A Derivatized Gas Chromatographic-Mass Spectrometric Method for the Analysis of Both Isomers of Resveratrol in Juice and Wine

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A gas chromatographic-mass spectrometric method has been developed and validated for the analysis of *cis*- and *trans*-resveratrol simultaneously in matrices such as wine and grape juice. Solid phase extraction of resveratrol isomers on a C-18 column was followed by derivatization with bis-[trimethylsilyl]-trifluoroacetamide under optimized conditions followed by gas-liquid chromatography of an aliquot (1 µL) on a DB-5HT column. Selective ion monitoring was performed at ion mass 444 for quantification and using ions at mass 445 and 446 as qualifiers. Unlike other methods previously reported, this method utilizes only 1 mL of sample, has an instrument analysis time of 16 minutes, is simple, and has a detection limit as low as 10 µg/L. The utilization of the Mass Selective Detector makes it a very specific method. A survey of Ontario wines indicated that the red wines have higher concentrations of *trans*-resveratrol than those reported by previous investigators for wines of other regions. In about half, even higher concentrations of *cis*-resveratrol were measured. Since the latter was not detected in grape skins or juices, it appears to be formed from the isomerization of *trans*-resveratrol or break-down of resveratrol polymers (viniferins) during skin fermentation.

KEY WORDS: phytoalexin, gas chromatography, mass spectrometry, bis-[trimethylsilyl]-trifluoroacetamide, Cabernet Sauvignon, Pinot noir

Resveratrol (3,5,4'-trihydroxystilbene) is a phytoalexin found in various parts of the vine, including the grape skin, as well as in some other plant species (3,12) in which it appears to confer resistance to fungal infection. It is synthesized by a unique enzyme using as substrates malonyl CoA and coumaroyl CoA which also serve as substrates for a related enzyme, chalcone synthase (29,35). In vines, its synthesis is initiated in response to stress, especially interaction of the plant with pathogens such as *Botrytis cinerea* and *Plasmospora viticola* (3,14,24,27), by a regulatory mechanism which under these conditions favors resveratrol synthase at the expense of chalcone synthase. Resveratrol has been identified as one of the active ingredients of Japanese and Chinese folk medicines used for treating hyperlipidemia, arteriosclerosis, allergies, inflammations and other diseases (1,16,17,22,30).

It is widely accepted that moderate consumption of alcoholic beverages reduces the incidence of atherosclerosis and death from myocardial infarction (11,19,20,34). Wine seems to be the most potent cardio-protective beverage (18), and some epidemiological (33,38) and human experimental evidence (36) exists to support this concept. Moreover, Klurfeld *et al.* (21) have shown that red wine reduced the incidence of coronary artery atherosclerosis in rabbits that were fed a high cholesterol diet more effectively than any other alcoholic beverage tested. Frankel *et al.* (6) described *in vitro* studies demonstrating that red wine phenolics devoid of alcohol have potent antioxidant properties and greatly inhibit the production of oxidized human low-density lipoproteins (LDL) which appear to be more atherogenic than native LDL (5,39,40). It has been proposed that these antioxidant properties are, at least in part, contributed by the presence of resveratrol, and some preliminary evidence to support this notion has been presented (7).

Although resveratrol exists as *cis* and *trans* isomers, all the available information is restricted to *trans*-resveratrol. It is this form that has been used exclusively in biological studies aimed at elucidating its potential action upon lipids and eicosanoids in whole animals and cell culture systems (1,7,16,17,32). Demonstration of the presence of resveratrol in grapes and its role in resistance to fungal infection has likewise been restricted to the *trans* isomer.

