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Liquid Chromatography−Electrospray Tandem Mass Spectrometry of *cis*-Resveratrol and *trans*-Resveratrol: Development, Validation, and Application of the Method to Red Wine, Grape, and Winemaking Byproducts

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The application of liquid chromatography−electrospray tandem mass spectrometry (LC-ESI-MS/MS) was investigated for the analysis of *trans*-resveratrol in red wine, grape skin, grape pomace, and winemaking byproducts. Chromatographic elution performed under isocratic reversed-phase conditions using a C18 narrow-bore LC column allowed retention times lower than 12 min to be obtained. Qualitative analysis was performed in negative-ion (NI) full-scan and product-ion scan acquisition modes, whereas method validation in terms of linearity, detection limits, accuracy, and robustness was carried out under NI selected reaction monitoring conditions. The matrix-matched detection limit was calculated in the low parts per billion range (10 μg/L). Results of the application of the method to red wine, grape, and winemaking byproduct samples were compared with those obtained using an LC-UV/DAD technique. Determination of *trans*-resveratrol in the samples investigated showed that the highest concentration was found in red wine, whereas wine made from grape pomace contained the lowest content.

KEYWORDS: ***trans*-Resveratrol; *cis*-resveratrol; wine; winemaking byproducts; liquid chromatography−tandem mass spectrometry**

INTRODUCTION

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a naturally occurring phytoalexin, a class of antibiotic compounds produced as a part of a plant defense system against disease. It came to scientific attention as an antioxidant (*1*−*3*), an anticancer agent (*4*), and a phytoestrogen (*5*). Although present in other plants, such as eucalyptus, spruce, and lily, and in other foods such as mulberries and peanuts, the most abundant natural sources of resveratrol are *Vitis vinifera*, labrusca, and muscadine grapes, which are used to make wines. It occurs in the vines, roots, seeds, and stalks, but its highest concentration is in the skin, which contains 50−100 μg/g (*6*).

The resveratrol content of wine is related to the length of time the grape skins are present during the fermentation process. Thus, the concentration is significantly higher in red wine than in white wine, because the skins are removed earlier during white wine production, lowering the amount that is extracted (*7*). Grape juice, which is not a fermented beverage, is not a significant source of resveratrol. A fluid ounce of red wine averages 160 μg of resveratrol, compared to peanuts, which average 73 μg/oz (*8*). Because wine is the most notable dietary source, it is the object of much speculation and research. The concentrations in the form of *trans*- and *cis*-isomers of aglycon and glucosides are subject to numerous factors. In red wine, the concentrations of the *trans*-isomer, which is the major form, generally range between 0.1 and 15 mg/L (*9*−*12*).

trans-Resveratrol is known to exhibit important physiological and biological activities; many studies suggest that consuming alcohol (especially red wine) may reduce the incidence of coronary heart disease (CHD) (*13*−*15*).

The growing interest in the determination of resveratrol in food products is directly connected with the necessity to develop a sensitive and robust method for their analysis. As reported in the literature dealing with the analytical chemistry of fruit polyphenols (*8*, *9*, *14*), the usually proposed methods for the determination of these compounds are based on HPLC-UV and HPLC−fluorescence detection. The use of capillary electrophoresis and electrochemical detection has been also described (*12*−*16*). On the other hand, various reports attest to the power of mass spectrometry (MS) detection with electrospray (ESI) ionization as an identification and confirmation method in the characterization of resveratrol (*6*, *17*, *18*).

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In a research program dealing with the use of hyphenated techniques in food chemistry, we evaluated the application of liquid chromatography−electrospray tandem mass spectrometry (LC-ESI-MS/MS) for the analysis of *trans*-resveratrol in red wine, grape, and winemaking byproduct samples. In particular, this study was aimed at the validation of a robust method for the identification and determination of this compound in red wine, and the results of the application of the developed method to real samples are presented in this paper.

In a previous study, we reported the use of the LC-ESI-MS technique as a confirmation method for the identification of *trans*-resveratrol in red wine samples (*19*). In this work, in addition to the molecular mass information, usually obtained by the ESI ionization technique, the MS/MS technique was used to obtain valuable information on the fragmentation pattern of the analyte and higher selectivity and sensitivity on its determination.

