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Analytical Methods

Post-column on-line photochemical derivatization for the direct isocratic-LC-FLD analysis of resveratrol and piceid isomers in wine

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Abstract

Resveratrol is a stilbene produced by plants, e.g. grapes, in response to stress. Resveratrol is extracted during winemaking, being present in wine as 3-*O*-β-D-gluocoside (piceid) and as aglycone. Both, resveratrol and piceid exist in two isomeric forms, *trans* and *cis*. Resveratrol and piceid are weakly fluorescent in both of their isomeric forms, but highly fluorescent compounds are obtained when the original molecules are UV-irradiated. A chromatographic method with post-column on-line photoderivatization, has been developed for the analysis of resveratrol and piceid isomers. The four analytes are firstly separated in a C18 column (150 mm × 3.9 mm i.d., 4 μm) by isocratic elution, at 15 °C, with a mobile phase consisting of a mixture acetonitrile:*o*-phosphoric acid (0.04%), 18:82, v:v, at 0.9 mL min^{-1}, and secondly they are on-line phototransformed into their fluorescent photoproducts in a 3 m PTFE tube coiled around a 4 W xenon lamp. The elution conditions have been chemometrically optimized by means of the experimental design and the response surface methodology. Linearity ranges from 0.10 to 1.50 and from 0.10 to 1.00 μg mL^{-1} and LOD around 0.001 and 0.01 μg mL^{-1} have been calculated for *trans*- and *cis*-isomers, respectively. The method has been satisfactorily applied to red and white wine samples by standard addition and external calibration, respectively.

Keywords: Resveratrol; Piceid; Post-column photochemical derivatization; LC; Response surface methodology; Wine

1. Introduction

Resveratrol (3,4′,5-trihydroxystilbene), is a stilbene produced by plants in response to fungal infection or abiotic stresses such, e.g. produced by heavy metal ions. It occurs in mulberries, peanuts and grapes. Grapes contain a large amount of different phenolic compounds in skins, pulp and seeds, that are partially extracted during winemaking (Jackson, 1994).

It is believed that the high level of this compound in red wine (0.1–15 mg/L, of total *trans*-resveratrol, i.e. aglycone and glucoside forms) (Fremont, 2000) is linked to the low incidence of heart diseases in some regions of France, the so-called "French paradox", i.e. despite high fat intake, mortality from coronary heart disease is lower due to the regular drinking of wine (Corder et al., 2001; Soleas, Diamandis, & Goldberg, 1997).

This compound has attracted considerable attention due to its various biological and pharmacological activities, including antioxidative and anticancer activities, being implied in the inhibition of cellular events associated with tumour initiation, promotion and progression (Savouret & Quesne, 2002).

Resveratrol-3-*O*-β-D-glucoside (piceid) is the main component of the *Polygonum cuspidatum* root, used in Japanese and Chinese folk medicine for the treatment of some cardiac ailments, including atherosclerosis and inflammation.

trans-Resveratrol, *trans*-piceid, and their *cis*-isomers have been found in wines (Lamuela-Raventós, Romero-Pérez, Waterhouse, & de la Torre-Bororat, 1995).

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c-Resveratrol, *t*-piceid, and *c*-piceid are physiologically as important as *t*-resveratrol. It seems that *t*-piceid can be more effectively absorbed than the aglycone; according to Hollman (1997), the absorption of some phenols from the diet is enhanced by conjugation with glucose.

Piceid has received such attention as resveratrol because, in grape products, the concentration of the glucoside is, usually, significantly higher than the aglycone (Lamuela Raventos & Waterhouse, 1999). The relative distribution between the glucosilated and aglycone forms in wines, is dependent on a number of factors such as fermentation and ecological conditions (Moreno-Labanda et al., 2004). It is a fact that resveratrol in all its forms is found in much higher concentration in red grape varieties compared with white grape ones.

On the other hand, due to the generally high ratio of *t*- to *c*-isomers found in wines, it has been suggested that the *c*-isomers could arise from light exposure of must or wine during the winemaking process or possibly from light exposure of wine bottles during storage (Lamuela-Raventós et al., 1995).

Numerous methods have been described to determine the concentration of the four resveratrol derivatives in wine, most of them, HPLC methods by acidic solvent gradient elution. Currently, HPLC detection procedures are based upon UV absorption (Abril, Negueruela, Pérez, Juan, & Estopañán, 2005; Castellari, Sartini, Fabiani, Arfelli, & Amati, 2002; Vitrac et al., 2005), fluorimetry (Bravo, Silva, Coelho, Vilas Boas, & Bronze, 2006; Piñeiro, Palma, & Barroso, 2006; Vitrac, Monti, Vercauteren, Deffieux, & Mérillon, 2002), electrochemistry (Bravo et al., 2006; Kolouchová Hanzlíková, Melzoch, Filip, & Smidrkal, 2004), and mass spectrometry (Bravo et al., 2006; Loredana La Torre, Saitta, Vilasi, Pellicanó, & Dugo, 2006; Pozo-Bayón, Hernández, Martín-Álvarez, & Polo, 2003).