Interest in resveratrol has led to the development of a variety of analytical approaches to its measurement. Previous methods published by Siemann and Creasy (37) and by Lamuela-Raventos and Waterhouse (23) appear to have high sensitivity, with detection limits of 1 ng/mL and 50 ng/mL, respectively, but utilize time-consuming procedures based upon multiple solvent extractions and one or more high performance liquid chromatography (HPLC) steps, as well as large volumes of sample. A gas chromatographic (GC) method developed by Jeandet *et al.* (13) utilizes a derivatization step with bis-[trimethylsilyl]-trifluoroacetamide (BSTFA)

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and provides high sensitivity. This method also requires time-consuming procedures based upon multiple solvent extractions. A radioimmunoassay developed by Hain *et al.* (10) tested only on tobacco has high sensitivity (detection limit = 10 ng/g), but the antibody used is raised in-house and it is not commercially available. Furthermore, only the method described by Jeandet *et al.* (13) assays for *trans*- and *cis*-resveratrol simultaneously.

In light of the potential importance of resveratrol and the desirability of improving the existing methods, we have developed an assay suitable for the analysis of *trans*- and *cis*-resveratrol in wine with very high sensitivity, which is inexpensive, quick, specific, selective and which utilizes minimal amounts of sample. We also report our initial experience of applying this assay to the analysis of resveratrol in Ontario wines, grape juices and grape skins with unexpected and potentially important results.

Materials and Methods

The *trans*-resveratrol standard used has a purity of 99% and was obtained from the Sigma Chemical Company (St. Louis, MO, USA). The standard was in the solid form, desiccated and stored in the dark at 4°C. The *cis*-resveratrol standard was prepared by UV-irradiation of *trans*-resveratrol under conditions that achieved >90% conversion of the latter to the former (see below).

All stock and working standards added to ethyl acetate eluates of wine were made in 100% ethyl acetate. Spiking standards added to raw wine were dissolved in absolute ethanol purchased from Reider Distillery (Grimsby, Ont., Canada). Other solvents used were purchased from Caledon Laboratories (Georgetown, Ont., Canada). BSTFA was purchased from Regis Chemical Company (Morton Grove, IL, USA) and was dissolved in pyridine ($d^{23}/_4 = 0.97$ g/mL).

All wine samples analyzed were from the South Eastern Ontario 1992 vintage. All red wines were skin-fermented and had undergone various clarification treatments.

Extraction and derivatization: One mL of wine was passed through a 3-mL Supelclean, LC-18 solid phase extraction cartridge (Supelco, Inc., Bellefonte, PA, USA) which was pre-conditioned with ethyl acetate, ethanol and 10% (v/v) ethanol. The SPE cartridge was dried for *ca* 45 minutes by maintaining a continuous gentle flow of nitrogen gas from the top and applying vacuum suction from the bottom. Resveratrol was eluted with 2 mL of ethyl acetate into a graduated centrifuge tube and the eluate was evaporated to dryness on an Organomation Nitrogen evaporator purchased from Meyer Organomation Assoc. Inc. (S. Berlin, MA, USA). The extract was further dried in an oven at 60°C for 30 minutes. The dried extract was then allowed to react with 1 mL of BSTFA in an incubator at 70°C for 60 minutes. SPE C-18 cartridges were re-conditioned with 60% (v/v) ethanol, stored in absolute ethanol and re-used up to three times before disposal.

Gas chromatographic/mass selective detector analysis: All equipment was purchased from Hewlett Packard (Canada), Ltd. (Mississauga, Ont., Canada). A HP 5890 GC equipped with an HP5971 Mass Selective Detector (MSD), coupled to an HP-G1034C MS-Chem Station was used for the analysis. Sample delivery was performed by an HP7673A autoinjector. Derivatized extract (1 µL) was injected on to a splitless injector heated at 280°C. The transfer line temperature was maintained at 300°C. The GC oven program was initially set at 110°C and was increased at a rate of 30°C/min to 250°C and immediately increased again at a rate of 5°C/min to 280°C with a maximum baking temperature at 300°C for two minutes. The column used finally was a DB-5HT (5% phenyl, 95% methyl) purchased from J & W Scientific (Folsom, CA, USA) 30 m long, 0.25 mm i.d. and 0.10 µm film thickness. However, our initial work was performed using a DB-5 column 30 m long, 0.25 i.d., and 0.25 µm film thickness purchased from the same source and which resulted in slower elution of the resveratrol isomers. Data were acquired on the Selective Ion Monitoring (SIM) mode using ion 444 for quantitation and ions 445 and 446 as qualifiers.

