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Analysis of *trans*-resveratrol by laser ionization mass spectrometry and HPLC with fluorescence detection Comparison between both techniques

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Abstract

A comparison between two analytical techniques is presented using *trans*-resveratrol as analyte and vine leaf as sample. The employed methods were: (a) laser desorption followed by resonance enhanced multiphoton ionization coupled with time-of-flight mass spectrometry (LD–REMPI–TOFMS), and (b) reversed-phase high performance liquid chromatography (RP-HPLC) with fluorescence detection. While both techniques show a similar range of linearity and reproducibility, marked differences were found in their sensitivity and required time for a single analysis. For example: (i) the chromatographic method required considerable less time (30 min) than the REMPI method to implement the analysis, (ii) the detection and quantification limits of the REMPI technique were 2.1 and 6.7 $\mu g\,L^{-1}$, respectively, while for the chromatographic method they were ten times minor, i.e. 20 and 67 $\mu g\,L^{-1}$, respectively. A critical assessment including advantages and drawbacks of each technique is presented.

Keywords: *Trans*-resveratrol; Laser ionization; Mass spectrometry; HPLC

1. Introduction

Phytoalexins are produced by plants as a defense response to fungal infection, mechanical damage and UV irradiation [1]. *Trans*-resveratrol (3,5,4′-trihydroxystilbene) is one of the major stilbene phytoalexins found in different families of plants such as the *Polygonum Cuspidatum*, a Chinese medicinal plant, whose extract contains *trans*-resveratrol of purity range from 10 to 99%. Also grapes, peanuts and their products are considered the most important dietary sources of resveratrol [2]. The amount of *trans*-resveratrol in red wines is higher than in rose or white wines, partly due to the winemaking process but also depending on the grape variety, environmental factors in the vineyard and wine processing techniques [3].

Trans-resveratrol has received attention in recent years due to its capacity to protect against global cerebral ischemic injury and to ameliorate oxidative damage; it can also inhibit cellular events associated with tumour initiation, promotion and progression [4].

Due to the beneficial health effects of *trans*-resveratrol, several methods have been developed for its detection and quantification. Monitoring of resveratrol and other phenolic compounds requires analytical methodologies capable of performing determinations at trace concentration levels, such as chromatographic techniques, and some pretreatment steps because usually the matrix is too complex.

Trans-resveratrol is usually analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) with standard bore columns. Most HPLC methods perform separation by acidic solvent gradient elution and detection with spectrophotometric UV [5], UV diode array (DAD) [6], or

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fluorimetry [7–9]. Fluorimetry, together with UV-DAD detection [10] and electrochemical detector [11], have been also applied to enhance the sensitivity of detection in HPLC. In addition, some work based on the application of LC coupled with MS [12] has been published. Methods based on gas chromatography mass spectrometry [13] have been proposed for *trans*-resveratrol analysis, but this technique has a major inconvenience: usually extraction, clean-up or a derivatization reaction are required prior to GC analysis of this substance and this handling can enhance the *trans* to *cis* isomerization of resveratrol. In all these techniques, the limiting step in *trans*-resveratrol analysis is the sample preparation, not only because of the need for costly and time consuming operations, but because of the error sources introduced during this analytical step. This has originated some controversy among different laboratories on their respective sample preparation procedures [14–16]. Revisions of some of the methods for the analysis of *trans*-resveratrol [17–19] showed a huge variability in the values published. This was attributed to a possible isomerization during the process of derivatization, losses due to oxidation, isomerization or hydrolysis during the extraction and separation processes, and the presence of some resveratrol derivatives that could interfere in the results. Several sample preparation methods used for the determination of *trans*-resveratrol by HPLC and a comparison of their main features have also been reviewed [20]. Direct analysis of *trans*-resveratrol using no chromatographic techniques as micellar electrokinetic capillary electrophoresis and a new technique based on the combination of laser desorption (LD) followed by resonance enhanced multiphoton ionization (REMPI) and time-of-flight mass spectrometry (TOFMS) has been also performed.