EXPERIMENTAL PROCEDURES

Chemicals. trans-Resveratrol was obtained from Sigma-Aldrich (Milan, Italy). Stock solution of *trans*-resveratrol (1 mg/mL) was prepared in methanol and stored in the dark at 4 °C.

Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q Element water purification system (Millipore, Bedford, MA). Acetonitrile, methanol, and 2-propanol (HPLC grade purity) were purchased from Carlo Erba (Milan, Italy). Analytical reagent grade formic acid was from Carlo Erba.

In-Line Liquid Chromatography−Photodiode Array UV−Tandem Mass Spectrometry. Analyses were performed using an in-line LC-UV-MS/MS system equipped with an electrospray ion source.

All of the LC separations were carried out at room temperature and in the isocratic mode using a Waters 2690 series Alliance quaternary pump (Waters, Milford, MA) equipped with a photodiode array (PDA) detector model 996. Sample injections were performed by a 120-vial capacity sample management system. Injection volume was 5 μL. The LC column was 250 × 2.1 i.d. mm Luna C18(2) packed with 3 μm particles (Phenomenex, Torrance, CA). A Luna C18(2) cartridge C18 (4 × 2 mm i.d.) was used as the precolumn. The mobile phase consisted of aqueous formic acid (pH 2.4)/acetonitrile/2-propanol (70:22:8, v/v/v) at the flow rate of 200 μL/min. The solvent delivered to the ESI interface was split in a 1:10 ratio, delivering ∼20 μL/min to the interface.

A Quattro LC triple-quadrupole instrument (Micromass, Manchester, U.K.) equipped with a pneumatically assisted electrospray interface was used. The system was controlled by Masslynx software version 3.4 (Micromass).

The nebulizing gas (nitrogen, 99.999% purity) and the desolvation gas (nitrogen, 99.998% purity) were delivered at flow rates of 55 and 500 L/h, respectively. Infusion experiments were conducted by infusing the standard solution of *trans*-resveratrol (0.1 μg/mL) with a Harvard syringe pump (Harvard Apparatus, South Natick, MA) at the flow rate of 5 μL/min. Full scan LC-MS analyses were carried out in positive-ion (PI) and negative-ion (NI) modes. Selected-reaction monitoring (SRM) experiments were performed by operating the mass spectrometer in NI mode.

Optimized conditions of the interface were as follows: electrospray voltage, −2.5 kV; cone voltage, −40 V; rf lens, 0.5 V; source temperature, 130 °C; desolvation temperature, 150 °C. Full-scan mass spectra were acquired over the scan range *m*/*z* 200−500 using a step size of 0.1 Da. For MS-MS experiments collision-induced dissociation (CID) was performed with a collision gas pressure of 1.3×10^{-3} mbar in the collision cell. Quantitative analysis was carried out under SRM conditions by monitoring the transition *m*/*z* 227/143 of the analyte.

Quantitation of *trans*-resveratrol in red wine, grape, and winemaking byproduct samples was done by constructing calibration graphs over the 6−600 μg/L range. Quantitative analysis of *cis*-resveratrol was performed by using the calibration curve of *trans*-resveratrol by supposing analogous ionization features and similar response factor.

For LC-UV analysis of *trans*-resveratrol, spectral data were acquired by scanning in the 220−450 nm range. Quantitative analysis of *trans*-resveratrol was carried out at 306 nm by performing calibration in the 6−600 μg/L range. LC-UV determination of *cis*-resveratrol was performed using the calibration curve of *trans*-resveratrol.

LC-MS/MS Method Validation. The validation process of the LC-MS/MS method was carried out following the EURACHEM guidelines (*20*).

Both instrumental and matrix-matched detection limits (y_D) and quantitation limits (y_Q) were evaluated for *trans*-resveratrol as signal based on the mean value ($\bar{y}_b$) and the standard deviation (s_b) of the blank signal ($n = 10$). Solvent solution was used as the blank for the instrumental calculation of y_D and y_Q, whereas a red wine in which *trans*-resveratrol was degraded was used as matrix.

The concentration values of detection limit (LOD) and quantitation limit (LOQ) were obtained by projection of the corresponding signals y_D and y_Q through a calibration plot $y = f(x)$ onto the concentration axis constructed in the 4−40 μg/L range close to concentration values expected.