Results and Discussion

The mass spectrum obtained from the GC/MSD analysis of the trimethylsilyl derivative of pure *trans*-resveratrol, acquired on the scan mode, revealed a number of fragments suitable to quantitate and qualify the presence of this compound (Fig. 1). The predominant molecular ion was at m/z = 444 (relative abundance, 100%). Other abundant ions were m/z = 429, 443, 445, 446, and 447.

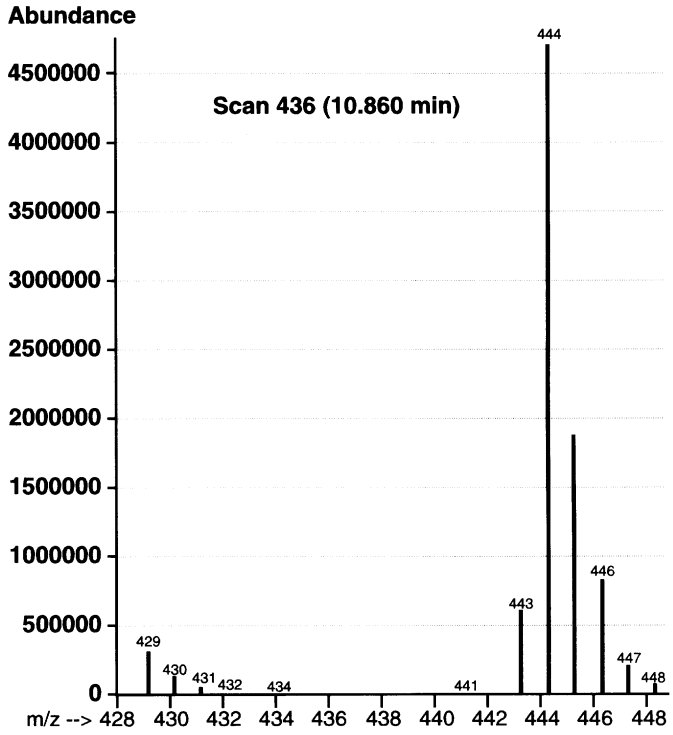

Fig. 1. Full scan mass spectrum of the trimethylsilyl derivative of *trans*-resveratrol. The molecular ion at m/z = 444 amu is the major peak. *Cis*-resveratrol exhibits the same mass spectrum and abundance ratio.

