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Sensitized synchronous fluorimetric determination of *trans*-resveratrol and *trans*-piceid in red wine based on their immobilization on nylon membranes

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ABSTRACT

It has been carried out the determination of *trans*-resveratrol and *trans*-piceid in red wine samples by using room temperature synchronous fluorescence, sensitized through their retention on nylon membranes, in front-face mode. These compounds are weakly fluorescent in solution but their retention allows using the native fluorescence of these compounds as analytical signal, due to the increase in the medium rigidity. To determine these compounds in red wine, a previous liquid–liquid extraction is necessary and in the case of *trans*-resveratrol it is also necessary a previous cleanup stage using C18 cartridges. Diethylether and ethyl acetate are the selected extractant solvents for *trans*-resvertarol and *trans*-piceid, respectively. The retention on nylon membranes was carried out by immersion of the membranes in solutions of these compounds. Variables involved in the retention and measurement processes were optimized, and the analytical figures of merit were obtained under optimal conditions. Ethanol:water 10:90 v:v and ethyl acetate were the solvents used for the retention of *trans*-resveratrol and *trans*-piceid, respectively and, for each case a immersion time of 300 and 600 s was selected. Satisfactory linear relation between fluorescence intensity and concentration was found in the intervals 0.040 and 0.242 mg L^{-1} of *trans*-resvertarol and 0.009 and 0.288 mg L^{-1} of *trans*-piceid. Concentration of 1.08 ± 0.21 mg L^{-1} for *trans*-resveratrol and 1.49 ± 0.36 mg L^{-1} for *trans*-piceid were found in a wine sample obtained from a pool of commercial red wines.

1. Introduction

Resveratrol (3,4′,5-trihydroxystilbene) is a stilbene-like phytoalexin which has been identified as a natural constituent in several plant species. Resveratrol in plants plays a role of natural pesticide since the plants uses it as a defence against external aggressions. Its production is stimulated by stress or fungal infection [1], among other causes. It occurs e.g. in mulberries, peanuts kernels and grapes. Also grapes contain a large amount of resveratrol and other phenolic compounds in skins, pulp and seeds, which are partially extracted during the winemaking process [2], making these compounds natural constituents of wine. Indeed, grapes and wine are considered the most important dietary sources of resveratrol for humans [3].

In wine, resveratrol occurs free and as resveratrol-3-β-D-glucoside, also called piceid. Isomers *trans* and *cis* of both compounds have been identified in wine. However, due to the generally high ratio of *trans* to *cis* forms in wines, it has been suggested that the *cis*-isomers could arise from light exposure of must or wine during the winemaking process or possibly from light exposure of wine bottles during storage [4]. On the other hand, the normal concentration of the glucoside in grape products is significantly higher than the concentration of the aglycone [3], and that is why piceid has received similar attention as resveratrol. The relative distribution between the glucosidic and the aglyconic forms in wine depends on several factors such as fermentation procedures or ecologic conditions in the vineyard [5]. The ratio glucoside/aglicone could be use in the future as a parameter for the control in the wine industry, especially to detect irregularities or even fraud in appellations.

The levels of resveratrol and piceid in red wines from different regions have been compared by Stervo et al. [6] and according to these authors, maximum levels of 14.3 and 29.2 mg L^{-1} for *trans*-resveratrol and *trans*-piceid, respectively, can be found in red wines. On the other hand, 100 μg L^{-1} has been reported [7] as the maximum concentration of resveratrol in white wines. The higher levels found in red wine are mainly due to the winemaking process, as the elaboration of red wine implies longer times of contact between the must and the grape-skins.