The most common samples analyzed for the determination of resveratrol are wine (mainly red wine), grapes, peanuts or peanut butters, although, the are also studies to a lesser degree in plants like tea and soy, and human tissues. For wine and grape juice samples, several methods have been developed [21–23] for the analysis of *trans*-resveratrol by direct injection on the HPLC system, but in most cases this leads to complex chromatograms which sometimes do not allow reliable identification and/or quantification of the peaks [20]. Micellar electrokinetic capillary electrophoresis has been also used in wine samples with a clear lack of sensitivity attributed to the need for preconcentration techniques [24]. In a previous paper, the analysis of *trans*-resveratrol in plant samples, namely in vine leaves by LD–REMPI–TOFMS was reported [25]. The technique has been further used for *trans*-resveratrol analysis in order to assess its relationship to grape disease resistance and to investigate its activity as a natural pesticide [26,27].

The present paper is dedicated to the comparison between RP-HPLC with fluorimetric detection and LD–REMPI–TOFMS techniques in the analysis of *trans*-resveratrol. Thus, the separation of *trans*-resveratrol from other phenolic compounds in vine leaf extract samples was performed using RP-HPLC. The same type of extract sample and compound was also analyzed by means of the REMPI technique. It should be pointed out that the REMPI technique can be applied without sample preparation; therefore, for the sake of the comparison with the HPLC the same extract sample was used in both experimental methods. After a brief description of those methods, their quality of the results and the relevant analytical parameters were compared and a critical assessment was done in order to establish the main advantages and drawbacks of each experimental method.

2. Experimental

2.1. Reagents and standards

HPLC-grade methanol, from Fluka (Switzerland), ethanol, from Scharlau (Barcelona, Spain), glacial acetic acid from Carlo Erba (Milan, Italy) and purified water with a Milli Q system from Millipore (Milford, MA, USA) were used.

A *trans*-resveratrol standard (99%) from Sigma Aldrich was used.

Standard solutions: 250 mg L^{-1} stock solution in ethanol was prepared. Working standard solutions were prepared by diluting the stock solution in ethanol. The standard solutions were stored at −4 °C in darkness.

2.2. Samples

Vine leaves were directly obtained from the vineyard after harvest in October. They were cut in pieces and introduced in ethanol (ca. 8 L for 4 kg of leaves) allowing 3 weeks of maceration to extract the *trans*-resveratrol. During maceration a sample of the solution was taken every 2 days to follow the extraction process by the evolution of the UV–vis absorption spectrum. The maceration process was carried out in darkness and at room temperature.

After maceration, the solution was filtered through cotton and the residue was analyzed by LD–REMPI–TOFMS to confirm the complete extraction of the *trans*-resveratrol from the leaves.

The obtained solution was directly used for the subsequent analysis. All samples were protected from light to avoid photon-induced isomerization during sample treatment

2.3. LD–REMPI–TOFMS

A full description of the REMPI technique based on the combination of laser desorption with REMPI–TOFMS for the analysis of *trans*-resveratrol in plants has been previously published [25], so only a brief report is given here. Essentially, it consists of two independent high vacuum chambers; the first chamber is used for both laser desorption and laser post-ionization of the sample followed by the ions acceleration towards the second chamber, basically a time-of-flight unit with a two microchannel plate detector. A few nanosecond

Table 1
Main analytical parameters for the analysis of *trans*-resveratrol by LD–REMPI–TOFMS

Linearity (mg L^{-1})	0–40
Repeatability (%)	4.9
Reproducibility (%)	4.4
Accuracy (%)	96
Detection limit (μg L^{-1})	2.0
Quantification limit (μg L^{-1})	6.7

laser pulses from the fundamental emission of a Nd:YAG laser (1.064 nm) are used for sample desorption. A frequency-doubled dye laser is then used to selectively ionise the desorbed neutral compound by resonant-enhanced multiphoton ionization. To this end active wavelength laser scanning is achieved with tunability from 230 up to 730 nm. In addition to the selective ionization due to REMPI, further selectivity is achieved by the use of mass spectrometry, which enables sample mass identification and makes the technique more sensitive and universal.