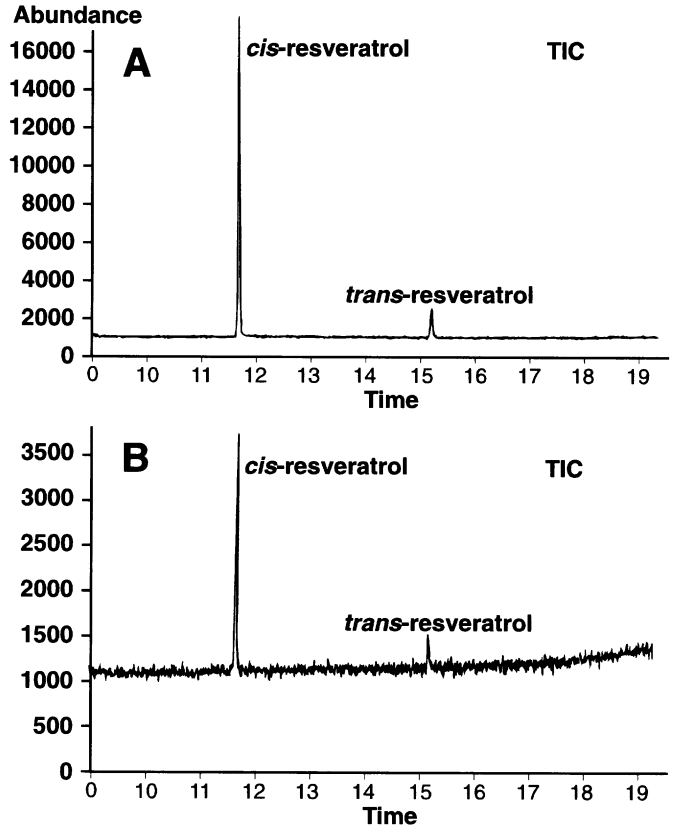

Fig. 2. (**A**) Total ion chromatogram of a 525 µg/L *trans*-resveratrol standard after 24-hour exposure to sunlight before derivatization; (**B**) Total ion chromatogram of the same standard derivatized after 96-hour exposure to sunlight. A DB-5 column was used for separation.

To detect and quantitate the trimethylsilyl derivatives of both *cis*- and *trans*-resveratrol when present in the same fluid, a pure standard of *trans*-resveratrol in ethyl acetate at a concentration of 525 µg/L was exposed to daylight for 12 hours, 24 hours and 96 hours prior to derivatization. The *cis*-resveratrol derivative appeared as a peak approximately three minutes earlier than that due to the *trans*-resveratrol derivative (using the DB-5 column) and with ions at m/z = 444,445 and 446 at relative abundances identical to those found in the *trans*-resveratrol peak (Fig. 1). After 24 hours of exposure, the conversion of *trans*-resveratrol to the *cis*-isomer was 90% to 95% complete (Fig. 2A). After 96 hours, the concentrations of both isomers were significantly reduced (Fig. 2B). Ultraviolet light is known to stimulate synthesis of *trans*-resveratrol in vines, as well as its conversion to molecular aggregates, the viniferins (3,4,8,9,12,15,25,27). Oxidative dimerization of related monohydroxy-stilbenes has also been demonstrated (2,26). On the other hand, UV-irradiation of *trans*-resveratrol in solution leads to its isomerization to *cis*-resveratrol (37), although the stoichiometry of this reaction has not previously been determined. Further studies with various other concentrations of *trans*-resveratrol revealed that after 24-hour irradiation the conversion to *cis*-resveratrol was consistently 90% to 95% complete, and the response as area counts was equivalent to that of *trans*-resveratrol at the same molar concentration.

Data were acquired with the SIM mode using the molecular ion m/z = 444 (relative abundance, 100%) as the target ion and ions m/z = 445 (relative abundance, 44%) and m/z = 446 (relative abundance, 19%) as the qualifying ions.

Preliminary analysis performed on wine, using this method, exhibited three major peaks. Two of these were peaks corresponding to *cis* and *trans*-resveratrol; they were consistently different in retention time by 2.34 minutes (Fig. 3A) and gave identical spectra with the same molecular ions and characteristic abundance ratios (Fig. 3B and 3C). The molecular ion m/z = 444 was