t-Piceid and *t*-resveratrol themselves are weakly fluorescent, but intense UV-irradiation of their alcoholic solutions, first induces the isomerization into *c*-isomers, also weakly fluorescent, which very quickly disappear in favour of highly fluorescent compounds (Roggero & García-Parrilla, 1995; Roggero, 2000). With the objective of exploiting this photochemical behaviour, in favour of the method sensitivity, a chromatographic method for the analysis of total (*t*- plus *c*-) resveratrol and piceid in wine, previous off-line irradiation of samples, has been proposed (Galeano-Díaz, Duran-Merás, & Airado-Rodríguez, 2007b). The aim of the present work was to design a system in which the photochemical process occurs on-line after the separation of isomers in the chromatographic column, i.e. an on-line post-column LC method.

An isocratic mobile phase has been optimized for wine analysis, by using the experimental design and the response surface methodology (RSM). The method has been validated in terms of linearity and limits of detection, and has been successfully applied to the analysis of wine samples.

2. Experimental

2.1. Chemicals and solutions

All solvents were gradient grade for liquid chromatography (Merck, VWR International Eurolab, SL; Carretera no. 152, km10; 08100 Mollet del Valles, Barcelona, Spain). All other chemicals were of analytical reagent grade and used without further purification. Ultrapure water, obtained from a Millipore Milli-Q System, was used throughout.

t-Resveratrol and *t*-piceid were obtained from Sigma and Aldrich Chem. Co. (Sigma Aldrich Química, SA; Avda. Valdelaparra, 53; 28100 Alcobendas, Madrid, Spain) respectively, and used as received. Ethanolic stock solutions (100 $\mu g\,mL^{-1}$) of each compound were prepared and stored at −4 °C in darkness. Fresh working standard solutions were prepared by appropriate dilution of stock solutions in ultrapure water.

c-Resveratrol and *c*-piceid were *in situ* obtained by exposure of solutions of their *t*-isomers containing 1.5 $\mu g\,mL^{-1}$ to the sunlight for a period of thirty seconds. The concentration of the obtained *c*-isomers was calculated through the decrease of the chromatographic peaks of their respective *t*-isomers.

2.2. Wine samples

Several samples of red and white wine, were obtained in the market. Samples were kept at 4 °C avoiding exposure to direct light.

2.3. Instrumentation and software

The chromatographic studies were performed on a Hewlett–Packard Mod. 1100 LC instrument, equipped with degasser, quaternary pump, manual six-way injection valve containing a 20 μL loop, UV–visible diode-array detector, rapid scan fluorescence spectrophotometer detector and the CHEMSTATION software package to control the instrument, data acquisition and data analysis. The analytical column used was a Nova-Pak C_{18}, 150 mm × 3.9 mm i.d., 4 μm particle size, and 60 Å pore size (Waters Millipore). The column temperature was controlled by a coil with re-circulating water, which temperature was selected through a thermostatic bath. A post-column photoreactor (Softron, Gynkotek HPLC, Germany), consisting of a PTFE tube (3 m × 0.3 mm I.D. × 1.6 mm E.D.) coiled around a 4 W xenon lamp, was placed between the UV–visible diode-array detector and the fluorescence detector. The mobile phase consisted of a mixture acetonitrile:*o*-phosphoric acid (0.04%), 18:82, v:v. Mobile phase components were filtered through a 0.22 μm membrane nylon filter and degassed by ultrasonication before use. The flow rate was 0.9 $mL\,min^{-1}$. Fluorescence detection was performed at 364 nm ($\lambda_{exc} = 260$ nm). A scan range from 200 to 500 nm was performed in DAD. Samples were filtered

through 0.20 μm membrane PTFE filters (Millex®-FG) before injection.

The software package THE UNSCRAMBLER® 6.11_b CAMO ASA (Unscrambler v. 6.11, CAMO A/S Olav Tryggvasonsgt, N-7011, Trondheim, Norway), running under Windows XP, was used for the application of chemometrics.

2.4. General procedure: calibration curves

Aliquots of *t*-resveratrol and *t*-piceid ethanolic solutions, containing between 1.0 and 10 μg of each one of them, were placed in 10.0 mL volumetric flasks, and ultrapure water was added up to the mark. Aliquots (20 μL) of each solution were injected into the chromatographic system, previous filtration through 0.20 μm membrane PTFE filters. The eluate was fluorimetrically monitored at 364 nm ($\lambda_{exc} = 260$ nm).