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The determination of phenolic compounds in wine is important as they play an important role in sensory properties such as colour, flavour, astringency and hardness [8]. Besides, the scientific community and the society in general, have shown an especial interest in resveratrol and piceid, due to their various biological and pharmacological activities. Among other effects, resveratrol has shown to reduce cell death from oxidative stress [9], and can inhibit cellular events associated with tumour initiation, promotion and progression [10]. Piceid is physiologically as important as resveratrol, indeed it can be more effectively absorbed than the aglycone, as according to Hollman [11], and the absorption of some phenols from the diet is enhanced by conjugation with glucose.

Liquid and gas chromatography and capillary electrophoresis have been widely exploited for the analysis of resveratrol and piceid in wine. RP-LC by acidic solvent gradient elution combined with DAD, fluorimetric (FLD), electrochemical or MS-detectors, has been the mostly employed methodology for the analysis of both compounds in wine [12]. The lower detection limits reported so far have been achieved by LC-chemiluminiscent detection [13], and LC-FLD with off-line [14] or on-line post-column photochemical derivatization [15]. Gas chromatography tandem MS [16], micellar electrokinetic chromatography (MEKC) with UV detection [17], and capillary zone electrophoresis (CZE) with DAD [18] or electrochemical detection [19], have been also employed for the analysis of resveratrol in wine. Pre-treatment stages for the extraction of the compounds of interest and cleaning-up of the extracts are common even prior to the chromatographic or electrophoretic separation, due to the complexity of the matrix. Liquid–liquid extraction followed by evaporation to dryness and re-dissolution [12], and solid-phase extraction (SPE) [20], have been traditionally employed. Additional and more complex cleaning-up procedures such as cation-exchange chromatography are also reported [21].

Fluorescence has been typically employed as detection system after chromatographic separation, however spectrofluorimetric methods for the determination of resveratrol and piceid in wine are scarce, and just two methods based on photochemically induced fluorescence, published by Galeano et al. [22] and Durán et al. [23], are the only ones found in the literature so far, for the analysis of resveratrol and piceid, respectively. In both methods a liquid–liquid extraction of wine is carried out prior to the fluorescence measurement.

Solid-Surface Room Temperature Fluorescence (SS-RTF) is based in the same fundamentals as fluorescence in solution, but in the case of SS-RTF the measurements are performed on a solid surface in which the analytes are retained. This implies an enhanced sensibility as a consequence of the increase in the medium rigidity and the preconcentration on the membrane. Several materials have been successfully used as solid supports, starch, sucrose, sodium acetate, ion-exchange resins, etc. Probably it has been filter paper the most traditionally employed support to induce luminescence, however it presents some drawbacks as providing high background signals. In this context, nylon has arisen as a new and promising solid support. Nylon presents a high sensitivity, it is easily managed, and it provides low background signals. Escándar et al. [24] and Bortolato et al. [25,26] have already demonstrated that nylon is a suitable support to induce fluorescence signals from different compounds retained on its surface, such as the pharmaceutical carbamazepine or polycyclic aromatic hydrocarbons (PAHs).

It is important to develop new, competitive and validated analytical methodologies for the accurate determination of resveratrol and piceid in wines, due to the current lack of official methods for this propose. The competitiveness should be contemplated in terms of sensitivity, selectivity, reproducibility and accuracy, consumption of solvents and time, complexity of the required apparatus and costs. Taking into account all of these requirements, a novel, sensible, simple and cheap method for the determination of these compounds in wines, is presented in this article. The method here described combines the sensitivity of the fluorimetric techniques and the ability of nylon membranes to retain resveratrol and piceid on its surface. The analytes, retained on the nylon surface are determined by synchronous front-face spectra, directly recorded on the surface of the solid support with an illumination angle of 34°. Very small volumes of organic solvents are required, and the involved equipment is very simple. The quality of the method is evaluated through its analytical figures of merit and by its performance on real wine samples.

2. Experimental method

2.1. Reagents

For all experiments, analytical reagent grade chemicals and solvents were used. Ultrapure water, obtained from a Millipore Milli-Q System, was used throughout.