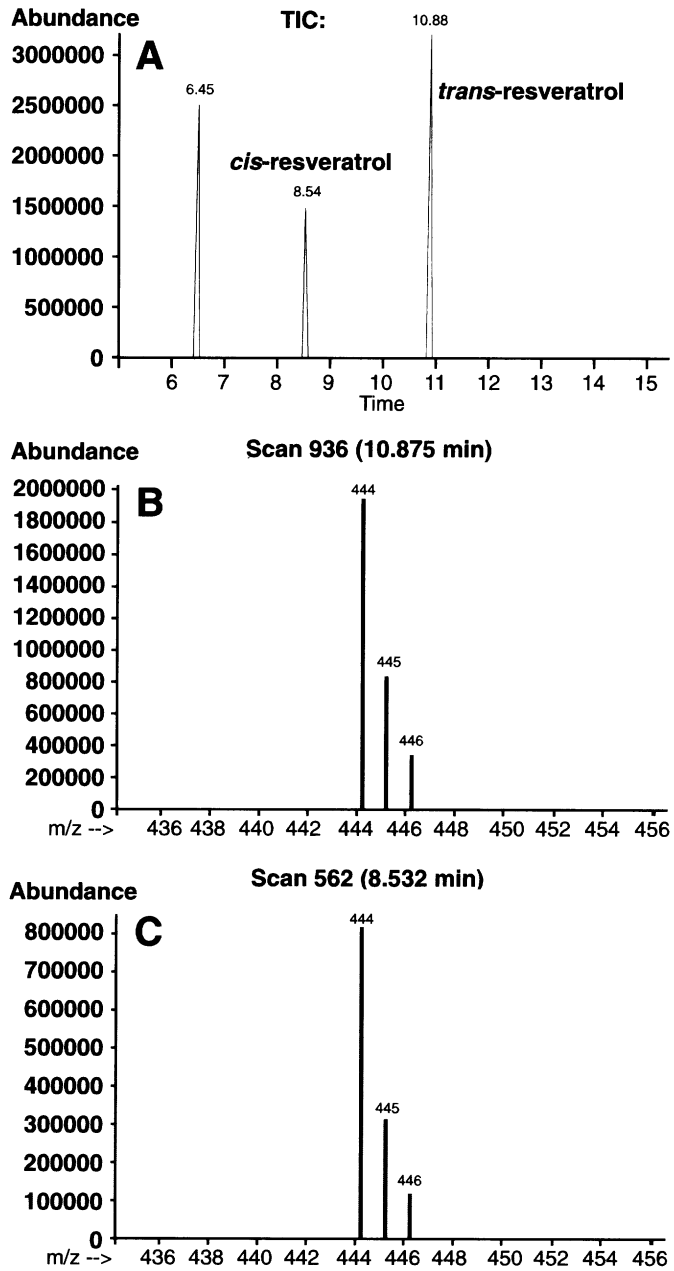

Fig. 3. (**A**) Total ion chromatogram of a Cabernet Sauvignon wine spiked with 2.0 mg/L of *trans*-resveratrol; (**B**) mass spectrum of *the trimethylsilyl derivative of trans*-resveratrol acquired by the SIM mode; (**C**) mass spectrum of the trimethylsilyl derivative of *cis*-resveratrol acquired by the SIM mode. Separation was carried out using a DB-5 HT column.

Table 1. Red wine was spiked with *trans*-resveratrol at 2mg/L and analyzed for *cis*- and *trans*-resveratrol after drying at time intervals of 0, 30, 60, and 90 minutes. Derivatization incubation period remained constant at 60 minutes.

Drying time (min)	*cis*-resveratrol (area counts)	SD (%)	*trans*-resveratrol (area counts)	SD (%)
0	18.8×10^6	8.4	50.2×10^6	11.0
30	18.2×10^6	3.0	50.1×10^6	5.4
60	18.5×10^6	5.5	50.2×10^6	6.0
90	18.4×10^6	7.6	49.7×10^6	7.6

* Mean of 6 samples at each level.

in both cases the major ion with relative abundance of 100%. The third peak, which elutes first (at 6.45 min, Fig. 3A), gave a different spectrum (not shown).

The incubation temperature and time for derivatization were set at 70°C after investigating a range of temperatures at 60°C, 70°C, and 80°C. A similar procedure was employed to set the incubation time at 60 minutes (data not shown). No significant difference was observed in the *trans*- and *cis*-resveratrol concentrations after drying the extract, before derivatization, for 60 or 90 min at a range of temperatures (*i.e.*, 50, 60, and 70°C). Due to the possibility of water competing with the hydroxyl groups of resveratrol for BSTFA, a drying step of 30 minutes at 60°C before derivatization was incorporated into the method, thus improving reproducibility (Table 1).

