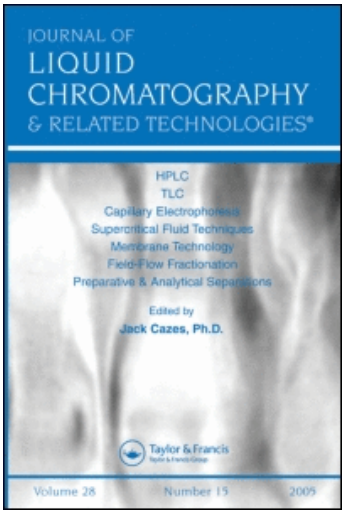

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A Rapid Method for Determination of Resveratrol in Wines by HPLC-MS

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Abstract: A new, sensitive, high throughput liquid chromatography coupled with mass spectrometry (LC-MS/MS) method for quantification of *trans*- and*cis*-resveratrol in wine samples was elaborated. During method development the influence of multiple factors on the ionization efficiency of resveratrol was studied. These parameters included ion source (APCI and ESI, both being operated in positive and negative ionization mode), percentage of aqueous and organic phase, type of organic solvent, and concentration of additives. The optimum conditions were as follows: a mobile phase consisting of a mixture of 1 mM ammonium acetate/acetonitrile (73/27, v/v) with a flow rate of 1 mL/min, MS/MS detection with an APCI interface, operated in negative ionization mode. The monitored ion transition was m/z 227→184.7. Calibration curves were generated for both isomers of resveratrol in the range of 10.47–837.86 ng/mL (*trans* isomer) and 9.12 – 730.14 ng/mL (*cis* isomer), respectively. The developed method has been successfully applied to the analysis and comparison of *trans*- and *cis*-resveratrol content of 20 different Romanian wine samples.

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Keywords: Atmospheric pressure chemical ionization, Electrospray ionization, Liquid chromatography, Mass spectrometry, Resveratrol, Wine

INTRODUCTION

Resveratrol (*trans-,* and *cis-3,5,4′*-trihydroxystilbene) (Figure 1) has been identified as the major active biological compound of the stilbene phytoalexins in wine. Dourtoglou et al.[1] discussed the problem to form *cis*-resveratrol derives from its *trans* isomer during vinification to rely on the reference data.[2–4]

Resveratrol was reported to have numerous health benefits including: cardiovascular protective and the reduction of the heart diseases,[5–11] anti-cancer and anti-inflammatory proprieties,[12–24] anti-oxidant activity,[25,26] antibacterial activity,[27] and was found to inhibit herpes simplex virus types 1 and 2 (HSV-1 and HSV-2).[28,29]

Generally the consumption of fatty food increased the rate of coronary heart disease, but data from France did not follow this pattern. This phenomenon has become known as "the French Paradox".[30] In 1992, Renaud and Lorgeril demonstrated that wine consumption was statistically the only factor correlated to the reduction in coronary heart disease.[31]

Trans-resveratrol also plays an important role in the organoleptic characteristics of wine, conferring astringency and structure to the beverage by formation of complexes with the proteins of saliva.[32] Its levels in white or red wine can be modified by winemaking practices, the effect of temperature, daylight, and grape *Botrytis* levels.

In recent years, some studies were focused on polyphenolic composition of wines by using high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS). The resveratrol being a polyphenol, was studied in some papers together with other polyphenols from red and white wines.

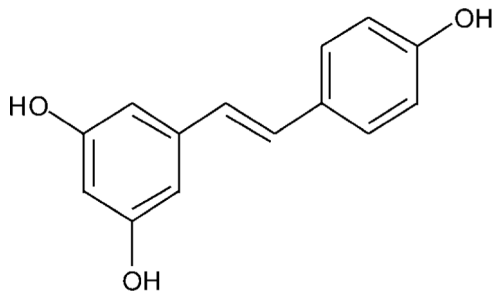

Figure 1. Chemical structure of *trans*-resveratrol.