Resveratrol and piceid (*trans*-isomers) were obtained from Sigma and Aldrich Chem. Co. (Sigma Aldrich Química, SA; Avda. Valdelaparra, 53; 28100 Alcobendas, Madrid, Spain) and used as received. 50.0 mg L^{-1} stock solutions were prepared in 250.0 mL coloured volumetric flask, by dissolving the suitable amount of the commercial products and diluting up to the mark with 96% ethanol. These solutions were stored at 4 °C, avoiding exposure to direct light. Fresh solutions of lower concentrations were daily prepared by appropriate dilution of the stock solution with the selected solvent.

Buffer solution of sodium hydrogen tartrate/disodium tartrate (total concentration 0.30 mol L^{-1}) was prepared by dissolving the suitable amount of disodium tartrate dihydrate in water and adjusting the pH of the resulting solution to 5.0 with small volumes (in the order of microlitres) of diluted hydrochloric acid.

13 mm ⌀, 0.22 μm pore size nylon membranes supplied by Teknokroma (Teknokroma, S. Coop. Ltda, Camí de Can Calders 14, Sant Cugat del Vallés, 08190 Barcelona, Spain), were used as received. Any conditioning of the membranes was not required and no significant background emission was found, in the measurement conditions employed in this work.

Sep-Pak Plus C18 cartridge (Sorbent Weight 360 mg; Particle Size 55-105 μm; Pore Size 125 Å) from Waters Cromatografia SA (Ronda de Can Fatjó 7-A, Parc Tecnològic del Vallès, 08290 Cerdanyola del Vallès, Barcelona, Spain) were utilized in sample preparation.

2.2. Apparatus

Fluorescence spectral measurements were performed using a Varian Cary Elipse fluorescence spectrophotometer, equipped with two Czerny-Turner monochromators and a xenon flash lamp, and connected to a PC microcomputer via an IEEE 488 (GPIB) serial interface. The Cary Elipse software was used for data acquisition.

The fluorescence spectrophotometer was equipped with a variable-angle front-face accessory, to ensure that reflected light, scattered radiation and depolarization phenomena were minimized. This accessory was equipped with a support for the retention of solid samples, as shown in Fig. 1. The angle of incidence, defined as the angle between the excitation beam and the perpendicular to the cell surface, was fixed at 34°.

When measurements in solutions were made, a 10-mm quartz cell at 15 °C (room temperature) was used.

The chromatographic studies were performed, utilizing a method previously developed by the authors [15] which allows separately determine *trans*- and *cis*-isomers, on a Hewlett–Packard Mod. 1100 LC instrument, equipped with degasser, quaternary

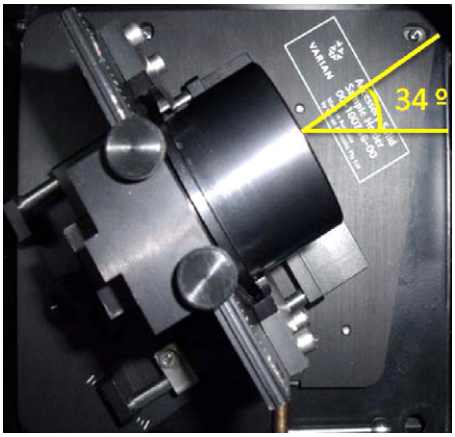

Fig. 1. Device utilized for the front-face fluorescence measurement on the nylon membrane.

pump, manual six-way injection valve with a 20 μL loop, UV–vis 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 C18, 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–vis 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 0.22 μm membrane nylon filters and degassed by ultrasonication before use. Since the photometric and fluorimetric detectors were both operative in the employed LC-instrument, the monitorization of the eluate was carried out photometrically and fluorimetrically all the time. The wavelengths 306, 290 and 260 nm were set in the photometric one for the detection of *trans*- and *cis*-isomers and fluorescent photoproducts, respectively. The fluorescence detection was performed at 364 nm (λ_{exc} = 260 nm) to look for photoproducts.