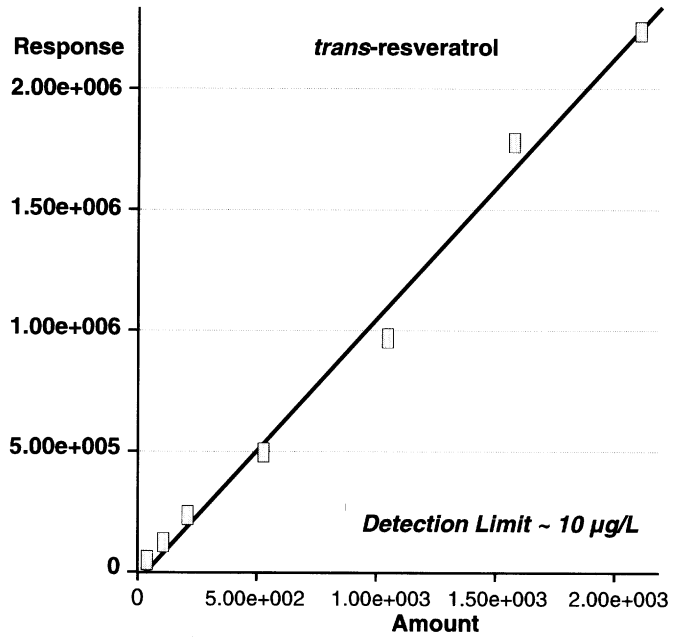

Fig. 4. GC-MSD calibration curve of synthetic *trans*-resveratrol analyzed by SIM at m/z = 444 of concentrations in the range of 52 to 2600 µg/L added to ethyl acetate eluate of unspiked wine, subtracting the wine matrix signal.

The linearity of the method for *trans*-resveratrol was tested up to 2600 µg/L and was found to be satisfactory (Fig. 4). The detection limit of approximately 10 µg/L (the lowest concentration that could be distinguished from zero at $p < 0.01$) was determined by analyzing 10 replicates of a wine sample having a mean *trans*-resveratrol concentration of 52 µg/L and multiplying the SD of the replicates by 3. *Cis*-resveratrol also demonstrated a linear response similar to that of the *trans* isomer (data not shown).

To establish the recovery of the method, white and red wines were spiked at three known levels of *trans*-resveratrol (210, 525, and 1050 µg/L) in ethanol, extracted and analyzed several times. Mean recoveries were in the range of 91% to 98% with a SD < 10% in all but one case.

For quantitation of wine samples, a calibration curve was prepared with known standards of synthetic *trans*-resveratrol in ethyl acetate at several concentrations ranging from 52 to 2600 µg/L added to an ethyl acetate eluate of wine low in resveratrol prior to derivatization and analysis, subtracting the signal due to the wine matrix. The possibility of matrix effects upon the standards was also studied by spiking the ethyl acetate eluates of white wines, red wines, and simulated wine (10% v/v alcohol, 5g/L glucose, and water) prior to derivatization and analysis and using them to prepare calibration curves. No significant differences were observed in the *cis*- and *trans*-resveratrol concentrations of 32 wine samples assayed independently using each of the three sets of different matrix-based calibrants (Table 2).

To determine the variance due to the instrument and detector, the same derivatized extract was injected 10 times in sequence. Coefficients of variation (CV) of 4.8% and 8.5% were obtained for *cis*- and *trans*-resveratrol at mean concentrations of 1.93 and 1.42 mg/L, respectively.

To determine the combined variance for the detector and derivatization, the ethyl acetate extracts of 12 samples were pooled and ten 1-mL aliquots of the combined extract were derivatized independently and analyzed. CVs of 8.4% and 13.1% were obtained for *cis*- and *trans*-resvera-

Table 2. Thirty-two red wine samples were analyzed for *cis*- and *trans*-resveratrol using white wine, red wine, and simulated wine standards for calibration. The three sets of assays were carried out at two-day intervals, with samples stored at 4°C in the dark.