Linearity of the LC-MS/MS method was studied in the 6−6000 μg/L range. Both solvent and matrix-matched calibration curves were established. Linearity studies were accomplished by verifying homoscedasticity by means of the Bartlett test and subsequently by calculating the goodness of fit of the calibration curve by applying the Mandel fitting test (*21*). A *t* test was carried out to verify the significance of the intercept (confidence level = 95%).

Precision was calculated on the matrix in terms of intraday and interday repeatability as relative standard deviation (RSD%) at two concentration levels. The confidence interval (CI) was also calculated at the 95% confidence level.

The matrix effect was investigated by performing spiking recovery experiments (*21*). For this purpose, first the calibration function of the fundamental analytical procedure was determined:

$$y = a_c + b_c x_c$$

An analytical calibration procedure was then performed on the matrix (a red wine in which *trans*-resveratrol was degraded) spiked with *trans*-resveratrol at six concentration levels. The analytical results x_f were then calculated using the found signal values y_f and the analysis function, that is, the calibration function solved for *x*:

$$x_f = y_f - a_c/b_c$$

By plotting the "found concentrations" (x_f) versus the original calibration concentrations (x_c), the recovery curve was calculated, which is mathematically described by the recovery function (linear regression line)

$$x_f = a_f + b_f x_c$$

In the ideal case, the recovery function results in a line with the intercept $a_f = 0$ and the slope $b_f = 1$ as well as a residual standard deviation that corresponds to the standard process deviation of the fundamental analytical procedure.

All statistical analyses and tests were carried out by using the statistical package SPSS 9.0 for Windows (SPSS, Bologna, Italy).

Sample Preparation. Nero d'Avola red wine, grape skins, grape pomace, and wine pomace samples were from the 2002 vintage. They were obtained directly from the winery as a kind gift of Eno Agricola "Pachino" (Pachino, Siracusa, Italy). Aliquots were obtained from two wine samples purchased in a supermarket (samples A and B) and from four samples collected from a wine produced during the whole vintage time and aged for 8 months in four different vertical stainless steel tanks (samples C, D, E, and F). All samples were contained in closed glass bottles. For red wine samples, triplicate *trans*-resveratrol analyses took place immediately after bottle opening. Samples were then diluted 10 times with deionized water and then filtered on 0.45 μm nylon syringe filters (Supelco, Bellefonte, PA) before LC analysis.

A red wine sample (sample C) was analyzed immediately after it was opened (time 0) and again after 15 and 30 days. Between each

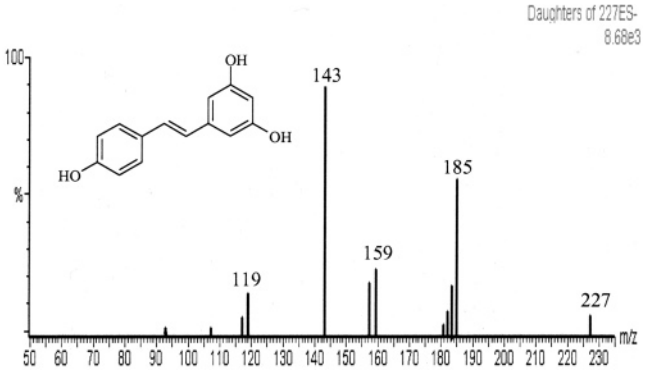

Figure 1. LC-ESI-NI-MS/MS product-ion mass spectrum of *trans*-resveratrol (standard solution of 0.1 μg/mL). (Inset) Chemical structure of *trans*-resveratrol.

analysis the glass bottle was closed and stored in the original bottle in the dark at 4 °C.

Sample Extraction Procedure. Extraction of *trans*-resveratrol from grape skins, grape pomace, and wine pomace was carried out according to the following sample extraction procedure previously reported (*19*): A 0.2−1 g portion of the sample, which had been previously lyophilized, was extracted with 25 mL of a mixture of methanol/ethanol (8:2, v/v) by ultrasonication for 15 min and shaking for 12 h at room temperature. After centrifugation, the remaining pellet was reextracted for 1 h using 5 mL of fresh extraction solvent. The combined extracts were evaporated under reduced pressure at 30 °C, and the residue was dissolved in 1 mL of methanol and submitted to chromatographic analysis. During sample preparation, extracts were constantly protected from light to avoid photochemical isomerization of *trans*-resveratrol to the *cis* form.