The characterization of nonvolatile phenolic compounds of a special Hungarian wine was performed by HPLC-MS using electrospray (ESI) and sonic spray (SSI) ionization.[33] Other groups of nonanthocyanin phenolic compounds in wines (vintage 2000, Navarra, Spain) from *Vitis vinifera* L. were studied by HPLC-diode array detection/ESI-MS.[34] The non colored phenolics in red wines were studied by compile LC/UV and MS/MS libraries using 39 phenolic standards and to apply them to investigate the phenols present in different wines samples.[35]

A simple procedure for the determination of resveratrol and piceid isomers, has been elaborated and validated by using LC-UV-VIS/MS analysis, with atmospheric pressure chemical ionization (APCI) and ESI ionization source.[36] The direct determination of some stilbenes in Italian wines by HPLC/MS was reported. For identification of piceatannol glucoside, because of the lack of a suitable standard, an HPLC/TOF/MS method was used. ESI in negative ion mode gives higher sensitivity for all the target compounds than APCI.[37]

The resveratrol is one of the most important compounds present in wines, which can be determined by LC/MS with ESI. The negative ion spectrum of *trans*-resveratrol showed pseudo molecular ion, $[M–H]^-$, as the most abundant ion, and low fragmentations corresponding to the losses of hydroxyl groups of the phenol nucleus.[38] The quantification of *trans*-resveratrol in wine samples performed by HPLC with three different detectors has been evaluated: florescence, UV-DAD, and tandem mass spectrometry MS-MS.[39] The application of LC-ESI-MS/MS was investigated for the analysis of resveratrol in red wines and results were compared with those obtained using an LC-UV/DAD technique.[40]

EXPERIMENTAL

Chemicals

Methanol and acetonitrile of HPLC gradient grade, ammonium acetate, formic acid, acetic acid of analytical grade were purchased from Merck (Merck KgaA, Darmstadt, Germany). Trans-resveratrol was purchased from Sigma (Sigma-Aldrich Chemie GmbH, Munich, Germany).

Preparation of Standard Solutions and Calibration Methodology

A methanolic stock solution (10 mg/mL) of the *trans*-resveratrol was prepared. The stock solution was kept at 4°C and protected from daylight. Before being used as a working solution, aliquots of the stock solution were appropriately diluted with bidistilled water. *Cis*-resveratrol was

obtained from a standard solution of *trans*-resveratrol after its irradiation for 10 minutes using an UV lamp (254 nm). First, two working solutions of *trans*-resveratrol with a concentration of 4.9 μg/mL were prepared. The first solution was used for generation of the calibration curve of *trans*-resveratrol in the range of 10.47–837.86 ng/mL (n = 7). The latter working solution was irradiated with UV light, as described above, and same subsequent dilutions were made as for *trans*-resveratrol. The two dilution series were chromatographed. The first series was used for generating the calibration curve for *trans*-resveratrol. Using this calibration curve, the concentration of residual (non converted) *trans*-resveratrol from the second series was calculated. Finally, the concentration of *cis*-resveratrol was obtained as the difference between the concentration of trans-resveratrol before and after irradiation, respectively. The analytical signal obtained for *cis*-resveratrol was plotted against its calculated concentration, thus generating the calibration curve for the cis isomer. This procedure was described earlier in the literature.[41–51]

When comparing the concentrations of *trans*-resveratrol with and without irradiation, the conversion yield of *trans*-resveratrol to *cis*-resveratrol, after 10 minutes of irradiation of *trans*-resveratrol was found to be about 90%. This way a calibration curve was constructed for *cis*-resveratrol in the range of 9.12 – 730.14 ng/mL.

Sample Preparation

The wine samples (15 red wines and 5 white wine samples, (Table 1) were diluted ten folds with bidistilled water, then centrifuged at 10.000 rpm for 5 minutes. An aliquot was then injected into the chromatographic system.

Apparatus and Chromatographic Conditions

The experiment was carried out using an Agilent 1100 HPLC Series system (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler G1311A.

For the separation, a reversed phase Zorbax SB-C18 analytical column (100 × 3.0 mm i.d., 3.5 μm particles) was used. The column was operated at 40°C in a G1316A oven. For the elution a degasser (G1322A), and a binary gradient pump (G1311A) were used.

The mobile phase was a mixture of 1 mM ammonium acetate/acetonitrile (73/27, v/v), isocratic elution. The flow rate was 1 mL/min and the injection volume 5 μL. All solvents were filtered through 0.5 mm (Sartorius) filters and degassed in an ultrasonic bath.