	White wine standard (mg/L)			Red wine standard (mg/L)			Simulated wine standard (mg/L)		
	cis-	*trans*-	Total	*cis*-	*trans*-	Total	*cis*-	*trans*-	Total
Mean	1.64	1.90	3.54	1.79	2.17	3.96	1.91	2.32	4.22
SD	1.04	1.11	2.03	1.11	1.30	2.31	1.19	1.39	2.48
Range	0.12-3.96	0.30-4.38	0.42-8.30	0.11-4.26	0.28-5.87	0.39-9.06	0.13-4.72	0.32-6.23	0.45-10.0

Table 3. Imprecision of the complete analysis of *cis*- and *trans*-resveratrol measured as the Coefficient of Variation (CV) at three different concentrations. Each wine was passed independently through a C-18 cartridge on 10 independent occasions and the ethyl acetate eluate was derivatized and injected into a GC/MSD.

	Mean	SD	CV (%)
		[a]*cis*-Resveratrol	
Wine 1	0.44	0.01	3.3
Wine 2	2.33	0.06	2.6
Wine 3	4.60	0.21	4.5
		[a]*trans*-resveratrol	
Wine 1	0.56	0.05	8.5
Wine 2	1.49	0.09	6.3
Wine 3	4.31	0.42	9.9

[a] All data in mg/L

trol at mean concentrations of 1.60 and 2.07 mg/L, respectively.

The within-day variance of the complete assay (imprecision) was determined by analyzing 10 replicate samples of each of three wines of resveratrol concentrations differing over a 10-fold range. Each replicate was extracted by passing 1 mL through the C-18 cartridge and was derivatized independently. The imprecision of the *cis*-isomer analysis was less than half that of the *trans*-isomer at all concentrations (Table 3). The between-day variance was measured by analyzing the same sample weekly over a period of seven weeks during which it was held at 4°C in the dark. The CVs for *cis*- and *trans*-resveratrol were 11.3% and 9.5% at concentrations of 1.79 and 2.37 mg/L, respectively, and both isomers were perfectly stable over this period. The derivatized extracts of *cis*- and *trans*-resveratrol were also stable at 4°C over a period of at least six weeks when held under these conditions prior to injection into the GC/MSD.

This method was then utilized for the analysis of several South Eastern Ontario wines from the 1992 vintage. The *cis*- and *trans*-resveratrol concentrations of each wine are presented in Table 4. All of the red wines analyzed contained both isomers of resveratrol, and in half, the concentration of *cis*-resveratrol was greater than that of *trans*-resveratrol (11 of 21). Concentrations ranging from 30 to 4590 µg/L of *trans*-

Table 4. A survey of *cis*- and *trans*-resveratrol in Ontario wines from the 1992 vintage.

Varietal	*cis*-Resveratrol (mg/L)	*trans*-Resveratrol (mg/L)
Cabernet Sauvignon	0.90	0.54
Cabernet Sauvignon	1.19	0.75
Cabernet Sauvignon	4.62	4.59
Pinot noir	1.35	0.54
Pinot noir	0.56	0.74
Pinot noir	0.73	1.12
Pinot noir	1.43	1.00
Cabernet franc	0.57	0.91
Cabernet franc	0.52	0.68
Cabernet franc	0.64	0.34
Cabernet franc	0.73	1.03
Gamay noir	0.30	0.82
Gamay noir	0.68	0.72
Gamay noir	0.77	1.56
Gamay noir	2.23	1.74
Merlot	0.94	1.15
Merlot	0.75	0.38
Marechal Foch	1.22	1.04
DeChaunac	0.10	0.04
Baco noir	0.90	0.03
Concord	0.03	0.19
Chardonnay	0.06	<0.01
Muscat (exp.)	<0.01	<0.01
Seyval blanc	0.04	0.02
Vidal	0.03	0.11

Table 5. A survey of *trans*-resveratrol in Ontario juices from the 1992 vintage.

