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Direct Injection Gas Chromatographic Mass Spectrometric Assay for *trans*-Resveratrol

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We have developed a novel method to measure the concentration of the trihydroxystilbene *trans*-resveratrol suitable for the analysis of wine and other biological materials. Solid-phase extraction is carried out on a reversed-phase disposable C-18 cartridge with elution of *trans*-resveratrol by ethyl acetate. From the first 1 mL of the eluate collected, 1 μL is injected directly into a gas chromatograph/mass spectrometer coupled through a DB-5 column. The molecular ion at a mass of 228 was quantitated by selective ion monitoring using as standard *trans*-resveratrol synthesized by a Wittig reaction. The method was linear up to 10 mg/L with a detection limit of 0.05 mg/L, which can be increased 10-fold if required. Recovery of added synthetic *trans*-resveratrol in wine ranged from 83 to 111% with a mean of 100%. Within-run precision was 5–7%. The method is faster and simpler than those previously published, which use organic-phase extraction, and yields higher values in wine presumably as a consequence of minimal loss during the solid-phase extraction. The *trans*-resveratrol concentration of wine is stable when protected from light for at least 6 weeks at 4 °C and for at least 1 week at room temperature.

trans-Resveratrol (3,5,4′-trihydroxystilbene) has been identified as a constituent of many plant species, including vitis vinifera.[1–4] It has potent antifungal properties and appears to be synthesized by vines in response to fungal infection.[5–9]

Biological interest in *trans*-resveratrol was stimulated by reports of its presence in Japanese herbal medications used for the treatment of fungal, inflammatory, and lipid disorders.[10] Subsequent experiments with purified *trans*-resveratrol demonstrated many biologically useful functions including modulation of hepatic cholesterol synthesis,[11] inhibition of lipoxygenase activity,[12] inhibition of anaphylactoid reactions,[13] and protection of lipoproteins against oxidative and free radical damage.[14] It has therefore been suggested that *trans*-resveratrol may be the active principles of red wines that have been shown in epidemiological,[15,16] clinical,[17] whole animal studies,[18] and in vitro studies[19] to confer protection against atherosclerosis and coronary heart disease.[20] For this reason, it may be important to know the resveratrol concentration in wines so that enological techniques to maximize its extraction during fermentation and further processing can be developed.

Currently, very few studies[20–23] have analyzed the *trans*-resveratrol content of wines and other biological materials. The present methods are cumbersome and time-consuming, require large sample volumes, and may have inherent errors due to incomplete recovery during the extraction procedures. We have therefore developed a novel assay for *trans*-resveratrol that may have wide application. It incorporates a solid-phase extraction followed by direct injection of the underivatized extract into a gas chromatograph/mass spectrometer (GC/MS) with the detector in the selective ion monitoring mode.

EXPERIMENTAL SECTION

Sample Preparation. Solid-phase extraction of *trans*-resveratrol was carried out on C-18 SPE bonded porous silica cartridges (500 mg, 3 mL volume) purchased from Supelco Inc. (Bellefonte, PA) and preconditioned as follows: 3 mL of