The detection of *trans*- and *cis*-resveratrol was performed using an Agilent Ion Trap VL mass spectrometer (Bruker Daltonik, GmbH,

Table 1. Romanian commercial wine samples

Wine sample	Grape variety	Vintage	Vineyard region
Red 1	Merlot	2005	Dealul Mare, Tohani
Red 2	Cabernet Sauvignon	2006	Dealul Mare, Tohani
Red 3	Feteasca noir	2005	Dealurile Munteniei
Red 4	Cabernet noir Sauvignon	2007	Dealul Mare, Tohani
Red 5	Cabernet	2007	Viile Banatului, Recas
Red 6	Merlot&Cabernet Sauvignon	2005	Dealurile Vrancei
Red 7	Primitivo	2007	Colinele Iasilor
Red 8	Feteasca noir	2007	Dealurile Moldovei, Bucium
Red 9	Feteasca noir	2007	Dealul Mare Tohani
Red 10	Tohani of noir	2007	Dealul Mare Tohani
Red 11	Feteasca noir	2006	Dealul Munteniei, Tohani
Red 12	Pinot noir	2006	Dealurile Vrancei
Red 13	Mixture	2007	Dealul Mare, Tohani
Red 14	Burgund	2007	Viile Banatului, Recas
Red 15	Busuiaca de Bohotin	2006	Dealurile Husilor
White 1	Feteasca	2007	Tarnave, Jidvei
White 2	Savignon Blanc	2006	Dealul Mare, Tohani
White 3	Grasa de Cotnari	2006	Cotnari, Jud.Iasi
White 4	Muscat Otonel	2004	Tarnave, Jidvei
White 5	Reisling	2007	Murfatlar, Jud. Constanta

Brehmen, Germany). The mass spectrometer was operated using an atmospheric pressure chemical ionisation (APCI) ion source in negative mode. The nitrogen was used as nebulising and dry gas. The APCI heater was set at 350°C, the nebulizer pressure 60 psi, dry gas flow was 5 L/min, and was heated at 250°C. The mass spectrometer operated in multiple reaction monitoring mode and was set to monitor the transition m/z 227→m/z 185. Chromatographic and mass spectrometric data acquisition were performed using Chemstation software (Agilent Technologies, Palo Alto, CA, USA), version B.01.03 and LC/MSD Trap Control (Bruker Daltonik, GmbH, Brehmen, Germany), version 5.3, while data processing was performed using LC/MSD Data Analysis and Quant Analysis software (Bruker Daltonik, GmbH, Brehmen, Germany), version 1.7.

RESULTS AND DISCUSSION

During the method development and optimization process the influence of multiple factors on the ionization efficiency of resveratrol was studied. The main factors, which could affect the MS signal, are the ionization source and the mobile phase composition.

In the case of the ionization source, a comparison of APCI-MS/MS and ESI-MS/MS (electrospray ionization) techniques was performed, each ion source being operated in both, positive and negative ionization mode. The monitored ion transitions were m/z 229→134.9 and m/z 227→184.7 for positive and negative ionization modes, respectively.

Figure 2 shows a typical full scan mass spectrum of *trans*-resveratrol and a MS/MS spectra (indicating the 229→134.9 transition) obtained through the fragmentation of the protonated molecular ion $m/z = 229$.

Figure 3 presents the full scan spectrum of the same analyte, but in the case of negative ionization mode and also a MS/MS spectra (indicating the 227→184.7 transition) corresponding to the fragmentation of the deprotonated molecular ion with $m/z = 227$.

Multiple changes were made during the optimization process in order to evaluate the influence of mobile phase composition. These changes were made regarding the percentage of aqueous and organic phase, type of organic solvent, and concentration of additives.

Regarding the additives in LC-MS/MS analysis, it is very important to use volatile buffer additives and to use organic acids.[52] The additives used in this study were acetic acid (0.1% and 0.2%), formic acid (0.1%), and ammonium acetate (1, 2, 5, and 10 mM).