A basic feature of the technique is the absence of any separation method for sample preparation. The main analytical parameters obtained after the validation of the technique are shown in Table 1. For additional experimental details, the reader is addressed to Ref. [25].

2.4. RP-HPLC

The chromatographic system consisted of the following components: a liquid chromatograph using PU 1580 pump, and LG-1580-02 gradient controller from Jasco (Tokyo, Japan), a Rheodyne 7725 injection valve furnished with a 20 μL loop, a Perkin-Elmer LS 30 luminescence spectrometer (Norwalk, USA) and a Biocrom worstation 2000-3.0 from Micron Analitica S.A. (Madrid, Spain). Separation was carried out using a Tecknokroma Cromasyl C_{18} column (150 × 4.6) mm, 5 μm, and a guard column of the same stationary phase, at room temperature. Membranes (Millipore) of 0.45 μm were used to filter solutions. A vacuum pump (Barna, USA) was also used.

The chromatographic separation was carried out using a linear gradient as mobile phase with methanol–acetic acid-water (10:2:88 v/v/v) as solvent A and methanol–acetic acid–water (90:2:8) as solvent B [7] at a flow-rate of 1.0 ml min^{-1} as shown in Table 2.

A wavelength of λ_{ex}, 330 nm and λ_{em}, 374 nm, characteristic for *trans*-resveratrol were used for fluorescence detector [7,8]. Chromatographic peaks were identified by comparing retention times of samples with that of the standard compound. Identification and quantification of *trans*-resveratrol in the standard and samples was carried out in a similar composition to the mobile phase by the addition and external standard techniques. The solutions for the external calibration points from 0.5 to 5 mg L^{-1} concentration range of *trans*-resveratrol and the samples were prepared using the same standard solution or sample volume and water–acetic acid solution (at pH 2.6).

Table 2
Linear gradient for separation of *trans*-resveratrol in leaf sample extracts

Time (min)	A (%)	B (%)
0	85	15
5	65	35
8	65	35
15	50	50
25	30	70

The standard addition calibration was established on the basis of 1 mL sample extract in ethanol, with 1 mL of ethanol, which contains different amounts of *trans*-resveratrol from 1.5 to 15 μg, and 2 mL of water–acetic acid solution at pH 2.6.

3. Results and discussion

3.1. Analysis by LD–REMPI–TOFMS

Fig. 1 displays a time of flight spectrum obtained from an sample of vine leaves extract under the usual experimental conditions (E_d = 40 mJ/pulse at 1064 nm; E_i = 550 μJ/pulse at 302.1 nm); the resveratrol peak as indicated is clearly noticeable showing how the combination of selective ionization plus the versatility of the time-of-flight spectrometry, allows to identification and analysis of one component with no interference from the rest of them present in the sample. This makes any separation process prior to the analysis unnecessary. This is, of course, one of the major advantages of the present technique.

For this sample the *trans*-resveratrol content has been determined using the standard addition method, i.e. adding known quantities of *trans*-resveratrol to several identical samples of vine leaf extract; the value obtained for the intercept with the *X*-axis gives the quantity of analyte in the blank.

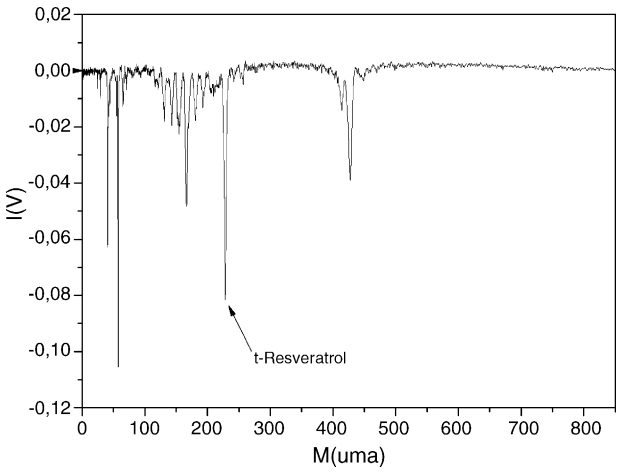

Fig. 1. TOF mass spectrum from a vine leaf extract sample obtained by LD–REMPI–TOFMS. See text for experimental details.