To obtain the calibration curves for the *c*-isomers, the procedure was: solutions containing 1.5 μg mL^{-1} of *t*-resveratrol and *t*-piceid were exposed to the sunlight for thirty seconds. The resulting solutions were injected into the chromatographic system and the concentration of *c*-resveratrol and *c*-piceid in this solution was calculated through the decrease observed in the chromatographic peaks corresponding to their *t*-isomers, quantifiable through their respective calibration curves, previously established. It was proven that the solutions containing *c*-isomers, kept in the dark, were stable at least for 24 h. Calibration curves for *c*-isomers were established from *c*-resveratrol and *c*-piceid samples, prepared by successive dilution of the sun-irradiated solutions, in the concentration range from 0.10 to 1.0 μg mL^{-1}. Aliquots (20 μL) of each standard solution were injected into the chromatographic system, and eluted and monitored as previously specified.

2.5. Procedure for the analysis of resveratrol and piceid isomers in wine samples

2.5.1. Red wines

The analysis of the four compounds in red wine was carried out by the standard addition method. Identical aliquots of wine were added into a set of flasks, and increasing volumes of a standard mixture of the four isomers, previously quantified, and ultrapure water were added to the mark. Aliquots (20 μL) of each one of these solutions were injected into the chromatographic system.

2.5.2. White wines

In this case, the analysis was carried out by external standard calibration. Aliquots of 5.0 mL of wines and ultrapure water up to the mark were added in a 10-mL volumetric flask. 20 μL of the resulting solutions were directly injected into the chromatographic system. The concentration of the four analytes in white wine was calculated from their respective calibration graphs.

3. Results and discussion

The study about the photoinduced spectrofluorimetric behaviour of resveratrol and piceid has been recently published (Durán Merás, Galeano Díaz, & Airado Rodríguez, 2008; Galeano Díaz, Durán Merás, & Airado Rodríguez, 2007a). In short, hydroethanolic solutions of *t*-resveratrol and *t*-piceid are weakly fluorescent, showing two excitation maxima centered to 225 and 318 nm & 230 and 300 nm, respectively, and a single emission maxima centered to 385 and 395 nm, respectively. It has been proven that intense UV-irradiation of those solutions, under a high pressure mercury lamp, totally transforms resveratrol and piceid into highly fluorescent compounds with a sharp excitation maxima at 260 nm, and two emission maxima at 364 and 382 nm & 361 and 380 nm, respectively.

On the other hand, this photochemical behaviour, has been also exploited for the chromatographic analysis resveratrol and piceid in wine, previous photochemical derivatization, (Galeano-Díaz et al., 2007b). However, the main drawback of that method is the fact that total amounts (*t*- plus *c*-isomer) of each one of the analytes are determined, and it is not possible to determine the relative isomeric distribution of each one of them, because no the original analytes present in the sample, but their photoproducts, are injected and separated into the chromatographic system. In the method reported in this paper, *t*- and *c*-isomers of resveratrol and piceid are eluted in their original state, and once eluted and photometrically detected, they are phototransformed into their respective fluorescent photoproducts, in order to increase the sensibility and selectivity in the detection.

Most of the previously proposed chromatographic methods for the analysis of these analytes in wine, are reverse phase methods, and carry the elution out by a gradient, being the mobile phases mixtures of diluted acid solutions and organic solvents. Acetic and formic acids, and methanol and acetonitrile, are the most common acidic and organic components of mobile phases, respectively. In the development of a chromatographic method in which a post-column derivatization reaction goes on-line, it is highly important the composition of the mobile phase not only for the separation, but for the detection. Thus, some previous assays were carried out by fluorescence spectroscopy. It was proven that the post-column photoreaction goes equally in neutral and acidic media when the pH was fixed by the addition of diluted hydrochloric acid. It was also proven the minor photoinduced fluorescent signals obtained in presence of any other acid, specially the organic ones, and finally *o*-phosphoric acid was chosen as a compromising solution. Regarding to the organic component of the mobile phase, it was proven that the rate of the photoreactions and the maximum photoinduced signal were similar in presence of different percentages of methanol or acetonitrile. Finally acetonitrile was chosen, mainly because of its lower viscosity, higher elution strength, and lower UV absorptivity.

A study was first done to evaluate the effect of the photoreactor on the fluorimetric detection of a mixture of

t-resveratrol and *t*-piceid. For this purpose, a mixture of both *t*-isomers was injected and eluted with a mobile phase consisting in acetonitrile:water 19:81, v:v, with the photoreactor turned OFF. Two chromatographic peaks were observed at 4.87 and 15.35 min, corresponding to *t*-piceid and *t*-resveratrol, respectively. A second injection under the same conditions, but with the photoreactor turned ON, showed that both compounds were greatly modified by photoreaction, and their fluorescent photoproducts, discussed above, had been obtained in a certain extension. Fig. 1A shows a comparison of the obtained FLD-profiles with and without post-column photoderivatization.

Besides, it was chromatographically proven that increasing the content of *o*-phosphoric acid in the mobile phase, implies a slight reduction in the retention times and a loss of sensitivity in *t*-resveratrol and *t*-piceid chromatographic peaks.