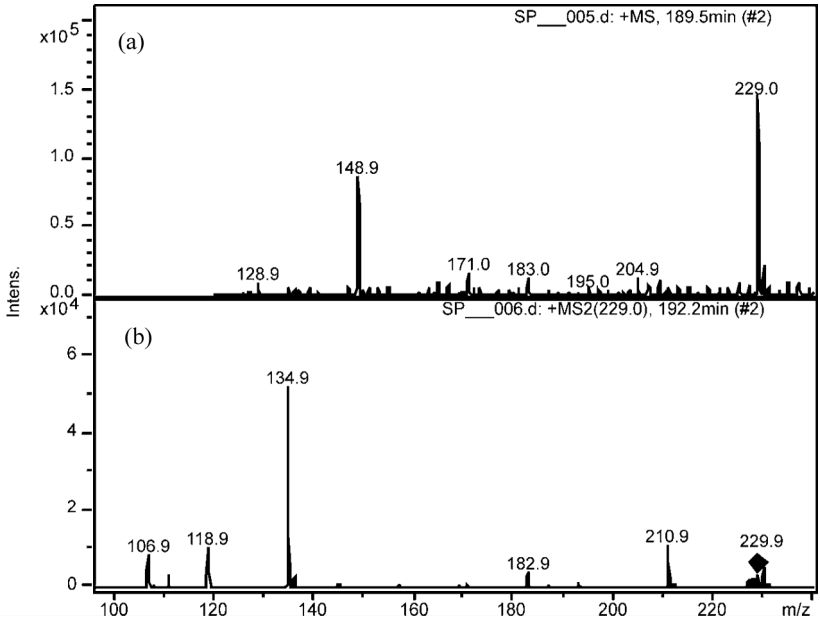

Figure 2. Full scan mass spectra of *trans*-resveratrol (a) and mass spectra of the pseudo-molecular ion $[M + H]^+$ at $m/z = 229$ (b), in positive ionization mode.

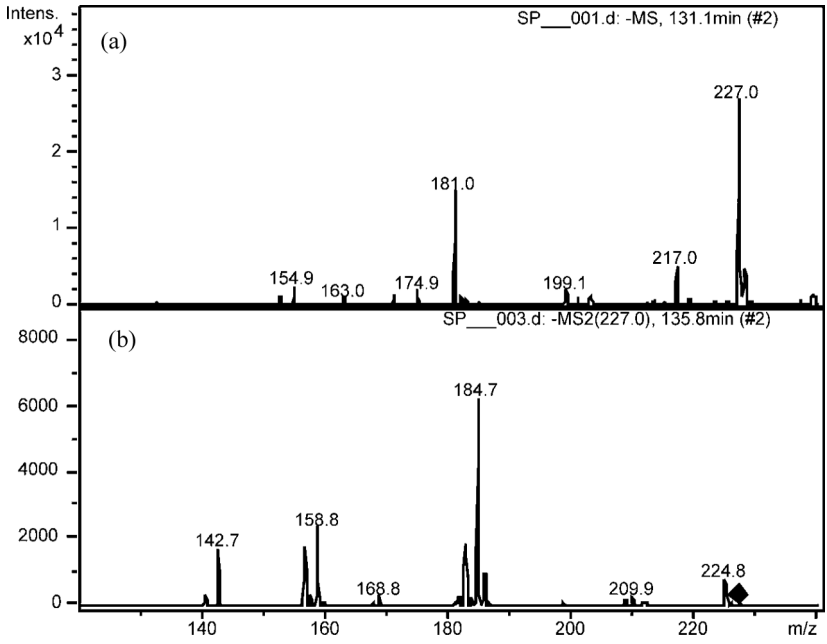

Figure 3. Full scan mass spectra of *trans*-resveratrol (a) and mass spectra of the pseudo-molecular ion $[M-H]^+$ at $m/z = 227$ (b), in negative ionization mode.

In order to find the optimum ion source (MS interface) and mobile phase composition the signal to noise (S/N) ratios were calculated for each injection. The obtained values are presented in Table 2.

A great difference was observed between methanol and acetonitrile concerning the type of organic solvent used in the mobile phase. In APCI, the use of methanol instead of acetonitrile produced an enhancement of detection sensitivity, but it is also considered to increase the long term stability of the analyte signal.[52] This is because acetonitrile in gas phase is a relatively strong base and, therefore, appears as competition between it and the analytes for protonation. In the case of APCI, in positive ionization mode, the greater S/N ratio obtained with methanol as the organic component of the mobile phase could be explained by the protic character of this solvent. Since in the case of APCI the ionization takes place in gas phase, another important aspect could be the higher content of organic component in the case of the mobile phase with methanol, which favors a faster/easier evaporation of the liquid phase.