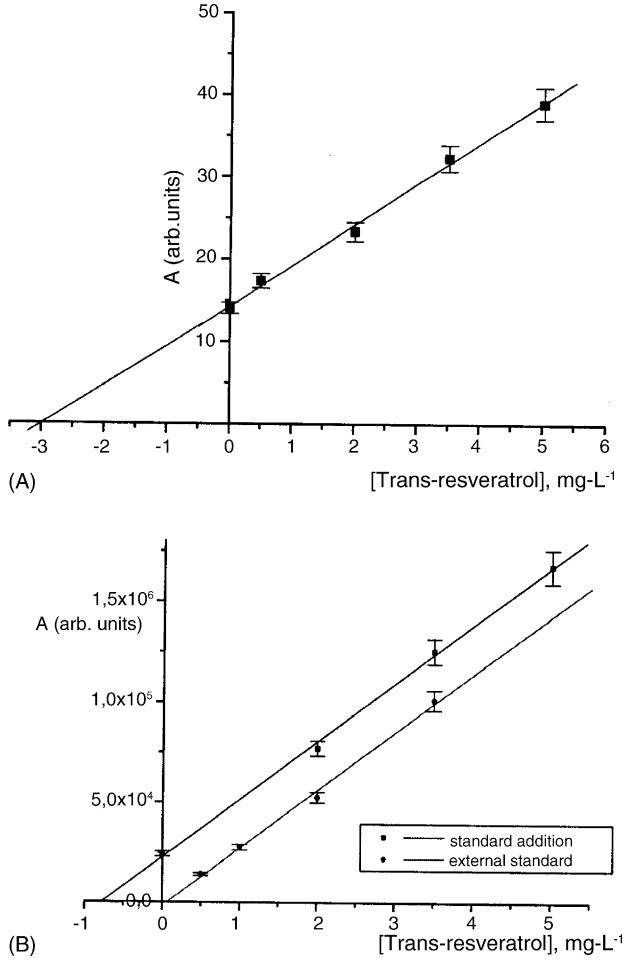

Fig. 2. (A) Standard addition calibration for *trans*-resveratrol from 0.5 to 5 mg L^{-1} using LD–REMPI–TOFMS. (B) Standard addition and external standard calibrations for *trans*-resveratrol from 0.5 to 5 mg L^{-1} using RP-HPLC.

Fig. 2A shows the standard addition curve; the concentration of *trans*-resveratrol in the vine leaves calculated from these values was 3.0 ± 0.2 mg L^{-1}. The limit of detection (LD, three times the noise to sensitivity ratio 3 N/s) and limit of quantification (LQ, 10 N/s) deduced from this spectrum are 2.1 and 6.3 μg L^{-1} respectively, which are consistent with the previously published values (see Table 1).

3.2. Analysis by RP-HPLC

Different mobile phase linear gradient programs for separation and identification of *trans*-resveratrol from other phenolic compounds in leaf sample extracts were studied according to the procedure already established for the analysis of wine samples by HPLC with fluorescence detection at its characteristic wavelengths, $\lambda_{ex} = 330$ nm and $\lambda_{em} = 374$ nm [7,8]. The optimal linear gradient is shown in Table 2. The elution of the sample was carried out directly without overlapping from other phenolic compounds including its isomer, *cis*-resveratrol. The retention time of *trans*-resveratrol was 11.09 ± 0.08 min. Fig. 3 displays a vine leaf sample extract chromatogram obtained by HPLC and fluorescence detection using the optimal mobile phase linear gradient program.

