) ferulic acid (0.60); (7) caffeic acid (0.52); (8) *cis*-resveratrol (0.94); (9) *trans*-resveratrol (0.55); (10) epicatechin (19.4); (11) catechin (24.6); (12) morin (1.90); (13) fisetin (100); (14) quercetin (1.75); (15) *cis*-polydatin (0.65); (16) *trans*-polydatin (0.69).

gooseneck glass insert, deactivated regularly, and a gold-plated injector seal increased sensitivity.

Extraction. Much of the initial work in developing this method was based on extracting 1 mL of wine sample using the Sep-Pak C_{18} (Waters Canada) and derivatizing with 1.0 mL of BSTFA. The very low recoveries of catechin and epicatechin, 27.2 and 45.8%, respectively, as well as the high relative standard deviation (RSD), up to 28.2%, was unacceptable. Furthermore, caffeic acid, gentisic acid, and quercetin displayed recoveries much greater than 100%, consistent with the notion of background or baseline interference.

In an attempt to improve the recoveries of catechin and epicatechin, we tested the Extrelut cartridge (diatomaceous earth) from BDH Chemicals (Etobicoke, ON, Canada), using the same amount of sample and BSTFA as previously. The same wine sample was extracted in triplicate each time with (i) 3 mL of ethyl acetate, (ii) 3 mL of 90% ethyl acetate/10% acetone (v/v), and (iii) 3 mL of methylene chloride. Most recoveries were much lower than with the Sep-Pak C_{18}, and some compounds were lost entirely. The thought that 12% (v/v) alcohol in wine may be retaining these phenolics before elution with ethyl acetate, thus explaining the low recoveries, triggered the idea of dealcoholizing wine prior to extraction. This entailed a lengthy evaporation on a rotary evaporator and a large volume of sample. In a comparison of the recoveries of dealcoholized red wine spikes using Sep-Paks C_{18} and C_8, the latter gave much better recoveries for quercetin, catechin, epicatechin, and *m*-coumaric acid. All other recoveries were very similar with both.

Use of the Sep-Pak C_8 offered the most promise and yielded exceptionally good recoveries, especially when the alcohol content of the wine was reduced by adding an equal volume of wter; this had the added advantages of reducing matrix interference, increasing recoveries, and decreasing the sample volume required to 0.5 mL.

Since some of these phenolics tend to polymerize once exposed to light, precautions were taken to avoid light exposure. Samples were kept in the dark, and the extraction apparatus was kept in a darkbox customized for this analysis.

Recovery and interference were not improved by acidification (pH 2.0), neutralization (pH 7.0), or dealcoholization of the wine prior to spiking. Use of a Sep-Pak C_1 was without benefit.

Derivatization. In an attempt to eliminate some interferences and improve recoveries, a comparison was made between different combinations of derivatizing agents: BSTFA only, BSTFA with 1% TMCS (v/v), and BSTFA/pyridine (1:1) by volume, using the Sep-Pak C_{18}. Some matrix interference was eliminated, and a significant increase in recoveries as well as a decrease in RSD was observed with the third.

Moisture is a major competitor of phenolic hydroxyl groups during derivatization with BSTFA/pyridine and can produce low recoveries. To avoid this problem, all glassware was washed in acetone during extraction; nitrogen passed through a moisture trap was introduced from the top of the Sep-Pak for the duration of the extraction; and methylene chloride was added to the dry extract and evaporated to dryness. The derivatized extracts were stable at 4 °C in the dark for at least 1 week.

Method Validation. *Linearity.* Data for each constituent were pooled from three experiments, in which the constituent was analyzed over a range of 6−9 concentrations in a mixture of all 15 dissolved in absolute alcohol, and added to a red wine matrix. The amplitude of the ranges varied from 0.23−1.86 (gentistic acid) to 0.84−33.40 mg/L (*trans*-resveratrol). Linear regression analyses were performed using the formula $y = mx + b$. The slope of

the calibration curve was almost perfectly linear for all compounds but catechin and epicatechin, and the square of the regression coefficient differed from unity by more than 0.020 only in the case of the former compounds (Supporting Information, Table SM-1).

Recovery. This was evaluated for each constituent by adding three concentrations (low, medium, high) to white wine and independently analyzing each wine six times. The overall recovery was obtained by pooling all data, i.e., $n = 18$. Excellent recovery was obtained which on average ranged from 90.7 ± 5.4% for *cis*-resveratrol to 104.6 ± 5.8% for caffeic acid. The exception was morin at 72.2 ± 9.7% (Supporting Information, Table SM-2).