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(1) Ingham, J. L. *Phytochemistry* **1976**, *15*, 1791–1793.
(2) Langcake, P.; Pryce, R. J. *Phytochemistry* **1977**, *16*, 1193–1196.
(3) Creasy, L. L.; Coffee, M. *J. Am. Soc. Hortic. Sci.* **1988**, *113*, 230–234.
(4) Jeandet, P.; Bessis, R.; Gautheron, G. *Am. J. Enol. Vitic.* **1991**, *42*, 41–46.
(5) Langcake, P.; Pryce, R. J. *Physiol. Plant Pathol.* **1976**, *9*, 77–86.
(6) Langcake, P.; McCarthy, W. V. *Vitis* 1979, *18*, 244–253.
(7) Langcake, P. *Physiol. Plant Pathol.* **1981**, *18*, 213–226.
(8) Pool, R. M.; Creasy, L. L.; Frackelton, A. S. *Vitis* **1981**, *20*, 136–145.
(9) Hanawa, F.; Tahara, S.; Mizutani, J. *Phytochemistry* **1992**, *31*, 3005–3007.
(10) Nonomura, S.; Kanagawa, H.; Makimoto, A. *Yakugaku Zasshi* **1963**, *83*, 988–990.
(11) Arichi, H.; Kimura, Y.; Okuda, H.; Baba, K.; Kozawa, M.; Arichi, S. *Chem. Pharm. Bull.* **1982**, *30*, 1766–1770.
(12) Kimura, Y.; Okuda, H.; Arichi, S. *Biochim. Biophys. Acta* **1985**, *834*, 275–278.
(13) Ragazzi, E.; Froldi, G.; Fassina, G. *Pharmacol. Res. Commun.* **1988**, *20* (Suppl. 5), 79–82.
(14) Frankel, E. N.; Waterhouse, A. L.; Kinsella, J. E. *Lancet* **1993**, *341*, 1103–1104.
(15) St. Leger ,A. S.; Cochrane, A. L.; Moore, F. *Lancet* **1979**, *1*, 1017–1020.
(16) Renaud, S.; De Lorgeril, M. *Lancet* **1992**, *339*, 1523–1526.
(17) Seigneur, M.; Bonnet, J.; Dorian, B.; Benchimol, D.; Drouillet, F.; Gouverneur, G.; Larue, J.; Crockett, R.; Boisseau, M.-R.; Ribereau-Gayon, P.; Bricaud, H. *J. Appl. Cardiol.* **1990**, *5*, 215–222.
(18) Klurfeld, D. M.; Kritchevsky, D. *Exp. Mol. Pathol.* **1981**, *34*, 62–71.
(19) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. *Lancet* **1993**, *341*, 454–457.
(20) Siemann, E. H.; Creasy, L. L. *Am. J. Enol. Vitic.* **1992**, *43*, 49–52.
(21) Lamuela-Reventos, R. M.; Waterhouse, A. L. *J. Agric. Food Chem.* **1993**, *41*, 521–523.
(22) Jeandet, P.; Bessis, R.; Maume, B. F.; Sbaghi, M. *J. Wine Res.* **1993**, *4*, 79–85.
(23) Mattivi, F. *Z. Lebensm. Unters. Forsch.* **1993**, *196*, 522–525.

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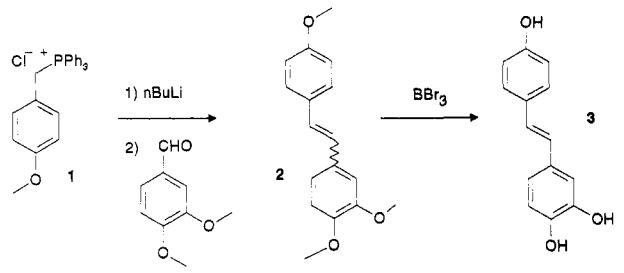

Figure 1. Overall chemical schema for the synthesis of *trans*-resveratrol with the following compounds enumerated as in the text: triphenylphosphine salt (1); trimethylresveratrol (2); *trans*-resveratrol (3).

ethyl acetate by gravity flow, followed by 3 mL of 96% (v/v) ethanol and twice with 3 mL of 10% (v/v) ethanol. A 1 mL sample was passed through the cartridge by gravity flow, and the cartridge was dried for 45 min under vacuum from a water aspirator. The absorbed resveratrol was eluted into a conical tube with 2 mL of ethyl acetate by gravity flow. The first 1 mL of eluent was collected and used for analysis. The cartridge can be reconditioned up to 10 times by washing with 60% (v/v) ethanol and storing in a container filled with 96% ethanol. No carryover was detected when reusing cartridges cleaned in this way immediately after extraction of wines with high resveratrol concentrations.

GC/MS Analysis. This was carried out using the Hewlett-Packard GC (Model 5890) with quadrupole MS detector (Model 5970) coupled through a DB-5 column (J&W Scientific, Folsom, CA), 30 m long, 0.25 mm internal diameter, and 0.25 μm film thickness. A 1 μL aliquot of the ethyl acetate eluent was introduced by an automatic sample injector (Hewlett-Packard Model 76733 GC/SFC). The temperature program was as follows: initial 150–160 °C held for 6 min; 20 °C/min to 290 °C, held for 2 min; 25 °C/min to 305 °C, held for 5 min. The total time per analysis for each eluate was 20.1 min. The molecular ion was detected and quantitated at a mass of 228, with qualifier ions at m/z 227 and 229.

Synthesis of Resveratrol as Primary Standard (Figure 1). *Principle.* *trans*-Resveratrol was synthesized by a Wittig reaction linking two appropriately substituted phenols through a styrene double bond utilizing the general concept proposed by Moreno-Manas and Pleixats[24] but with significant modifications. Methylated precursors were used to protect the OH groups. These were removed by boron tribromide with formation of only the trans isomer. This was most likely due to protonation of the product, which allows isomerization of the double bond to the thermodynamically favored trans isomer. All reagents were purchased from Aldrich (Milwaukee, WI); solvents were from Mallinckrodt (St. Louis, MO), and all reactions were carried out under nitrogen.