RESULTS AND DISCUSSION

ESI-MS and ESI-MS/MS Analysis of *trans*-Resveratrol. The ESI-MS behavior of *trans*-resveratrol was studied under both PI and NI modes. The PI ESI mass spectrum of this compound exhibited the protonated molecule $[M + H]^+$ (100% relative abundance) and ion signals corresponding to the sodium adduct $[M + Na]^+$ (22%) and the potassium adduct $[M + K]^+$ (8%), allowing confirmation of the molecular mass. Better sensitivity was achieved by operation in the NI mode, because under these conditions the ESI mass spectrum of *trans*-resveratrol was dominated by the $[M - H]^-$ ion. On the basis of these findings, MS/MS experiments were carried out in NI mode. As illustrated in **Figure 1**, the NI product-ion mass spectrum of *trans*-resveratrol showed an abundant ion at *m*/*z* 143 (100% relative intensity) and further peaks at *m*/*z* 185 (60%), 159 (25%), and 119 (20%). On the basis of these spectral results, as above-described the transition 227/143 was monitored in SRM mode for validation purposes and quantitative assays.

Validation of the LC-ESI-MS/MS Method. As reported in a previous work, we were able to obtain chromatographic elution of this compound in <13 min under reversed-phase isocratic conditions (*19*). Furthermore, in these conditions, the peak of *trans*-resveratrol was well resolved from the *cis*-resveratrol signal.

The primary goal of this work was to validate a sensitive and robust LC-MS/MS method for the analysis of *trans*-resveratrol. For this purpose, studies on detection and quantitation limits, dynamic and linear range, and accuracy were performed. To achieve optimum sensitivity, all experiments were carried out under NI SRM conditions. The detection and quantitation limits of *trans*-resveratrol were found to be 10 and 16 μg/L, respectively (**Table 1**), thus proving excellent detectability of this substance in the red wine matrix. The LC-ESI-MS/MS dynamic range of *trans*-resveratrol was explored over 3 orders of magnitude of concentration in the 6−6000 μg/L range. Using least-squares regression, the equation reported in **Table 2** was calculated. After testing significance of the intercept (p value < 0.05 at the 95% confidence level), linearity was mathematically verified by applying the Mandel fitting test. A p value of <0.05 (i.e., 0.001) demonstrated that the best data fit could be obtained using a second-order regression model. The dynamic range of linearity was then demonstrated in the 6−3600 μg/L range. Homogeneity of variance of replicates at different concentration levels was proved at the 95% confidence level (p > 0.05). Furthermore, because the intercept was demonstrated to be not significant, the best fit was obtained using a linear regression model: $y = b_1x$ (**Table 2**). From the results of the Mandel test performed on the regression data, significance values of >0.05 indicated a good linear fitting in this concentration range.

Table 1. Solvent and Matrix Limits of Detection and Quantitation of *trans*-Resveratrol bu LC-ESI-MS/MS

	LOD[a] (μg/L)	LOQ[b] (μg/L)
solvent	3	4
matrix	10	16

[a] Calculated as concentration corresponding to signal: $y_D = x_b + 2ts_b$. [b] Calculated as concentration corresponding to signal: $y_Q = x_b + 10s_b$.

Method accuracy was tested in terms of both precision and trueness. In particular, the intraday repeatability of the LC-ESI-MS/MS method provided RSD values between 0.9 and 1.2%, showing an excellent precision at two concentration levels (**Table 3**). By performing replicate injections over 5 days, good intermediate precision was obtained (**Table 3**).

As for trueness, a calculation of the recovery function was performed to ascertain the influence of the matrix for the determination of *all-trans*-resveratrol in red wine samples. In particular, the t test performed on the intercept provided a p value of >0.05 (p = 0.145) at the 95% confidence level, attesting that the intercept was not significantly different from 0. In the case of the slope, the calculated t (127) was found to be higher than the t tabulated at the 95% confidence level (1.86), proving that the slope of the recovery function was significantly different from 1. These findings suggested the presence of matrix effect, because the calibration curve obtained by spiking the red wine sample was significantly different from that obtained using standard solutions (**Table 2**). The matrix effect could be explained on the basis of coeluted compounds present in such a complex sample, resulting in systematic proportional errors owing to a competitive ionization in the ESI process and in a decrease of signal intensity proportional to the analyte concentration. To overcome the matrix effect and avoid time-consuming sample purification treatments, the red wine sample was diluted 4 and 10 times, respectively. When a matrix-matched calibration curve was calculated using diluted samples, no matrix effect was observed. In particular, a significant decrease of the ionization suppression effect was evidenced when a 10-time dilution was performed (**Table 2**). These results confirmed that when LC-ESI-MS/MS technique is applied, the external standard calibration method can be used to quantify resveratrol in red wine with good accuracy after suitable sample dilution.