The photomicrographs of a 0.22 μm pore size nylon disc, after its immersion in the corresponding solvents or in solutions of each one of these compounds, were realized with a Scanning Electron Microscope (SEM) model HITACH S-3600N using a voltage of 15 kV and a secondary electrons detector (ED).

2.3. Wine samples

The analyzed red wine sample was obtained from a pool of red wines obtained in the market and it was kept at 4 °C avoiding exposure to direct light.

2.4. Retention of trans-piceid and trans-resveratrol on the nylon surface. Calibration curves

To obtain the calibration curve for resveratrol, aliquots of 2.00 mL of *trans*-resveratrol solutions in ethanol:water, 10:90, v:v, containing between 0.040 and 0.242 mg L^{-1} of it, were placed in 25 mL beakers and nylon membranes were immersed in these solution with magnetic agitation constant during 5 min. Subsequently, membranes were removed from the solution and were completely dried with a nitrogen stream. For SS-RTF measurements, these membranes were placed in the holder of the front-face solid sample accessory and the synchronous spectra were collected with an incident angle of 34°, with a $\Delta\lambda$ = 46 nm, being the excitation and emission slit widths 2.5 nm. The fluorescence measurements were made at $\lambda_{exc}/\lambda_{em}$ 331/377 nm.

The procedure for piceid was similar: aliquots of 2.00 mL of *trans*-piceid solutions in ethyl acetate, containing between 0.009 and 0.288 mg L^{-1} of it were placed 25 mL beakers. From this point, the above procedure was followed but in this case the immersion time was of 10 min.

2.5. Procedure for the analysis of trans-resveratrol in red wine

In a separatory funnel, 0.50 mL of red wine at pH 5.0, fixed by the addition of 5.0 mL of sodium hydrogen tartrate/disodium tartrate buffer solution (0.3 mol L^{-1}), are diluted with ultrapure water up to 10.0 mL. This aqueous phase is extracted with 5.00 mL of diethylether by shaking for 60 s. The upper organic phase is separated and evaporated to dryness by a stream of nitrogen at room temperature. The residue is re-dissolved with 0.20 mL of ethanol, and water is added approximately up to 10.0 mL. The resulting solution is transferred to a C18 cartridge, previously conditioned with 5.0 mL of methanol and flushed with 20.0 mL of water. Once the sample is deposited, the cartridge is washed with 10.0 mL of an aqueous solution of ethanol (10%) and finally, resveratrol is eluted with 1.0 mL of ethanol:water 50:50, v:v. The eluate is collected in a 10.0 mL volumetric flask and 0.50 mL of ethanol and ultrapure water up to the mark, are added. Independent 2-mL aliquots of this solution are placed into 25 mL beakers and fortified with *trans*-resveratrol in a final concentration up to 0.20 mg L^{-1}. Nylon membranes are submerged under magnetic stirring during 5 min in each one of them. Then, membranes are dried with a nitrogen stream and, once situated in the holder, the front-face synchronous spectra are registered, being the *trans*-resveratrol concentration calculated by means of the standard addition method.

2.6. Procedure for the analysis of trans-piceid in red wine

In a separatory funnel, 0.10 mL of red wine at pH 5.0, fixed by the addition of 5.0 mL of sodium hydrogen tartrate/disodium tartrate buffer solution (0.3 mol L^{-1}), are diluted with ultrapure water up to 10.0 mL. This aqueous solution is further extracted with 5.0 mL of diethylether by shaking for 60 s. The upper organic phase is separated and the aqueous phase is again extracted with 10.0 mL of ethyl acetate by shaking for 150 s. The organic phase is isolated and evaporated to dryness at 35–40 °C in a rota-evaporator. The residue is re-dissolved in 2.00 mL of ethyl acetate. The nylon membrane is submerged and kept for 10 min under magnetic stirring into the resulting solution. After this, the membrane is dried with a nitrogen stream and situated in the holder. The front-face synchronous spectrum is registered, and the concentration of *trans*-piceid is calculated through the standard addition calibration method, using independent wine samples fortified with concentrations of *trans*-piceid up to 6.0 mg L^{-1}.