Lastly it is too important to highlight that nylon filters cannot be used to filter solutions containing resveratrol or piceid because it was proven that 100% of the analyte was retained in the membrane. Hydrophobic fluoropore (PTFE) filters are used throughout this work.

3.1. Optimization of the chromatographic conditions, by the RSM, for the sensitive analysis of wine samples

Separation and photoreaction results have to be taken into account to select the best mobile phase. Thus the nature of the mobile phase components was chosen looking for the best environment for the reaction course, namely acetonitrile as organic modifier and *o*-phosphoric acid to fix the pH, were finally selected, as said above. On the other hand, in the present case, a photochemical reaction is going to take place, which implies that ultraviolet light could be considered as one of the reactants, and its concentration will be controlled through the residence time of the analytes in the photoreactor, that is, the mobile phase flow. Because of this, the sensitivity of the method will be mainly controlled through the mobile phase flow.

An isocratic mobile phase has been looked for, and for the optimization of its composition, i.e. the percentage of acetonitrile and *o*-phosphoric acid, and its flow rate, the experimental design, namely a central composite design, has been used with the aim of calculating simultaneously the effect of the change in each one of the variables, and also their possible interactions. Five levels were fixed for each variable. There are also three central samples, giving rise to a total of 17 experiments. The assayed levels for each one of the variables were: % vol. acetonitrile: 25.1, 23.0, 20.0, 17.0, 15.0; % vol. H_3PO_4: 0.05, 0.04, 0.03, 0.02, 0.01; flow: 1.50, 1.30, 1.00, 0.70, 0.50. This design is rotatable, and therefore the precision in the calculation of the response is uniform over the whole experimental field.

A standard solution containing 0.50 μg mL^{-1} of *t*-resveratrol and *t*-piceid, respectively, and a sample containing 7.50 mL of a pool of commercial red wines, fortified with 13 μg of *t*-resveratrol and *t*-piceid, respectively, in a final volume of 25.0 mL, were eluted under the conditions indi-

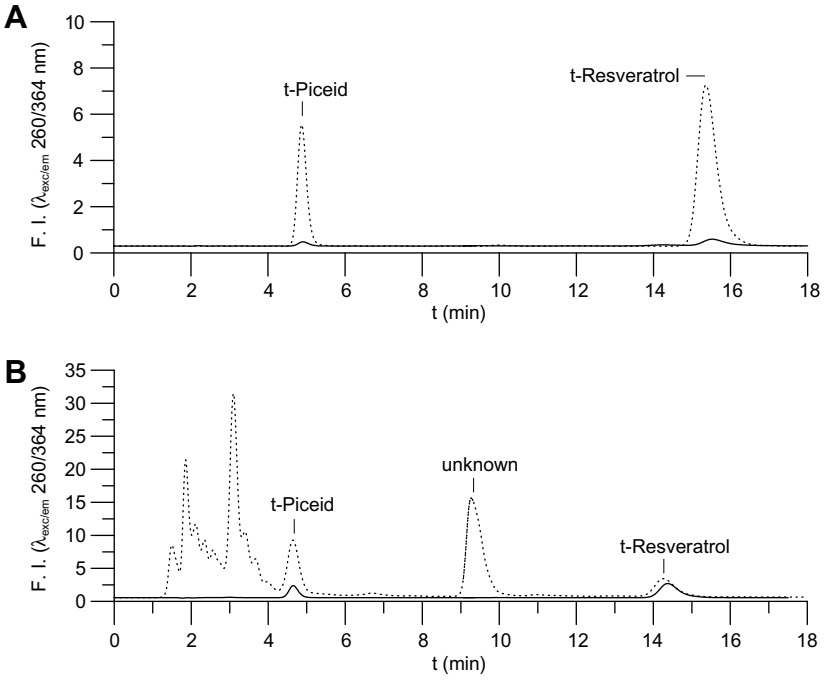

Fig. 1. (A) Chromatograms corresponding to a mixture containing 1.00 μg mL^{-1} of *t*-resveratrol and *t*-piceid, obtained with the photoreactor turned OFF (——) and ON (····). Mobile phase acetonitrile:water 19:81, v:v, 0.9 mL min^{-1}; FLD at $\lambda_{exc/em}$ 260/364 nm. (B) FLD-profiles (FLD $\lambda_{exc/em}$ 260/364 nm) corresponding to a standard sample containing 0.50 μg mL^{-1} of *trans*-resveratrol and *trans*-piceid, respectively (——), and a sample containing 7.50 mL of a pool of commercial red wines, fortified with 13.0 μg of *trans*-resveratrol and *trans*-piceid, respectively, in a final volume of 25.0 mL (····), eluted under conditions predicted as optimum and submitted to post-column photoderivatization.

cated by the design. The photoreactor was turned ON during all the experiments, and the eluate was photometrically and fluorimetrically monitored at 306 nm, and $\lambda_{exc/em}$ 260/364 nm, respectively.