Varietal	No. of samples	*trans*-Resveratrol[c]
Juices[a]		***Mean, µg/L***
Aurore	6	12
Muscat	6	3
Vidal	6	4
Seyval blanc	6	3
Cabernet Sauvignon	6	9
Chambourcin	6	13
Cabernet franc	6	6
Gamay	6	6
Merlot	6	9
Riesling	6	15
Skins[b]		***Mean, µg/g***
Baco noir (ON)	2	0.32
Baco noir (BC)	1	1.37
GR-F	1	0.36
Merlot	1	5.64
Marechal Foch	4	2.16
DeChaunac	4	0.77
Concord	3	0.93
Cabernet franc	2	3.54
Cabernet Sauvignon	1	2.75
Seyval blanc	6	1.84
Elvira	1	1.31
Niagara	4	<0.3
Chardonnay	5	1.62
J. Riesling	3	1.98
Vidal (ON)	5	1.20
Vidal (BC)	2	2.64

[a]Juices were obtained by lightly crushing the grapes and filtering through Whatman No. 1 paper prior to analysis.

[b]After extensive crushing to remove juice, skins were dried, weighed and macerated exhaustively with 30 volumes of 80% methanol followed by filtration through Whatman No. 1 paper prior to analysis.

[c]Many of these samples contained < 10 µg/L (detection limit) and were consequently assigned a value of zero.

resveratrol and 30 to 4620 μg/L of *cis*-resveratrol were found in red wines. All white wines were low in both isomers. Since our laboratory has taken all necessary precautions to avoid exposure of the samples to light and oxygen, we can exclude the possibility of partial UV-induced isomerization of *trans*- to *cis*-resveratrol during analysis. These requirements have recently been emphasized (31).

It is interesting to note from Table 4 that one Cabernet Sauvignon wine had by far the highest concentrations of *cis*- and *trans*-resveratrol. Generally, the *trans*-resveratrol concentrations in Ontario red wines were higher than those reported by Siemann and Creasy (37) and by Lamuela-Raventos and Waterhouse (23) for American wines as well as those of the Burgundy wines reported by Jeandet *et al.* (13). Higher *trans*-resveratrol concentrations were found in Italian red wines by Mattivi (28) who used a solid phase extraction technique followed by HPLC, but he did not measure the *cis*-resveratrol concentrations in these wines.

A number of South Eastern Ontario pressed grape juices were also analyzed for *trans*-resveratrol (Table 5). The very low concentrations found in juices are consistent with previous studies showing that *trans*-resveratrol is present in the skins of the grapes and is very low or absent from the berry flesh (3,12). Only one previous report describes the presence of *cis*-resveratrol in wine: Jeandet *et al.* (13) found higher concentrations of the *cis* than of the *trans* isomer in red Burgundy wines (derived from Pinot noir). It has up to now never been measured in grapes or grape juices. We were unable to detect its presence in the grape juices listed in Table 5 or in the skins after extensive maceration and extraction. *Cis*-resveratrol therefore does not seem to occur naturally in grapes. A similar observation has been made by Jeandet *et al.* (personal communication). Its presence in wine may occur as a consequence of fermentation due to a yeast enzyme (*e.g.,* isomerase) which converts *trans* to *cis*-resveratrol, or to the breakdown of viniferins (polymers of resveratrol) to the free monomer. Alternatively, similar non-enzymatic reactions may occur due to the effects of light and/or oxygen, although fermentation is carried out under conditions to minimize these influences. We are actively investigating the origin of *cis*-resveratrol in wines as well as the intriguing question as to its biological activity in comparison with that of *trans*-resveratrol.

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