Procedure. *p*-Methoxybenzyl chloride (10 g, 63.9 mmol) and triphenylphosphine (18.4 g, 70.3 mmol) were added to dry benzene (150 mL). A few crystals of KI were added, and the solution was refluxed for 1 week. The solution was filtered, and the precipitate was collected and dried under vacuum to yield the triphenylphosphine salt (**1**), 22.4 g, 53.4 mmol, 83% yield, of which 7.3 g (17.4 mmol) was suspended in ~150 mL of anhydrous diethyl ether. *n*-Butyllithium (1 M in hexane) was added until a persistent orange color appeared; a further

(24) Moreno-Manas, M; Pleixats, P. *Anal. Quim.* **1985**, *81*, 157–161.

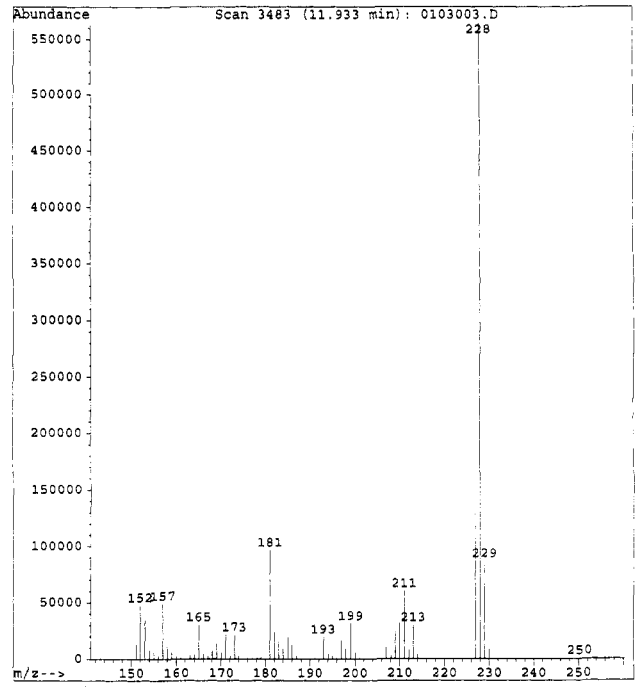

Figure 2. Mass ion spectrum of ethyl acetate solution of synthetic *trans*-resveratrol obtained by direct injection.

15 mL was added, followed by 3,4-dimethoxybenzaldehyde (2.9 g, 17.7 mmol). The solution was stirred for 12 h. Water was added and the volatile solvent was evaporated. The residue was dissolved in dichloromethane and the water separated after adding ~0.5 g of sodium dihydrogen phosphate. The aqueous layer was extracted once more, and the organic solution was dried over sodium sulfate. The solution was diluted with an equal volume of petroleum ether and filtered through a short silica gel column ("150 mL") which had been packed in 1:1 dichloromethane and petroleum ether. Evaporation yielded 3.83 g, 14.2 mmol, 82% yield of trimethylresveratrol (**2**).

Trimethylresveratrol (3.3 g, 13.6 mmol) was dissolved in 150 mL of dichloromethane on a dry-ice bath, and BBr_3 (10.2 g, 41 mmol) was added. The orange solution was poured onto ~500 mL of ice. When the ice had melted, stirring yielded a precipitate and two liquid phases, aqueous and organic. The three-phase mixture was extracted twice with ethyl acetate and once with chloroform, and the combined organic solutions were dried with sodium sulfate. This solution was evaporated in vacuo. Thin-layer chromatography showed one major spot at R_f 0.4 on silica in 9:1 (v/v) $CHCl_3$/MeOH. An off-white solid (2 g) appeared on evaporation. The solid was dissolved in the minimum volume of boiling ethanol, and chloroform was added to yield a precipitate. This was filtered to yield 1.2 g of white solid (5.26 mmol, 38% yield). The structure of *trans*-resveratrol (**3**) was confirmed by NMR spectroscopy[24] and by UV spectroscopy.[20]

RESULTS

Mass Spectrum of *trans*-Resveratrol. Figure 2 shows the mass ion spectrum of pure *trans*-resveratrol dissolved in ethyl acetate directly injected into the GC/MS. The largest signal was provided by the molecular ion (mass 228) with the next highest attributable to ion M – H (mass 227), which was