Two techniques of calibration, addition and external standard, were applied prior to determine *trans*-resveratrol in plant sample extracts, and all standard solutions of *trans*-resveratrol were prepared in darkness. For the determination of *trans*-resveratrol in the sample of vine leaf extract by the standard addition calibration the same concentrations as in the LD–REMPI–TOFMS technique were used. Fig. 2B shows the obtained curve ($r = 0.9993$): as it can be seen, the intercept with the *X*-axis is 0.78; considering the dilution ratio 1:3, a 3.1 ± 0.1 mg L^{-1} mean concentration value of *trans*-resveratrol was obtained from the vine leaf extract. A linearity study applied to the same *trans*-resveratrol concentration range using external standard calibration was carried out A plot of the signal versus the *trans*-resveratrol content in the standard solution, shown in Fig. 2B, gives a linear fit with a regression coefficient of 0.998. It is very satisfactory to note that the slope obtained by standard additions is very similar to that obtained by external additions; thus, no matrix effect was observed.

Four samples were analyzed using the external calibration method. The samples as indicated in Section 2 were prepared using the same sample volume and water–acetic acid solution at pH 2.6. Each sample was injected four times into the chromatographic system. A mean 3.2 ± 0.1 mg L^{-1} concentration value of *trans*-resveratrol was obtained in the vine leaf sample extracts.

The precision of the chromatographic method was evaluated by repeating the sample analysis in the same day, and

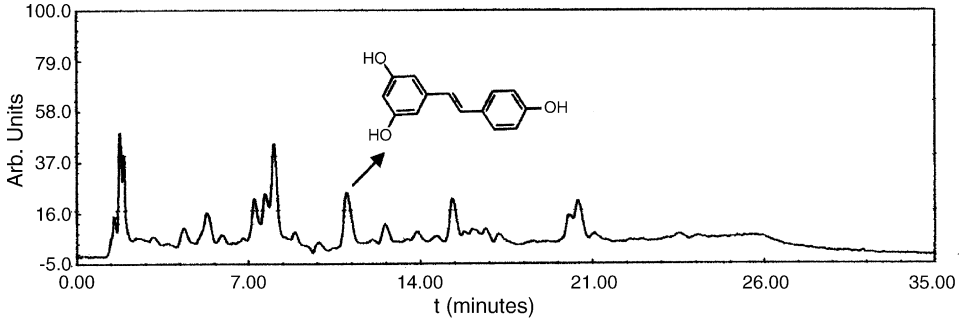

Fig. 3. Chromatogram of a vine leaf extract sample using fluorimetric detection (λ_{ex}, 330 nm and λ_{em}, 374 nm). See text for experimental details.

Table 3
Main analytical parameters for the analysis of *trans*-resveratrol in leaf sample extracts

Analytical parameter	RP-HPLC	LD–REMPI–TOFMS
Linearity ($mg\,L^{-1}$)	0–15	0–20
Reproducibility (%)	4	4.4
Detection limit ($\mu g\,L^{-1}$)	20	2.1
Quantification limit ($\mu g\,L^{-1}$)	67	6.3
Time necessary for 1 measurement (min)	30	45
Mean and confidence interval ($mg\,L^{-1}$)	3.2 ± 0.1	3.0 ± 0.2

in different days and operators at $1.5\,mg\,L^{-1}$ concentration level of *trans*-resveratrol ($n = 4$). The repeatability and reproducibility, expressed as RSD, were 2.6 and 4.0% for four samples which were injected two and four times, respectively.

Table 3 lists the analytical characteristics: calibration, limit of detection (LD, three times the noise to sensitivity ratio 3 N/s) and limit of quantification (LQ, 10 N/s).

3.3. Comparison between both techniques

The comparison between both analytical techniques can be best made with the aid of Table 3. There, the main analytical parameters are included, namely: linearity range, reproducibility, detection and quantification limits, necessary time for one measurement, and confidence interval as an accuracy measurement.