Identification and Determination of Resveratrol in Red Wine Samples. To verify the applicability of the method proposed, a qualitative and quantitative assay of *trans*-resveratrol

Table 2. Solvent and Matrix-Matched Calibration Curves Established in Red Wine Using LC-ESI-MS/MS Method

	concn range (μg/L)	homoscedasticity p^a	Mandel test p^a	$b_0 \pm s_{b0}$	$b_1 \pm s_{b1}$	r^2 ($n = 18$)
solvent	6–600	0.059	0.07		11.90 ± 0.08	0.997
	6–3600	0.067	0.13		11.54 ± 0.06	0.999
	6–6000	0.017	0.001	1101 ± 405	10.90 ± 0.11	0.998
matrix: red wine	6–600	0.106	0.05		3.68 ± 0.05	0.996
diluted 1:4	6–600	0.059	0.068		4.44 ± 0.15	0.998
diluted 1:10	6–600	0.062	0.124		10.46 ± 0.08	0.997

[a] 95% confidence level.

Table 3. Precision: Intraday Repeatability ($n = 6$) and Intermediate Precision ($n = 30$) of the LC-ESI-MS/MS Method

concn	intraday repeatability[b]		intermediate precision	
level (μg/L)	$X_m \pm CI^a$	RSD%	$X_m \pm CI^a$	RSD%
120	110 ± 7	1.2	119 ± 9	3.7
480	462 ± 23	0.9	486 ± 27	4.3

[a] Confidence level of 95%. [b] Repeatability limits of 95%.

was carried out on different samples of Nero d'Avola red wine and of grape skins, grape pomace, and wine pomace. **Figure 2** shows the LCsUV, the LC-MS full-scan total-ion chromatogram (TIC), the LC-MS/MS SRM chromatogram, and the NI mass spectra of the compounds identified in the samples, that is, *trans*-resveratrol and *cis*-resveratrol.

Under full-scan conditions, the extracted-ion chromatogram of the ion current at *m*/*z* 389, corresponding to the $[M - H]^-$ ion of the monoglycosylate form of resveratrol (piceid, MW