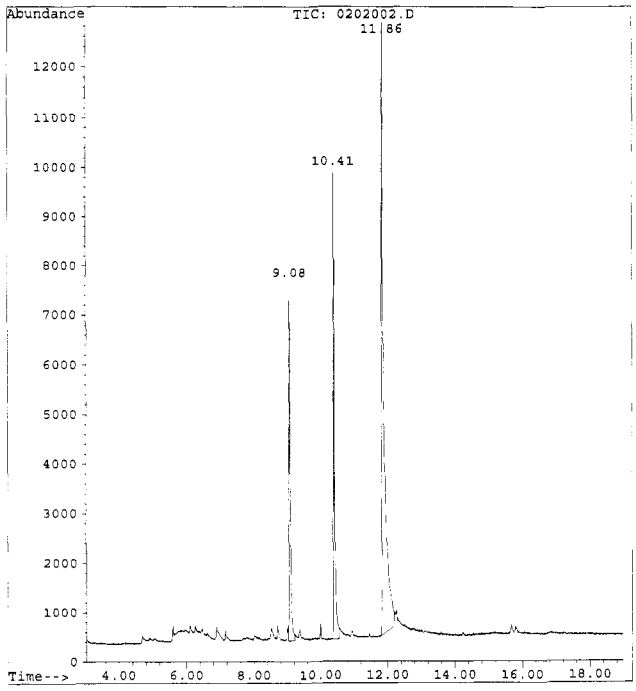

Figure 3. Total ion chromatogram of red wine obtained by adding the abundance of ions of masses 227, 228, and 229 of the ethyl acetate eluate from a C-18 SPE column directly injected into the gas chromatograph. The peak at 11.86 min is *trans*-resveratrol, corresponding to a concentration of 2.2 mg/L. The ion ratios of the other two peaks are not characteristic of *trans*-resveratrol.

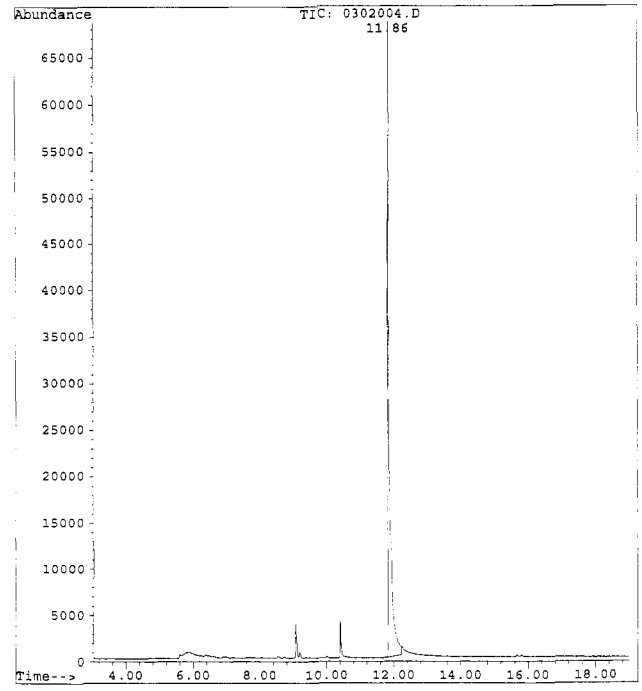

Figure 4. Total ion chromatogram of same wine as in Figure 3 (*trans*-resveratrol content, 2.2 mg/L) spiked with 10.5 mg/L purified *trans*-resveratrol, obtained by adding the abundance of ions of masses 227, 228, and 229. The peak at 11.86 min is *trans*-resveratrol.

therefore employed as a qualifier. For a second qualifier, we selected the ion at mass 229 rather than the slightly more abundant ion at mass 181 because more consistent ratios between the first three ions were obtained to characterize *trans*-resveratrol. The other ions displayed in Figure 2 provided signals too low to be practically useful. Figure 3 shows the total ion chromatogram for masses 227, 228, and 229 of an ethyl acetate eluate of red wine directly injected into the gas chromatograph; the relative abundance is based upon the peak area. Figure 4 shows the total ion chromatogram when the ethyl acetate eluate from the same wine was spiked with 10.5 mg/L purified *trans*-resveratrol. After several weeks use, increased baseline and some degradation of peak contours occur, but the original quality is restored by breaking off the first 50 cm of the column and reusing. This procedure may be repeated at lease one more time before a new column is required.