The distinct range of linearity of both techniques is not very significant as it merely reflects the limits employed in this investigation. It is very likely that using the RP-HPLC method a linearity range of 0–40 $mg\,L^{-1}$ or higher could also be obtained. The reproducibility of both methods is also of the same order of magnitude.

The most remarkable differences have been found in the detection and quantification limits obtained for each technique: i.e. 20 and 67 $\mu g\,L^{-1}$, respectively, for the chromatographic analysis and 2.1 and 6.3 $\mu g\,L^{-1}$ for the REMPI technique. This may be due to the fact that the RP-HPLC analysis is carried out without previous sample preparation, injecting directly the vine leaf extract in the chromatograph, hence obtaining a lower sensitivity than one would expect by using sample preparation techniques. The clean up of the sample or the use of the more adequate interphase can improve the LOD or LOQ values of this method. In the case of the LD–REMPI–TOFMS technique, the combination of the laser to desorb the sample with the selectivity of resonant ionization plus the mass spectrometric detection makes sample preparation unnecessary to determine *trans*-resveratrol with a good sensitivity.

Due to the direct injection in the chromatograph, the time needed to perform a single analysis is only 30 min. For the REMPI technique, it is necessary to have a good vacuum (better than 3×10^{-6} mbar) in the chamber for the mass spectrometry detection; in our system it takes ca. 30 min to reach this pressure, although this time could be reduced with additional vacuum pumps. Thus, the RP-HPLC allows a quicker determination of the *trans*-resveratrol content in plant extracts.

In summary, direct RP-HPLC analysis is recommended for *trans*-resveratrol fast routine analysis with detection and quantification limits within the $\mu g\,L^{-1}$ range. For analysis requiring lower values of these analytical parameters (within the low $\mu g\,L^{-1}$ range) the LD–REMPI–TOFMS technique, though slower, is recommended.

4. Concluding remarks

This paper was dedicated to the analysis of *trans*-resveratrol content in vine leaf sample extract using two well known techniques, namely: laser desorption coupled with resonance enhanced multiphoton ionization and time-of-flight mass spectrometry and RP-HPLC with fluorescence detection. One of the main conclusions of the investigation is the capability of both analytical methods to implement the analysis of this polyphenol in spite of the inherent difficulties such as its thermal unstability or its photon-induced isomerization.

Indeed, in the HPLC method, the *trans*-resveratrol degradation for chromatographic separation was prevented by preparing the standard solutions and the samples without daylight, and by keeping them at $-4\,^{\circ}C$.

The main goal of the present investigation was to critically compare both experimental methods for determination of *trans*-resveratrol. From such a comparison, the main conclusions that can be drawn are:

(i) The range of linearity and reproducibility of both methods is of the same order of magnitude.
(ii) The REMPI technique has shown to be more sensitive that the liquid chromatography, mainly due to the fact that the latter requires more sample pre-treatment.
(iii) The direct injection RP-HPLC allows a quicker determination for routine analysis in plant extracts, while the REMPI technique is slower due to the requirements of high vacuum conditions for sample analysis.
(iv) The analysis by HPLC requires previous sample preparation which needs longer time and higher consumption of solvent, sample volume and may suffer from the possibility of losses of analyte during the analytical process. In contrast, these drawbacks are absent in the LD–REMPI–TOFMS technique.