In the selection of the response function (RF), sensitivity and sensibility parameters have been taken into account. The selected response function was

$$RF = R^*_{s(t\text{-Pic})} + \left(\frac{PA_{t\text{-R}(h\upsilon)} + PA_{t\text{-P}(h\upsilon)}}{2}\right)_{norm} \quad (PA = \text{Peak Area})$$

The first term represents the performance of the chromatographic separation, and the second one, the method sensitivity. The resolution of the *t*-piceid peak with respect to the wine interferences is taken as representative of the performance. In some samples of the design, this peak appears resolved from the pre-eluted or the post-eluted interferences, and in some other points it is completely co-eluted with wine interferences. So, $R^*_{s(t\text{-Pic})}$ is the less favourable resolution value for the peak corresponding to *t*-piceid, multiplied by two when the other resolution value for this peak is higher than 1.5. The peak corresponding to *t*-resveratrol was fully resolved in all of the assayed conditions, and because of this, the resolution of this peak was not included in the response function. The second term, evaluates the sensitivity through peak areas values. These depend on the photoreaction yield and fluorescence quantum yield of the on-line generated photoproducts. This term has been obtained by normalizing the mean value of the peak areas of phototransformed *t*-resveratrol and *t*-piceid, measured in the standard mixture chromatograms. The strategy of normalizing the second term has been carried out to give a similar importance to both terms. The retention times were not considered, as in any case they were not longer than 30 min, an acceptable analysis time for an isocratic method.

The optimization of the model was made with THE UNSCRAMBLER software package (Unscrambler v. 6.11, CAMO A/S Olav Tryggvasonsgt, N-7011, Trondheim, Norway), and the results were interpreted with the Response Surface Methodology. In the corresponding analysis of variance (ANOVA) a second-grade quadratic model is assumed (model check quadratic, *p*-value (95%) = 0.0008). It was proven that the effects % ACN (*p*-value = 0.0310) and flow (*p*-value = 0.0067) and the interactions % ACN–% ACN (*p*-value = 0.0019), % H_3PO_4–% H_3PO_4 (*p*-value = 0.0002), and flow–flow (*p*-value = 0.0001) contribute significantly to the model for a 95% confidence level. Nevertheless, the effect % H_3PO_4 (*p*-value = 0.0765), and the interactions % ACN–% H_3PO_4 (*p*-value = 0.1348), % ACN–flow (*p*-value = 0.1013), and % H_3PO_4–flow (*p*-value = 0.1725) are not significant to the model for the considered confidence level. On the other hand, the *p*-value (95%) calculated for lack of fit was 0.0583, which means that the model describes the true shape of the response surface. Fig. 2 shows the response surfaces estimated by the model, for each pair of variables. In these surfaces, the maximum value of RF corresponds to the values of variables: 18% ACN, 3.3×10^{-2} % H_3PO_4, and flow 0.9 mL min^{-1}. Thus, the mobile phase selected as optimum was acetonitrile:*o*-phosphoric acid (0.04%), 18:82, v:v, at a flow rate of 0.9 mL min^{-1}. Under these conditions, both analytes are