Concerning ESI (negative ionization mode), the use of acetonitrile resulted in slightly greater sensitivity when compared with the results obtained with methanol.

Table 2. Results obtained during method optimization (signal/noise ratio)

Mobile phase					
Aqueous phase	Organic solvent	Composition (%,v/v)	Ionization source	Polarity	S/N
1 mM Ammonium acetate	ACN	73/27	APCI	negative	790
2 mM Ammonium acetate	ACN	73/27	APCI	negative	753
5 mM Ammonium acetate	ACN	73/27	APCI	negative	378
10 mM Ammonium acetate	ACN	73/27	APCI	negative	370
1 mM Ammonium acetate	ACN	73/27	APCI	positive	5
2 mM Ammonium acetate	ACN	73/27	APCI	positive	4
5 mM Ammonium acetate	ACN	73/27	APCI	positive	3
10 mM Ammonium acetate	ACN	73/27	APCI	positive	2
0.1% acetic acid	ACN	73/27	APCI	negative	80
0.2% acetic acid	ACN	73/27	APCI	negative	60
0.1% acetic acid	ACN	73/27	APCI	positive	9
0.2% acetic acid	ACN	73/27	APCI	positive	31
0.1% formic acid	ACN	73/27	APCI	negative	14
0.1% formic acid	ACN	73/27	APCI	positive	88
1 mM Ammonium acetate	MeOH	60/40	APCI	negative	401
2 mM Ammonium acetate	MeOH	60/40	APCI	negative	798
5 mM Ammonium acetate	MeOH	60/40	APCI	negative	538
10 mM Ammonium acetate	MeOH	60/40	APCI	negative	391
1 mM Ammonium acetate	MeOH	60/40	APCI	positive	274
2 mM Ammonium acetate	MeOH	60/40	APCI	positive	252
5 mM Ammonium acetate	MeOH	60/40	APCI	positive	196
10 mM Ammonium acetate	MeOH	60/40	APCI	positive	140
0.1% acetic acid	MeOH	60/40	APCI	negative	389
0.2% acetic acid	MeOH	60/40	APCI	negative	230
0.1% acetic acid	MeOH	60/40	APCI	positive	838
0.2% acetic acid	MeOH	60/40	APCI	positive	1088
0.1% formic acid	MeOH	60/40	APCI	negative	18
0.1% formic acid	MeOH	60/40	APCI	positive	842
1 mM Ammonium acetate	ACN	73/27	ESI	negative	190
2 mM Ammonium acetate	ACN	73/27	ESI	negative	160
5 mM Ammonium acetate	ACN	73/27	ESI	negative	123
10 mM Ammonium acetate	ACN	73/27	ESI	negative	84
0.1% acetic acid	ACN	73/27	ESI	negative	322
0.2% acetic acid	ACN	73/27	ESI	negative	204
0.1% formic acid	ACN	73/27	ESI	negative	23
1 mM Ammonium acetate	MeOH	60/40	ESI	negative	167
2 mM Ammonium acetate	MeOH	60/40	ESI	negative	130
5 mM Ammonium acetate	MeOH	60/40	ESI	negative	82
10 mM Ammonium acetate	MeOH	60/40	ESI	negative	78
0.1% acetic acid	MeOH	60/40	ESI	negative	164
0.2% acetic acid	MeOH	60/40	ESI	negative	189
0.1% formic acid	MeOH	60/40	ESI	negative	29

In the case of LC-MS/MS, when additives are used in the mobile phase, it is important to use a buffer concentration as low as possible: maximum 10 mM in ESI and below 100 mM in APCI.[52] The S/N ratios obtained in this experiment confirmed this theory. Independently of the MS interface and the positive or negative ionization mode, a significant decrease of S/N ratio could be observed as the concentration of ammonium acetate increased. The presence of ammonium acetate in the mobile phase increased sensitivity in the negative ionization mode, with significantly lower signal to noise ratio values for positive ionization (tested only for APCI).