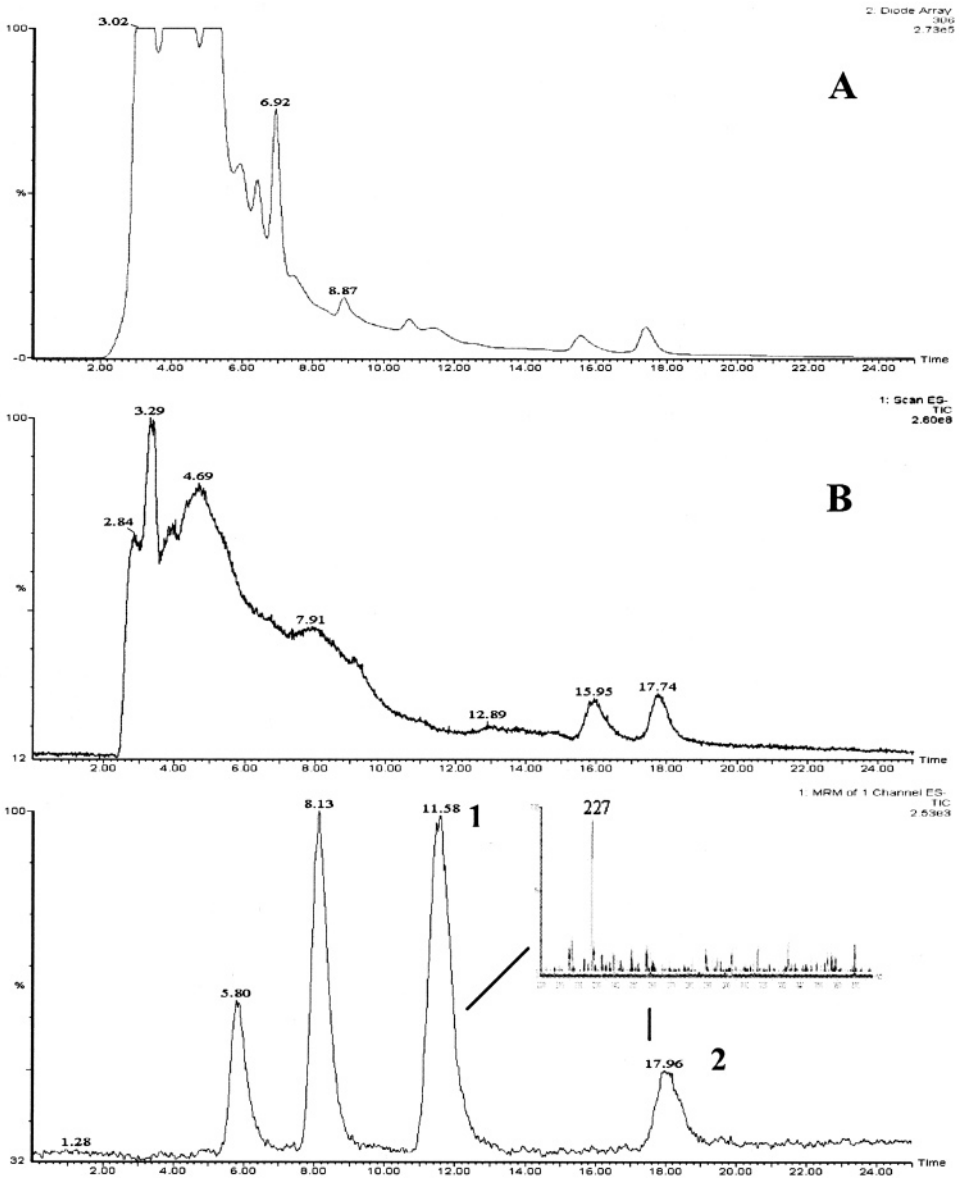

Figure 2. LC-UV/DAD (**A**), full-scan LC-MS (**B**), and LC-(SRM)MS/MS (**C**) chromatograms of a red Sicilian wine sample (Nero d'Avola, 2002 vintage). (Inset) NI full-scan mass spectra of *trans*- and *cis*-resveratrol identified in the red wine sample. Compounds: **1**, *trans*-resveratrol (433 ± 10 μg/mL); **2**, *cis*-resveratrol (173 ± 13 μg/mL). For chromatographic conditions see Experimental Procedures.

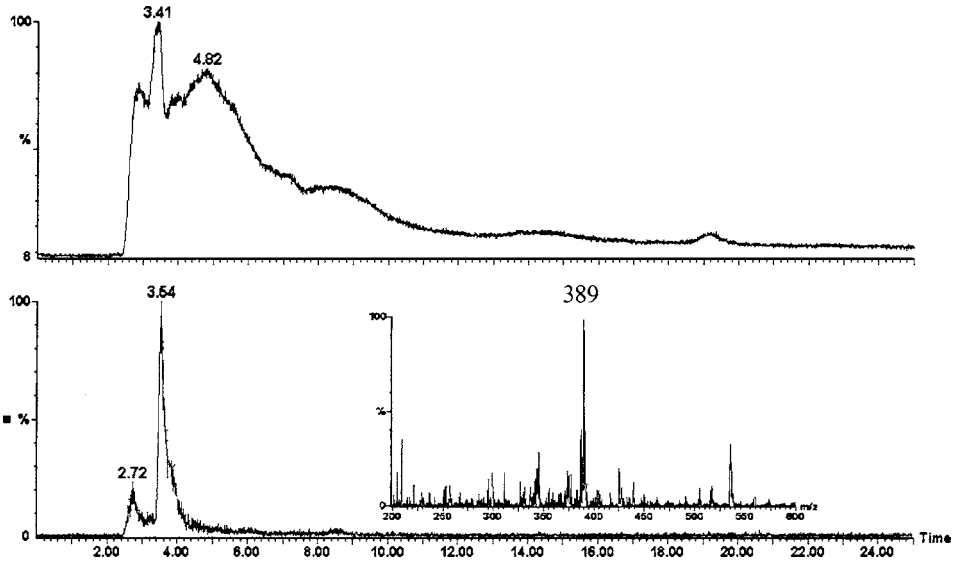

Figure 3. LC-MS (**A**) and LC-MS extract-ion (*m*/*z* 389) (**B**) chromatograms of a red Sicilian wine sample (Nero d'Avola, 2002 vintage). (Inset) NI full-scan mass spectrum of *trans*- and *cis*-piceide identified in the red wine sample.