Calibration. Five standards of *trans*-resveratrol covering the range 0.1–10.0 mg/L were routinely made up in the ethyl acetate eluate of red wine from the C-18 SPE cartridge and analyzed in duplicate. Calibration curves constructed after subtracting the matrix signal due to the unspiked eluate consistently showed excellent linearity, with the correlation coefficient between *trans*-resveratrol concentration and ion abundance routinely in the range 0.987–0.998. Moreover, the paired standards rarely differed by >5%, in line with the good precision of the method (see below).

Recovery. This was determined for the overall assay by adding varying quantities of synthetic *trans*-resveratrol in the range 0.1–20.0 mg/L to a wine sample before passage through the C-18 SPE cartridge and comparing the signal on direct injection with that obtained when the same quantities were added to the ethyl acetate eluate of the same wine. The mean recovery over the range 0.1–10 mg/L was 100.5%. At higher concentrations, a modest reduction occurred to 87.5% at 15 mg/L and 86.4% at 20 mg/L. For this reason, we recommend that the sample be diluted and the assay repeated if an initial result of >10.0 mg/L is obtained. In our experience, this is a rare occurrence (<2% of wines assayed by us to date). The validity of dilution has been confirmed by analyses on samples containing 5–10 mg/L *trans*-resveratrol.

Detection Limit. Zero signal was obtained when pure ethyl acetate was injected six times. The detection limit was therefore determined by performing six complete replicate analyses of a wine low in *trans*-resveratrol. The standard deviation (SD) of the replicates, multiplied by 3, gave a value corresponding to 0.049 mg/L *trans*-resveratrol from the current calibration curve. We take 50 μg/L to be the lowest concentration significantly different from zero (P <0.01).

Precision. This was determined by performing 10 replicate analyses of each of three wines of varying *trans*-resveratrol content, with each of the replicates passed independently through the C-18 SPE cartridge prior to elution with ethyl acetate. The results in milligrams per liter as mean ± SD (CV%) were as follows: 0.64 ± 0.03 (5.3); 2.48 ± 0.14 (5.6); 4.56 ± 0.34 (7.4).

Scope of Method. More than 1000 wines have so far been analyzed. Very little *trans*-resveratrol (typically <0.1 mg/L) was found in white wines, whereas red wines had concentrations ranging from 0.1 to 12.0 mg/L) with lowest concentrations in wines from California, Australia, and Italy, and highest in wines from Oregon, Canada, and from various regions of France.[25] We have also applied this method to grape juices, jams, and jellies and have found low concentra-

(25) Goldberg, D. M.; et al. *Am. J. Enol. Vitic.*, in press.

Table 1. Concentrations of *trans*-Resveratrol in 30 Red Wines Stored in the Dark at Room Temperature and 30 Red Wines Stored at 4 °C on Initial Analysis and Again after 1 and 6 Weeks

	concn,[a] mg/L		
temp	initial	after 1 week	after 6 weeks
~20 °C	2.16 ± 0.61	2.18 ± 0.74	1.80 ± 0.59
4 °C	3.47 ± 0.87	3.56 ± 0.76	3.59 ± 0.91

[a] Mean ± SD.

tions of *trans*-resveratrol (<0.15 mg/L) in these materials. Preliminary studies show that the method is feasible for serum and urine, but problems with matrix effects and protein binding have still to be overcome.

Stability. Because *trans*-resveratrol is known to isomerize on exposure to UV light,[20] a number of wines, after the initial analysis, were stoppered in dark glass vials, covered with silver foil, and kept in the dark for up to 6 weeks at room temperature and at 4 °C (Table 1). No change in the means occurred at the latter temperature, or at room temperature for 7 days, and the differences for individual samples were random and within the variance of the method; however, the mean value declined to 82% of the initial value after 6 weeks at room temperature. The paired *t*-test showed that this decline, which occurred in 23 of the 30 samples, was statistically significant ($P < 0.01$). Ten red wines were also analyzed initially and then split into four aliquots: two were stored at 4 °C and two at room temperature in the dark for 48 h and then reanalyzed; at each temperature, one of the pair was left uncapped to test the influence of exposure to air (oxygen) upon the stability of *trans*-resveratrol. Only trivial and random differences not exceeding 8.0% occurred when the two temperatures and the aliquots exposed to and protected from air were compared.