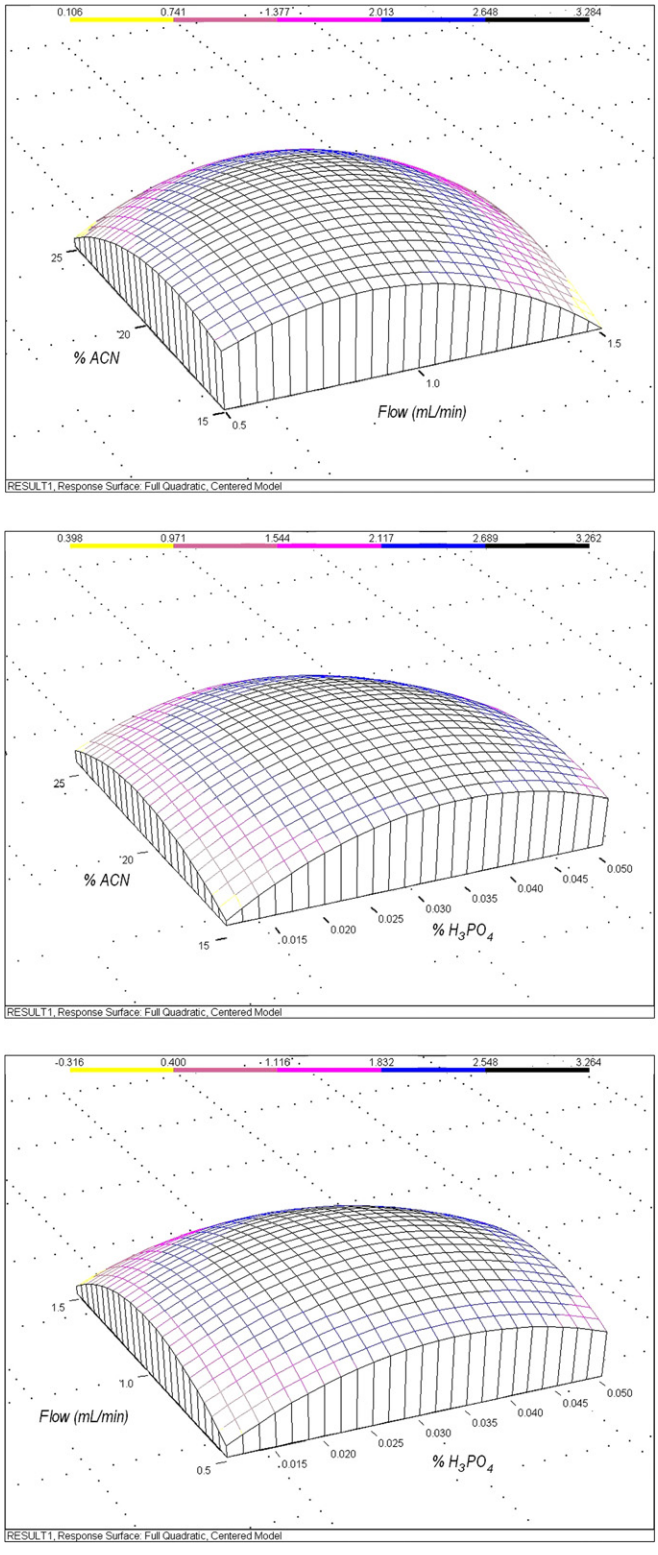

Fig. 2. Estimated response surfaces for each pair of variables.

resolved to the baseline in less than 15 min, as shown in Fig. 1B, and the retention times and capacity factors are 4.65 and 14.37 min, and 2.1 and 8.58, respectively, for *t*-piceid and *t*-resveratrol, respectively.

3.2. Influence of the temperature

In a study of the interday precision, a poor reproducibility in the retention times for both compounds, and in the resolution of the peak corresponding to *t*-piceid with regard to the wine interferences, was observed when injections were carried out without fixing the column temperature, and because of this, the influence of the temperature was thoroughly studied at this point.

Thus, a standard sample containing 1.0 μg mL^{-1} of *t*-resveratrol and *t*-piceid, and a commercial red wine sample three-times diluted with ultrapure water, were eluted under optimum conditions, keeping constant the column temperature by a coil with re-circulating water, which temperature was controlled through a thermostatic bath. The assayed values were 10, 15, 20, 25, and 30 °C.

An increase in the capacity factors of both compounds was observed as decreasing the temperature. On the other hand, it was found that when the temperature was higher than 15 °C, a wine interference coeluted with *t*-piceid. Finally, a temperature of 15 °C was selected as optimum, and it was fixed for further analysis.

3.3. cis–trans Isomery

It has been described in the literature (Roggero, 2000; Roggero & García-Parrilla, 1995), and spectrophotometrically proven (Durán Merás et al., 2008; Galeano Díaz et al., 2007a), the *cis–trans* photoisomery of resveratrol and piceid.

c-Resveratrol and *c*-piceid are originated by exposing the corresponding *t*-isomers to sunlight, as shown in Fig. 3A. When the sunlight-exposed sample is eluted under conditions previously deducted as optimum, two new peaks are observed at 11.30 and 35.04 min, corresponding to *c*-piceid and *c*-resveratrol, respectively, apart from peaks corresponding to *t*-resveratrol and *t*-piceid. The establishment of the nature of those peaks has been carried out by obtaining the spectra in the peak with the diode-array detector. The peaks corresponding to *c*-isomers are totally resolved with regard to the corresponding to the *t*-ones. On the other hand, *c*-isomers are less polar than the *t*-ones, probably due to the intra-molecular interaction type hydrogen-bond between hydroxyl groups.

In Fig. 3B, the FLD-chromatogram corresponding to a commercial red wine sample 20-times diluted, and a standard mixture of *t*-resveratrol and *t*-piceid 30 s exposed to the sunshine, are shown. In both cases the photoreactor is turned ON, and the corresponding fluorescent photoproducts from *t*- and *c*-isomers are successfully obtained.