The acetic acid showed superior results than formic acid when used to acidify the mobile phase ($pH < 7$). These differences were more obvious in case of negative ionization mode, independently of the MS interface used (ESI or APCI). A possible explanation is that acetic acid is a more volatile compound and a weaker acid than formic acid.

The maximum signal to noise ratio (1088) was obtained in the case of APCI, operated in positive ionization mode, with a mobile phase of 0.2% acetic acid/methanol (60/40, v/v) and using these conditions a red wine sample was analyzed. A third peak appeared between the peaks corresponding to *trans*- and *cis*-resveratrol, as can be seen in Figure 4. The resolution between the trans-resveratrol and the new peak was not good enough to allow an accurate quantification of the analyte. Resveratrol

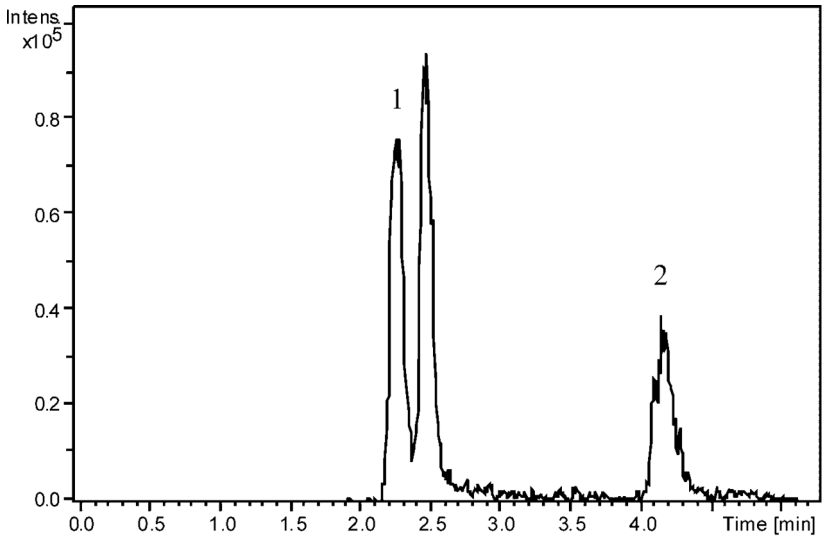

Figure 4. Chromatogram of a red wine sample obtained with a mobile phase 0.2% acetic acid/methanol (60/40, v/v) and MS/MS detection with APCI (+) (peak 1 = *trans*-resveratrol; peak 2 = *cis*-resveratrol).

can be found in wines as esters with organic acids. At the temperature of the ion source or due to the molecular collisions at the pressure of this source, a uncontrolled fragmentation may occur, which could result in the regeneration of some molecular ions characteristic to free resveratrol (i.e., the adduct of resveratrol with proton). This phenomenon is known as in source CID (collision induced dissociation) and occurs frequently in case of molecules which contains labile chemical bonds. This way, after the fragmentation from the ion trap, a new peak may occur due to the same fragments characteristic to *trans*-resveratrol, but since the free *trans*-resveratrol and the esterified *trans*-resveratrol does not have the same retention time, two peaks appeared in the chromatogram with very close retention times. The first peak at 2.2 min corresponded to the ion transition m/z 229→134.9 of the protonated free *trans*-resveratrol from the wine sample, while the peak at 2.45 min corresponded to the same transition, but of the protonated free *trans*-resveratrol generated from the esterified form of the analyte.

For acetonitrile as the organic component of the mobile phase, the maximum signal to noise ratio (790) was obtained as a mixture of 1 mM ammonium acetate/acetonitrile (73/27, v/v), with MS/MS detection using APCI ion source in negative ionization mode. In a second step, the same red wine sample as in Figure 4 was analyzed but with this new LC-MS/MS condition (Figure 5). The new mobile phase guaranteed a very good separation of free *trans*- and *cis*-resveratrol (retention times of 1.5 min and 2.3 min, respectively), but also of the ions (peaks) generated due to CID of resveratrol esters and glycosides.

In conclusion, the final conditions chosen to analyze all wine samples were as follows: a mobile phase consisting in a mixture of 1 mM ammonium acetate/acetonitrile (73/27, v/v), and MS/MS detection with an APCI interface, operated in negative ionization mode. The monitored ion transition was m/z 227→184.7 (Figure 5).