3. Results and discussion

3.1. Selection of retention mode on the nylon membrane and optimization of the retention time

According to the data previously reported in the literature by our research team, it is known that both, *trans*-resveratrol and *trans*-piceid themselves are weakly fluorescent in solution. *trans*-Resveratrol shows two excitation maxima centred at 225 and 318 nm, and a single emission maximum centred at 385 nm, in ethanol:water 40:60, v:v [22]. Hydroethanolic solutions of *trans*-piceid present excitation and emission maxima centred at 230 and 300, and 395 nm, respectively [23]. Here, it has been proved

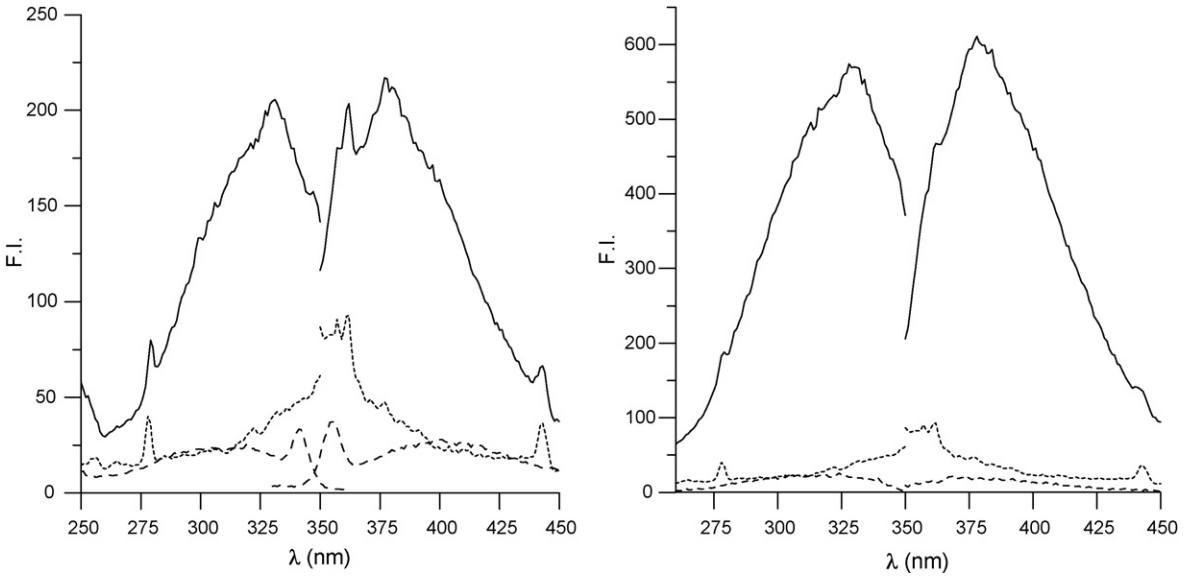

Fig. 2. Fluorescence spectra corresponding to: (left) 0.081 mg L^{-1} *trans*-resveratrol solution in ethanol:water 10:90 v:v (dashed line), the *trans*-resveratrol retained on the nylon membrane (solid line) and the nylon membrane treated similarly (dotted line); (right) 0.49 mg L^{-1} *trans*-piceid solution in ethyl acetate (dashed line), the *trans*-piceid retained on the nylon membrane (solid line) and the nylon membrane treated similarly (dotted line).