Table 4. Quantitative Analysis of Resveratrol in Nero d'Avola Red Wine Samples ($n = 3$)

	trans-resveratrol (μg/L)		*cis*-resveratrol (μg/L)	
sample	HPLC-UV/DAD	HPLC-ESI-MS/MS	HPLC-UV/DAD	HPLC-ESI-MS/MS
red wine				
A	770 ± 13	735 ± 11	221 ± 9	203 ± 8
B	787 ± 16	776 ± 11	232 ± 5	209 ± 6
C	499 ± 6	421 ± 4	134 ± 6	121 ± 8
D	479 ± 12	468 ± 12	132 ± 8	119.0 ± 0.6
E	458 ± 11	433 ± 10	180 ± 12	173 ± 13
F	395 ± 5	376 ± 34	189 ± 10	182 ± 8
grape skins				
A	17.5 ± 0.5	11.7 ± 0.7	0.75 ± 0.02	0.64 ± 0.05
B	16.12 ± 0.02	10.74 ± 0.04	1.05 ± 0.03	0.82 ± 0.08
grape pomace				
A	10.2 ± 0.04	9.2 ± 0.3	2.4 ± 0.08	1.5 ± 0.2
wine from grape pomace				
A	0.82 ± 0.02	0.039 ± 0.004	0.56 ± 0.02	0.027 ± 0.007
B	1.25 ± 0.05	0.314 ± 0.004	0.85 ± 0.03	0.16 ± 0.03

390), showed the presence of two peaks, attributable to *trans*- and *cis*-piceid, at the retention times of 2.72 and 3.54 min, respectively (**Figure 3**). The identity of these compounds was then confirmed by the fragmentation pattern obtained by acquiring the LC-MS/MS chromatogram under product-ion scan mode (data not shown).

For quantitative analysis, the red wine, grape skins, and wine pomace samples were further analyzed under SRM mode. In the red wine samples, in addition to the peak corresponding to *trans*-resveratrol, the MS/MS chromatograms showed the presence of other three peaks at the retention times of 5.80, 8.13, and 17.96 min, respectively (**Figure 2**). In particular, the peak at 17.96 min was attributed to *cis*-resveratrol, taking into account also the more hydrophobic nature of the *cis*-isomer on a reversed phase compared to *trans*-resveratrol. In addition, the product-ion mass spectrum of this compound showed a fragmentation pattern similar to that obtained for the *trans*-isomer. Also, the product-ion scan mass spectra of the signals at 5.80 and 8.13 min showed a fragmentation pattern similar to that observed for *trans*-resveratrol. To identify these compounds, a standard solution of *trans*-resveratrol (5 μg/mL) was degraded under UV radiation (254 nm) for 3 h and then analyzed using the LC-MS/MS method. The SRM profile of the degraded solution and the product-ion mass spectra of the observed peaks were comparable to those obtained for the red wine samples investigated, suggesting the presence of polymerized forms of resveratrol in the samples.

In the SRM chromatographic profile of grape skins, the presence of *trans*- and *cis*-resveratrol was observed with a predominance of the *trans* form, whereas no signal was observed at lower retention times (**Figure 4**). Concerning grape pomace and wine made from pomace, the four peaks attributable to *trans*- and *cis*-resveratrol and to the hypothesized polymerized forms was found to be present with different relative intensity (**Figure 4**) and at lower concentrations than those found in wine (**Table 4**). These results could be explained on the basis of both the different distribution of *trans*-resveratrol in the various grape components and a different exposure of such grape or pomace to light and oxygen in the winery during the wine-making process (*22*).

By data comparison, the results obtained by application of the LCsUV technique were systematically higher than those obtained by using the LC-MS/MS method. These results could be explained on the basis of an improved selectivity of the MS/MS detection toward matrix interferences with respect to UV detection and demonstrate the highest accuracy of the present MS/MS method developed for resveratrol analysis.