DISCUSSION

The method herein described is rapid and sensitive and has good precision. The analysis is performed with only 1 mL of wine, and of the 1 mL of ethyl acetate eluate, only 1 μL is injected into the GC without prior derivitization. With this routine, we are able to quantitate *trans*-resveratrol to concentrations as low as 50 μg/L; for greater sensitivity, the eluate is reduced to dryness with reconstitution in 0.1 mL of ethyl acetate and manual injection of 1 μL, giving a lower limit of 5 μg/L for quantitation. The solid-phase extraction can be batched, and automatic sampling of the eluates can be employed with an assay time of 20.1 min for each eluate.

Of those methods available for the measurement of *trans*-resveratrol to date, most have employed organic-phase extraction followed by high-performance liquid chromatography (HPLC). Because of the multiple steps employed to obtain an extract sufficiently enriched in *trans*-resveratrol while being low in interfering compounds, large initial sample volumes have typically been used. In the method described by Siemann and Creasy, 100 mL of wine is required, the extraction procedure involves eight independent manipulations followed by two independent HPLC runs separated by a period of UV-light exposure to convert *trans*-resveratrol to the cis isomer.[20] With this method, the highest *trans*-resveratrol concentration of any of the wines tested by these authors was 0.65 mg/L. The method of Lamuela-Raventos and Waterhouse[21] starts with an initial volume of 500 mL of wine and requires 11 individual steps involving extraction, evaporation, washing, and filtration before the sample is ready for HPLC. The highest value reported with this method was 0.15 mg/L. Jeandet et al.[22] started with 100 mL of wine, and their method requires eight individual steps prior to silylation with bis-(trimethylsilyl trifluoroacetamide) (BSTFA); separation and quantitation of resveratrol by GC is then performed. The highest value they reported with this method was 2 mg/L.

The method of Mattivi[23] requires 50 mL of wine. As in our method, a solid-phase extraction on a C-18 cartridge was performed, but this was followed by HPLC analysis. Concentrations spanning a range from 1.20 to 7.17 mg/L were present in red wines analyzed by this investigator. These values are in the range that we have found in a global survey of wines from many regions and countries.[25] It is worth noting that, apart from a solid-phase extraction (as with our method), Mattivi used an internal standard (*trans*-4-hydroxystilbene), and it is probable that his method was less susceptible to losses (which were in any case allowed for) than the previously published assays.

An interesting method was described by Hain et al.[26] This involved an enzyme-linked immunosorbent assay (ELISA) using an antibody raised in rabbits and conjugated with horseradish peroxidase. A test sensitivity of 10 ng/mL was reported. However, the method was used in plant extracts, which are much less complex than wines, and the reagents are not commercially available. It is to be expected that raising antibodies "in-house" would lead to variability between laboratories, since these antisera would recognize different epitopes. The GC/MS method presented in this paper should have a specificity at least equal to that of immunoassay and can be performed without derivitization by any laboratory with this readily available instrumentation. It is superior in terms of sensitivity, accuracy, and simplicity to all previously published methods.

Both linearity and recovery were excellent up to concentrations of 10.0 mg/L, the latter averaging 100% over this range, but beyond this value it is advisable to dilute the sample and reassay, although this is a rare requirement for analyses on wines. Calibration is accomplished by diluting the synthetic standards into an ethyl acetate eluate of red wine followed by direct automatic sample injection of 1 μL into the GC/MS. Because of the excellent recovery routinely obtained when wines are spiked prior to solid-phase separation on the C-18 cartridge, we consider this calibration technique to be appropriate, and it allows the calibration curve to be prepared while the samples to be analyzed are going through this chromatographic procedure. The time to dry the column (45 min) is not a disadvantage since this is a batch procedure (up to 20 samples at a time are handled) and the limiting time factor is the 20 min required to complete the GC/MS analysis. The good recovery, combined with the unique specificity of mass ion detection and quantitation based upon the characteristic ratios between the molecular ion and two qualifier ions, renders use of an internal standard or further correction for recovery in individual samples or calibrants redundant.

(26) Hain, R.; Bieseler, B.; Kindl, H.; Schroder, G.; Stocker, R. *Plant. Mol. Biol.* **1990**, *15*, 325–335.

Our stability experiments have shown that, once opened, wines that are capped and protected from light are stable for at least 1 week at room temperature and at least to 6 weeks at 4 °C. Under these conditions, exposure to air up to 48 h has no deleterious effect. These conditions meet the working requirements of most analytical laboratories. This new method should have the ability to determine *trans*-resveratrol concentrations in body fluids as well as in wine, with the potential of clarifying its biological effects in animal and human experiments.

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