Precision. Six replicate analyses of four red wines (different cultivars) with varying concentrations of each constituent were performed. *cis*-Resveratrol was not detected in one, morin in two, and *cis*- and *trans*-polydatin in two of the four samples. The overall mean RSD generally ranged from 4.0 (gentisic acid) to 10.3% (*trans*-resveratrol). Morin and quercetin were the exceptions with overall mean RSDs of 16.1 and 16.0% respectively (Supporting Information, Table SM-3).

Combined Variance for the Detector and Derivatization. The extracts of 12 red wine samples were pooled together. Ten 1-mL aliquots of the combined extract were derivatized independently and analyzed for all 15 phenolic constituents. The RSD ranged from 2.0 to 10.2% apart from the highest single value for quercetin at 15.9% (Supporting Information, Table SM-4).

Detection Limit. This was based on 3 SDs of the mean assay value of each phenolic compound analyzed at the lowest of the three concentration levels, satisfying both qualifier and target ions and the correct abundance ratio. These limits were as follows: vanillic acid, 0.063 mg/L; gentisic acid, 0.024 mg/L; *m*-coumaric acid, 0.051 mg/L; *p*-coumaric acid, 0.117 mg/L; gallic acid, 0.048 mg/L; ferrulic acid, 0.063 mg/L; caffeic acid, 0.111 mg/L; *cis*-resveratrol, 0.111 mg/L; *trans*-resveratrol, 0.084 mg/L; epicatechin, 0.324 mg/L; catechin, 0.336 mg/L; morin, 0.309 mg/L; quercetin, 0.843 mg/L; *cis*-polydatin, 0.015; *trans*-polydatin, 0.132 mg/L.

Day-to-Day Variation. To study the day-to-day variation of the method and the instrument, seven bottles of red wine picked from the same case were stored in the dark and analyzed freshly on seven separate occasions (Supporting Information, Table SM-5). The RSD for all polyphenols ranged from 4.7 (catechin) to 12.5% (*trans*-resveratrol). The values were not significantly different from the RSD data for the simultaneously analyzed replicates (Supporting Information, Table SM-6).

DISCUSSION

Up to the present, HPLC technology has been the most widely used analytical approach to assay phenolics either individually or in combination.[2−10,17] The most recently published paper in this context presented a method to measure eight polyphenols simultaneously in wine samples and described its analytical performance in extensive details.[10] Although GC/MS analysis has been used to measure resveratrol,[12,14,16] and some other polyphenols in wine,[19] the present method is the first fully developed with this instrumentation to permit simultaneous quantitative determination of a wide array of compounds including most of those that have been shown to possess significant biological properties. The details provided reveal certain key principles that guided the development of the method (such as the matrix dilution to reduce ethanol content rather than distillation; use of C_8 rather than the more popular C_{18} cartridge for solid-phase extraction; incorporation of pyridine in the derivatization reagent) and should be useful to other investigators who want to undertake similar applications with other compounds or matrixes. All analytical characteristics required for a thorough evaluation of the method have been provided for each constituent analyzed.

Few investigators have reported the use of MS methods to analyze the polyphenol content of other beverages and foodstuffs, a task for which HPLC has more usually been employed.[20−24] Recent exceptions include thermospray LS/MS analysis of polyphenols from tea,[25] a similar approach to screen for polyphenols in plant extracts,[20] and a pyrolysis GC/MS technique which has been proposed as applicable for analysis of wine polyphenolics but not yet validated.[19] We have used this present method to analyze the same polyphenols in extracts of solid vitaceous materials such as stems, leaves, skins, and pips after exhaustive pulverization and homogenization in ethanol and adjustment of the final concentration of the latter to 5% (v/v) prior to solid-phase separation. It should be equally suitable for analyzing these polyphenols, and potentially many others, in any plant or food material provided that extraction is complete and possible matrix interference by the solvents employed on the solid-phase separation and derivatization steps are excluded or circumvented. Furthermore, the excellent sensitivity and selectivity coupled with the small sample volume required (0.5 mL) for this assay render it useful for the analysis of biological fluids.

SUPPORTING INFORMATION AVAILABLE

Tables SM-1 to -6 of recoveries, precision of assay, and variance for 15 phenolic constituents of wine (6 pages). Ordering information is given on any current masthead page.

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