that the fluorescence behavior of *trans*-resveratrol and *trans*-piceid drastically varies when these compounds are retained on a solid surface as the nylon membrane, and their fluorescent quantum yields greatly increase when they are immobilized on it. This phenomenon can be appreciated Fig. 2, showing the fluorescent spectra of both compounds in solution and retained on nylon according to procedure described in Section 2.4. Excitation maxima of *trans*-resveratrol and *trans*-piceid retained on the nylon membrane are centred at 331 nm and emission maxima are centred at 380 and at 377 nm, respectively. It can be appreciated that about a 10-fold increase in fluorescence signal for *trans*-resveratrol, and a 30-fold increase in fluorescence signal for *trans*-piceid is produced when they are retained on nylon membrane. It is also important to notice the very low background signals coming from nylon in the measurement conditions, which is an important advantage of this material. Still, this background signal was subtracted from all obtained for solutions containing *trans*-resveratrol or *trans*-piceid.

Different approaches were assayed to carry out the retention of the analytes on nylon membranes and the results were compared. Firstly, membranes were placed in a holder coupled to a syringe which was utilized to force a volume of 5.0 mL of diluted solutions of the analyte through the membrane. When this deposition mode was employed, an unequal distribution of the analytes in the membrane was obtained, as a consequence of the irregular internal structure of the membrane holder. This unequal distribution was visualized by having a look at the membranes placed under an UV-lamp. Also, a high irreproducibility was found when registering emission spectra at different points of the membrane. Secondly, the direct deposition of analyte solutions on the membrane, by using a Hamilton syringe was assayed. 10.0-μL aliquots of suitable concentration solutions were used for these experiments. This method would give rise to highly sensitive fluorescent signals by concentrating the analyte in a little spot on the membrane. Nevertheless, the results were not satisfactory, due mainly to the lack of reproducibility. Finally, a third mode of retention was assayed, by immersing the membranes into solutions containing the analytes during a suitable time, and keeping them inside under magnetic stirring. To carry out these immersion experiments, vol-

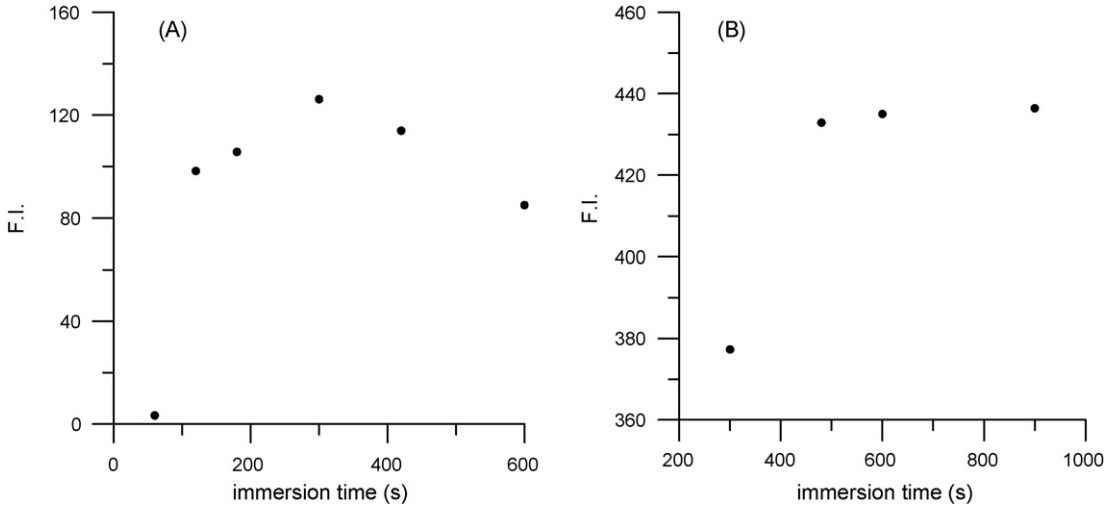

Fig. 3. Influence of immersion time in the fluorescence intensity. (A) Solution in ethanol:water 10:90 v:v, resulting of a wine sample fortified with 0.195 mg L^{-1} of *trans*-resveratrol and submitted to the cleaning-up stages; (B) 0.307 mg L^{-1} *trans*-piceid solution in ethyl acetate. $\lambda_{exc}/\lambda_{em}$ = 331/377.