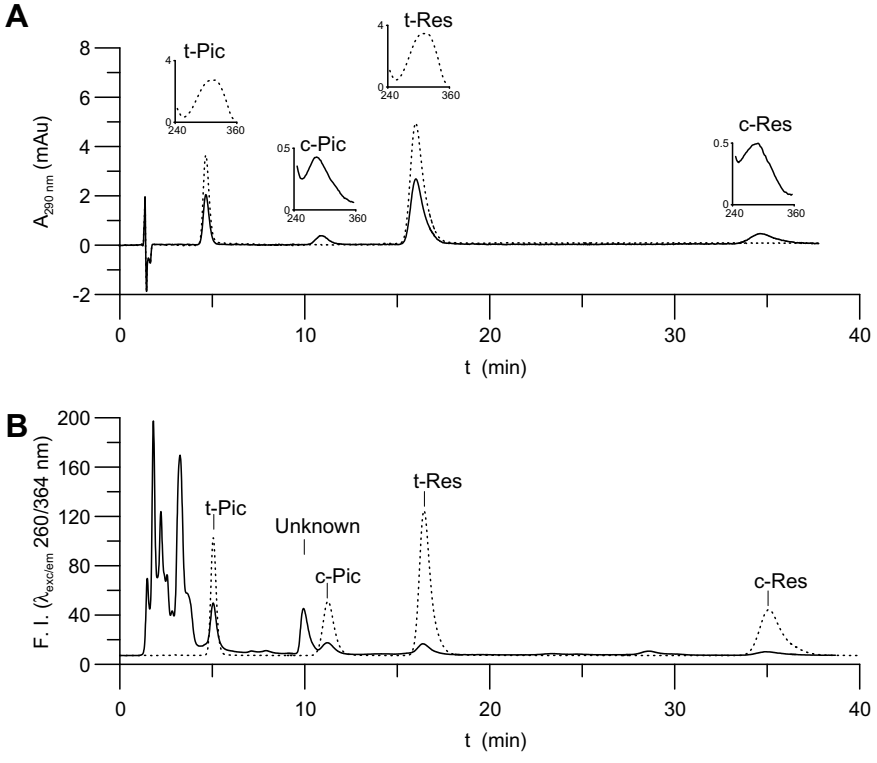

Fig. 3. (A) $A_{290\ nm}$-profiles corresponding to a sample containing 0.80 μg mL^{-1} of *trans*-resveratrol and *trans*-piceid before (····) and after (—) a 30 s exposure to the sunshine, and apex-spectra on-line registered. (B) FLD-profiles corresponding to a sample containing 1.40 μg mL^{-1} of *trans*-resveratrol and *trans*-piceid 30 s exposed to sunshine (····) and a commercial red wine 20-times diluted (—).

As it is observed, the four isomers are present in the real sample. It is also observed a total resolution between the four compounds of interest and the wine interferences, and because of that it was not considered to change the mobile phase.

It has been proven that intense ultraviolet irradiation, under a high pressure mercury lamp, totally transform *t*- and *c*-resveratrol and *t*- and *c*-piceid in highly fluorescent photoproducts. A single photoproduct (FR) is originated from resveratrol, and two photoproducts (FP1 and FP2), isomers between them, from piceid (Galeano-Díaz et al., 2007b). It is very important to highlight that the exposition to the sunshine has to be carried out for a time not so long in order to avoid the appearance of the corresponding fluorescent photoproduct FP1, FP2, and FR, and thus avoiding errors in the *c*-quantification. It has been proven that only *c*-isomers, and not the fluorescent forms, are generated when samples are exposed for 30 s under the sunshine, by comparing the chromatograms obtained for a sample irradiated under a high pressure mercury lamp in the necessary conditions to obtain the fluorescent compounds, and the corresponding to a 30 s exposed to the sunshine. This fact has also been proven by following the photoreaction at the wavelength (275 nm) corresponding to the isobestic point of the on-line obtained spectra for each pair of *t*- and *c*-isomers. This isobestic point is shown in Fig. 4, where the spectra obtained in the peak apex for the pair *c*- and *t*-piceid are represented. Since at this wavelength the molar absorptivity of both isomers is the same, the peak area vs. concentration ratio is also the same for the two peaks. Thus, the total area must be kept constant as the concentration of each pair *cis–trans* does. This has been proven to be true in the present case, and it was also confirmed by the absence of any other photoproducts.

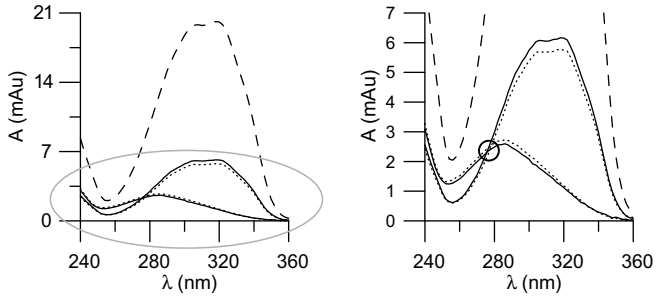

Fig. 4. Apex-spectra on-line obtained corresponding to a solution containing *trans*-piceid before exposure to sunshine (—), and after a exposure of 30 s (—) and 60 s (····). Isobestic point, at 275 nm, is rounded in the right figure.