Using these LC-MS/MS conditions twenty (15 red and 5 white) Romanian wine samples were analyzed. In order to quantify *trans*- and *cis*-resveratrol calibration curves were constructed for both analytes as described in the Preparation of Standard Solutions and Calibration Methodology section and using the selected LC-MS/MS conditions. A chromatogram corresponding to a standard mixture of *trans*- and *cis*-resveratrol at LOQ level is presented in Figure 6. The accuracy corresponding to the different concentration levels used for calibration are presented in Table 3.

The results are summarized in Table 4. All white wines were demi-dry, while in red wines 5 were dry, 7 demi-dry, and 3 sweet.

As can be seen in Table 4, the *cis*- and *trans*-resveratrol content is significantly lower in the white wine samples. In all red wines the *trans*-resveratrol content is higher than the *cis*-resveratrol content, while

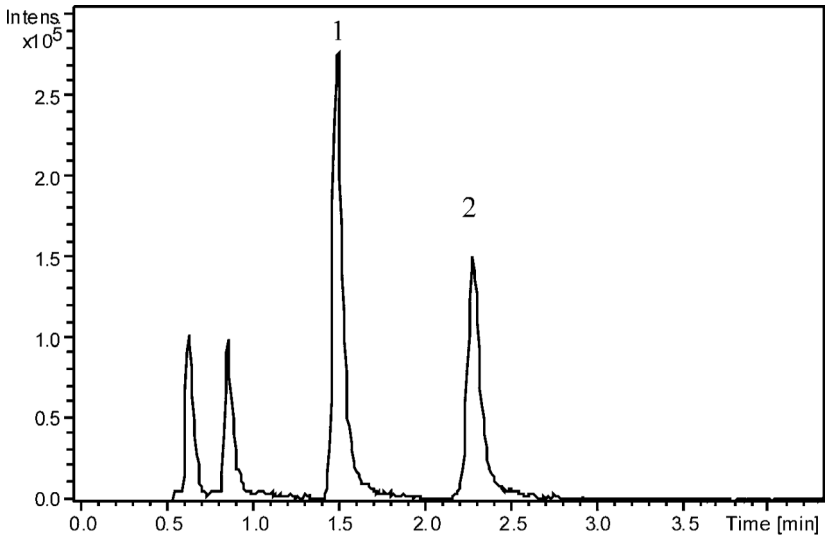

Figure 5. Chromatogram of a red wine sample obtained with a mobile phase 1 mM ammonium acetate/acetonitrile (73/27, v/v) and MS/MS detection with APCI (−) (peak 1 = *trans*-resveratrol; peak 2 = *cis*-resveratrol).

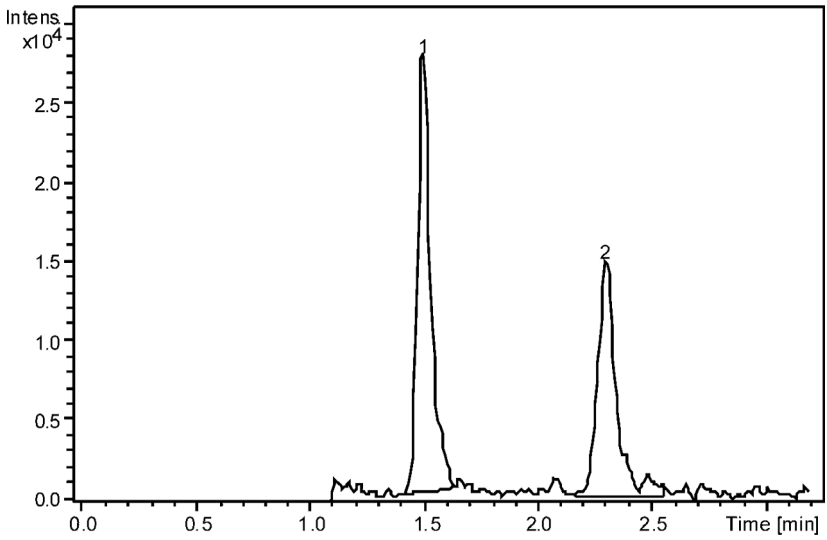

Figure 6. Chromatogram of a *trans*- and *cis*-resveratrol mixture at LOQ level (peak 1 = *trans*-resveratrol; peak 2 = *cis*-resveratrol).