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References

[1] P. Langcake, Physiol. Plant Pathol. 18 (1981) 213.
[2] S.S. Lee, S.M. Lee, M. Kim, J. Chun, Y.K. Cheong, J. Lee, Food Res. Int. 37 (2004) 247.
[3] S. Kallithraka, I. Arvanitoyannis, A. El-Zajouli, P. Kefalas, Food Chem. 75 (2001) 335.
[4] M. Jang, L. Cai, G.O.V. Udeani, K. Slowing, C.F. Thomas, C.W.W. Beecher, H.S.S. Fong, N.R. Farnsworth, A.D. Kinghorn, R.G. Mehta, R.C. Moon, J.M. Pezzuto, Science 275 (1997) 218.
[5] N. Ratola, J.L. Faria, A. Alves, Food Technol. Biotechnol. 42 (2004) 125.
[6] M. López, F. Martínez, C. del Valle, C. Orte, M. Miró, J. Chromatogr. A 922 (2001) 359.
[7] M.A. Rodriguez-Delgado, S. Malovana, J.P. Pérez, T. Borges, F.J. García-Montelongo, J. Chromatogr. A 912 (2001) 249.
[8] G. Stecher, C.W. Huck, M. Popp, G.K. Bonn, Fresenius J. Anal. Chem. 371 (2001) 73.
[9] M.A. Rodriguez-Delgado, G. González, J.P. Pérez-Trujillo, F.J. García-Montelongo, J. Food Chem. 76 (2002) 371.
[10] P. Jeandet, A.C. Breuit, M. Adrian, L.A. Weston, S. Debord, P. Meunier, G. Maume, R. Bessis, Anal. Chem. 69 (1997) 5172.
[11] I. Kolouchová-Hanzlíková, K. Melzoch, V. Filip, J. Smidrkal, Food Chem. 87 (2004) 151.
[12] T. Luan, G. Li, Z. Zhang, Anal. Chim. Acta 424 (2000) 19.
[13] M. Careri, C. Carradini, L. Elviri, I. Nicoletti, I. Zagnoni, J. Agric. Food Chem. 51 (2003) 5226.
[14] J. Pazourek, G. González, A.L. Revilla, J. Havel, J. Chromatogr. A 874 (2000) 111.
[15] D.M. Godberg, E. Ng, A. Karumanchiri, J. Yan, E.P. Diamandis, G.J. Soleas, J. Chromatogr. A 708 (1995) 89.
[16] R. Pezet, V. Pont, P. Cuenat, J. Chromatogr. A 663 (1994) 191.
[17] D.M. Goldberg, E. Tsang, M. Levesque, G.J. Soleas, J. Liq. Chromatogr. Rel. Technol. 22 (1999) 1843.
[18] G.J. Soleas, D.M. Goldberg, E. Ng, A. Karumanchiri, E. Tsang, E.P. Diamandis, Am. J. Enol. Vitic. 48 (1997) 169.
[19] J.P. Roggero, P. Archier, S. Coen, in: T.R. Watkins (Ed.), Wine, Nutritional and Therapeutic Benefits. ACS Symposium Series, No. 661. American Chemical Society, Washington, DC, 1997, p. 6.
[20] S. Malovaná, F.J. García Montelongo, J.P. Pérez, M.A. Rodriguez Delgado, Anal. Chim. Acta 428 (2001) 245.
[21] A.I. Romero Pérez, M. Ibern-Gómez, R.M. Lamuela-Raventós, M.C. de la Torre-Boronat, J. Agric. Food Chem. 47 (1999) 1533.
[22] D.M. Goldberg, E. Tsang, A. Karumanchiri, E.P. Diamandis, G.J. Soleas, E. Ng, Anal. Chem. 68 (1996) 1688.
[23] K.D. McMurtrey, J. Minn, K. Pobanz, T.P. Schults, J. Agric. Food Chem. 42 (1994) 2077.
[24] Q. Chu, M. O'Dwyer, M.G. Zeece, J. Agric. Food Chem. 46 (1998) 509.
[25] J.M. Orea, C. Montero, J.B. Jiménez, A. González Ureña, Anal. Chem. 73 (2001) 5921.
[26] A. González Ureña, J.M. Orea, C. Montero, J.B. Jiménez, J.L. Gonzalez, A. Sánchez, M. Dorado, J. Agric. Food Chem. 51 (2003) 82.
[27] C. Montero, S.M. Cristescu, J.B. Jiménez, J.M. Orea, S. Te Lintel Hekkert, F.J.M. Harren, A. González Ureña, Plant Physiol. 131 (2003) 129.