With the aim of quantifying the four isomers in wine, the corresponding calibration graphs for the *c*-isomers were obtained by relating the area of the new peak attributed to the *c*-isomers, with the difference between the concentrations of *t*-isomers, prior and after exposure to sunshine.

3.4. Method validation: analytical parameters

The linearity of the method, was assessed by preparing calibration standards with concentration from 0.10 to 1.50 μg mL^{-1} of *t*-resveratrol and *t*-piceid and from 0.10 to 1.00 μg mL^{-1} of *c*-resveratrol and *c*-piceid, as previously explained. Each standard was introduced in the chromatographic system in triplicate, and eluted under optimum conditions. Calibration curves for each one of the four analytes, were built by plotting the mean of the obtained area for the phototransformed analytes as a function of the standard concentration. The results obtained are summarized in Table 1.

The limits of detection (LOD) and quantification (LOQ) were obtained as 3 and 10 times, respectively, the ratio signal/noise. So, the average amplitude of the baseline of standard chromatograms, measured in different zones, for time intervals around 2 min, was multiplied by 3 or 10, and the concentrations of the respective analyte to these signals, were calculated with the respective calibration curves constructed employing the peak height as analytical signal. The obtained values are summarized in Table 1.

3.5. Determination of resveratrol and piceid isomers in wine samples

The optimized method was applied to the analysis of wine samples.

Firstly, a standard addition was carried out on commercial red and white wines pools in order to examine the possible matrix effect. Within the assayed whites wines it was proven the absence of matrix effect, and because of that, further analysis were carried out by external calibration.

Table 1
Analytical and statistical parameters for the chromatographic determination of resveratrol and piceid isomers (FLD $\lambda_{exc/em}$ 260/364 nm)

	t-Resveratrol	*c*-Resveratrol	*t*-Piceid	*c*-Piceid
Linearity range (μg mL^{-1})	0.10–1.50	0.10–1.00	0.10–1.50	0.10–1.00
Calibration curve	$y = (10581 \pm 355)x$	$y = (2820.2 \pm 185.1)x$	$y = (4366.9 \pm 112.6)x$	$y = (1902.5 \pm 241.5)x$
(peak area vs. μg mL^{-1})	$-(673 \pm 340)$	$+(320.3 \pm 104.7)$	$-(432.9 \pm 117.0)$	$+(166.4 \pm 116.7)$
r	0.9944	0.9957	0.9967	0.9843
LOD[a] (μg mL^{-1})	0.0012	0.014	0.0014	0.0071
LOQ[b] (μg mL^{-1})	0.0041	0.046	0.0047	0.023

[a] S/N = 3.
[b] S/N = 10.

Table 2
Concentration of resveratrol and piceid isomers found in the analyzed wines. Analysis was done in triplicate in all cases

	t-Piceid ($\mu g\ mL^{-1}$)	*c*-Piceid ($\mu g\ mL^{-1}$)	*t*-Resveratrol ($\mu g\ mL^{-1}$)	*c*-Resveratrol ($\mu g\ mL^{-1}$)
Commercial red wines POOL 1	6.5 ± 0.5	2.9 ± 0.3	1.4 ± 0.1	0.21 ± 0.12
Commercial red wines POOL 2	1.2 ± 0.1	1.5 ± 0.1	0.070 ± 0.050	0.060 ± 0.020
Commercial white wines POOL	0.099 ± 0.013	0.42 ± 0.02	0.054 ± 0.009	0.068 ± 0.010
Noble rot white wine (*Tokaji*)	0.29 ± 0.05	0.70 ± 0.02	0.070 ± 0.010	0.064 ± 0.050

In red wines, unlike in the white ones, it was found the existence of matrix effect, which implies that the analysis has to be carried out by the standard addition method. Besides it was deducted that the origin of this matrix effect is in the photochemical process, because no matrix effect was observed when peak areas were measured in the DAD-profile, being this detector before the photochemical reactor in the experimental device.

Two commercial red wines pools and a white one were analyzed. Besides a noble rot white wine, from Hungary (Tokaji) was also analyzed. Results are summarized in Table 2. Higher levels of the four compounds, mainly of piceid isomers, are found in this wine than in an ordinary white wine, because this one is made from *botrytized* grapes.

4. Conclusions

Resveratrol and piceid molecules are stilbenoids ones, and as such they suffer interesting photochemical reactions. Our research group following with the aim of exploiting this photochemical behaviour, proposes an isocratic-LC method with post-column on-line photoderivatization, which let us firstly separate the *cis–trans*-pairs, and then phototransform each compound in a highly fluorescent one. The photochemical reaction has been doubly exploited in this case, firstly to obtain the *c*-compounds, which are not commercially available, and secondly to improve the fluorimetric detection.

As far as we know this is the first time that an on-line post-column photoderivatization system is proposed for resveratrol and piceid isomers analysis.

The analysis of four compounds in wine samples has been carried out in a total time of 35 min, without sample treatment.

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