Table 3. Accuracy data for *trans*- and *cis*-resveratrol

trans-resveratrol		*cis*-resveratrol	
Calibration standard (ng/mL)	Accuracy	Calibration standard (ng/mL)	Accuracy
10.47	105.55	9.12	108.84
26.18	96.44	22.81	97.00
52.36	97.17	45.63	100.55
104.73	99.55	91.26	97.05
209.46	102.36	182.53	97.55
418.93	99.25	365.07	94.94
837.86	99.57	730.14	97.55

Table 4. The resveratrol content of some Romanian wine samples

Wine sample	Type	Vintage	Resveratrol content (μg/mL)		*trans* + *cis* resveratrol (μg/mL)	*trans* / *cis* Resveratrol Ratio
			trans-	*cis*-	$X \pm SD$	$X \pm SD$
Red 1	Dry	2005	1.413	0.795	2.208	1.777
Red 2	Dry	2006	8.807	3.285	12.092	2.680
Red 3	Dry	2005	2.763	1.785	4.548	1.547
Red 4	Dry	2007	1.257	1.047	2.304	1.200
Red 5	Dry	2007	1.995	1.858	3.853	1.073
			$X_1 = 3.246$	$X_2 = 1.754$	5.001 ± 4.089	1.66 ± 0.64
Red 6	Demidry	2005	3.524	2.011	5.535	1.752
Red 7	Demidry	2007	0.803	0.385	1.188	2.085
Red 8	Demidry	2007	1.007	0.415	1.422	2.426
Red 9	Demidry	2007	1.462	0.843	2.305	1.734
Red 10	Demidry	2007	0.875	0.523	1.398	1.673
Red 11	Demidry	2006	0.841	0.683	1.524	1.231
Red 12	Demidry	2006	1.683	0.585	1.268	2.876
			$X_1 = 1.456$	$X_2 = 0.778$	2.091 ± 1.469	1.97 ± 0.55
Red 13	Sweet	2007	1.374	0.560	1.934	2.453
Red 14	Sweet	2007	1.953	1.381	3.334	1.414
Red 15	Sweet	2006	0.854	0.400	1.254	2.135
			$X_1 = 1.393$	$X_2 = 0.780$	2.174 ± 1.060	2.00 ± 0.53
White 1	Demidry	2007	0.057	0.110	0.518	0.518
White 2	Demidry	2006	0.163	0.086	0.249	1.895
White 3	Demidry	2006	0.106	0.134	0.140	0.791
White 4	Demidry	2004	0.157	0.095	0.252	1.652
White 5	Demidry	2007	0.076	0.086	0.162	0.883
			$X_1 = 0.112$	$X_2 = 0.102$	0.264 ± 0.092	1.15 ± 0.59

X – average; SD – Standard deviation;.

in white wines two contained more *trans*-resveratrol and 3 presented more *cis*-resveratrol. From the three types of red wines analyzed, the average concentrations of *trans*- plus *cis*-resveratrol were found in the following order: dry ($4.902 \pm 3.741\ \mu g/mL$) > demidry ($2.091 \pm 1.469\ \mu g/mL$) ≥ sweet ($2.174 \pm 1.060\ \mu g/mL$). However, the *tans*-/*cis*-resveratrol ratio shows a reversed order: sweet (2.00 ± 0.53) ≥ demidry (1.97 ± 0.54 > dry (1.66 ± 0.64). The SD values are very high, meaning that values have a great dispersion, which can be explained by the wine varieties. In the case of the five white demi-dry wines studied, this couldn't be observed a rule as was observed for the red wines.

CONCLUSIONS

The optimum LC-MS/MS conditions were identified in this study for the quantification of *trans*- and *cis*-resveratrol from wine samples. The comparison of APCI-MS/MS and ESI-MS/MS techniques showed that APCI mode is more sensitive than ESI mode, and thus better suited for *trans*-resveratrol analysis. The optimum conditions were identified, which guaranteed high signal to noise ratio, and also the lack of any interference. The method was successfully used for *trans*- and *cis*-resveratrol quantification in a comparative study conducted on 20 Romanian wine samples.

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