In addition, despite the high selectivity of tandem mass spectrometry, the LC-MS/MS chromatographic profiles of red wine samples obtained in SRM mode suggest that flow injection

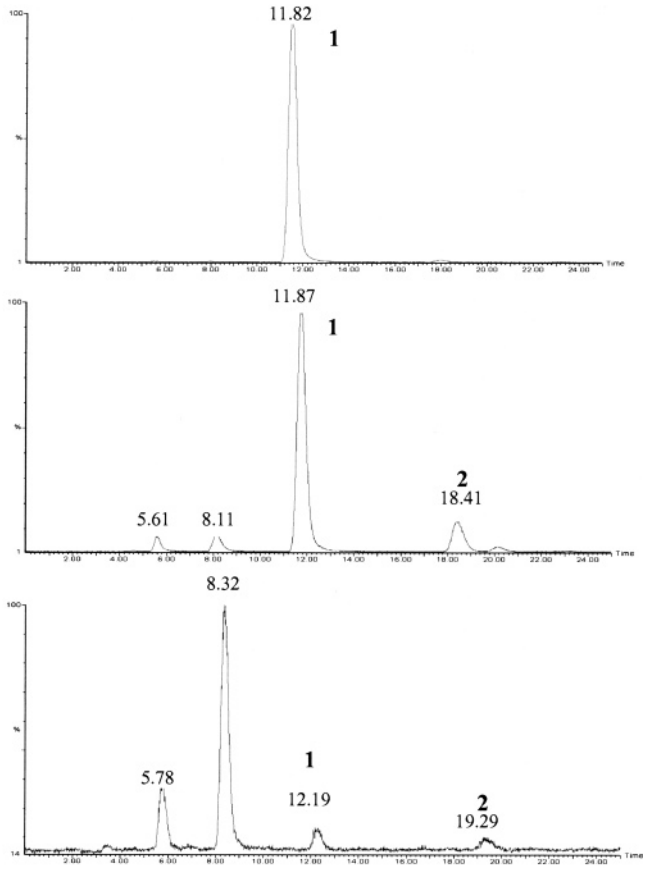

Figure 4. LC-SRM-MS/MS chromatograms of (**A**) grape skins, (**B**) wine pomace, and (**C**) wine from wine pomace. Compounds: **1**, *trans*-resveratrol; **2**, *cis*-resveratrol. For chromatographic conditions see Experimental Procedures.

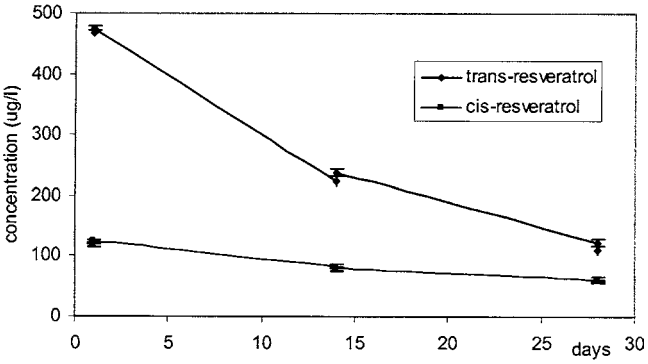

Figure 5. Variation of *trans*-/cis-resveratrol concentration versus time elapsed from the opening of the bottle (sample C).

analysis cannot be performed to determine *trans*-resveratrol in these kinds of samples.

Further investigations concerned the study of degradation of *trans*-resveratrol in one Nero d'Avola red wine (sample C) in a period of 1 month after the opening of the bottle, as reported under Experimental Procedures. The results obtained indicated that the concentrations of *trans*-resveratrol and *cis*-resveratrol decreased, respectively, with percentages of 74 ± 5 and 28 ± 7% with respect to their initial concentrations (**Figure 5**). These findings probably indicate that the *trans*-isomer rapidly converts to the *cis*-isomer and subsequently both isomers polymerize to form resveratrol oligomers.

Conclusion. This work demonstrates that LC-ESI-MS/MS under NI conditions may be a valuable tool for qualitative and quantitative analysis of resveratrol in red wine and winemaking byproduct samples. Validation results attest to excellent detectability, linearity, accuracy, and selectivity of the LC-ESI-MS/MS technique together with its capability to obtain unambiguous detection of resveratrol in red wine samples.

The LC-ESI-MS/MS method allowed us to identify the *cis*-isomer in all samples investigated.

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