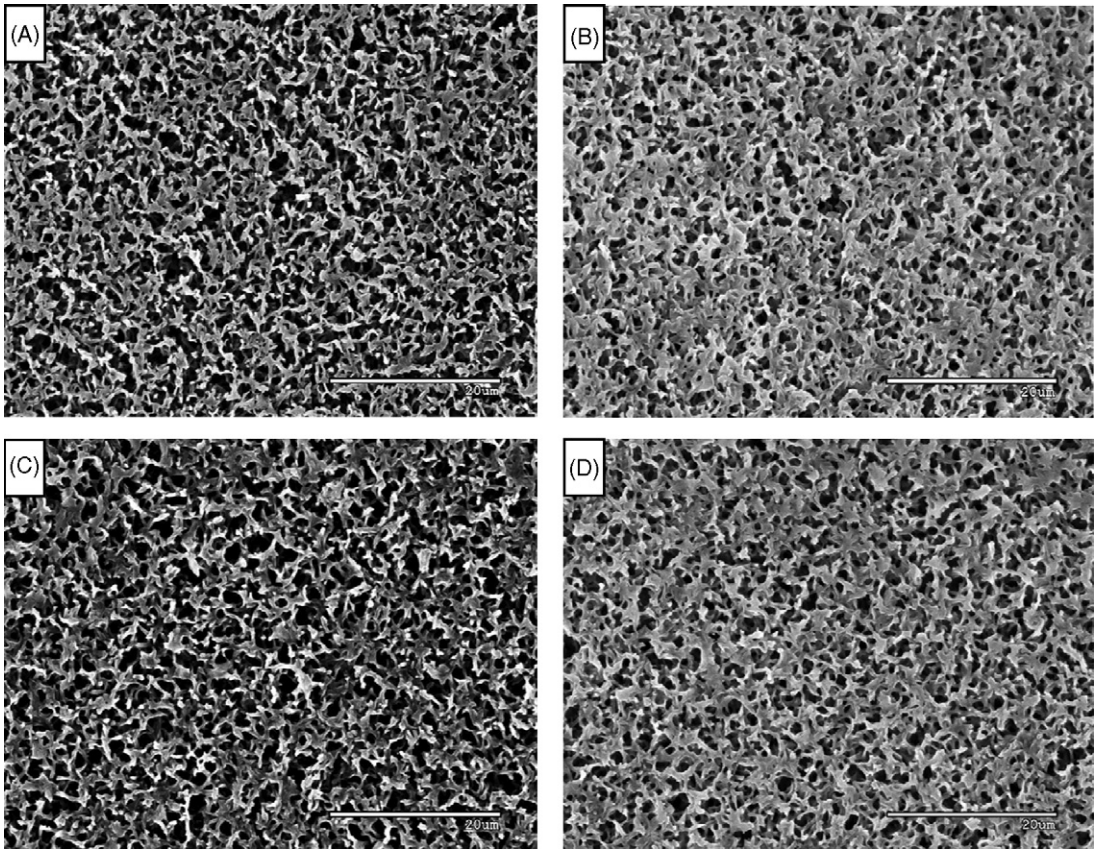

Fig. 4. Photomicrographs of a 0.22 μm pore size nylon disc after its immersion in a mixture of ethanol:water 10:90 v:v (A), when *trans*-resveratrol (0.501 mg L^{-1}) is retained on its surface (B), after its immersion in ethyl acetate (C) and the surface of this material when *trans*-piceid (0.501 mg L^{-1}) is retained on it (D). In all cases, the membranes have been dried with a stream of nitrogen, before taking the photographs. Magnification of ×2.0k.

umes of 2.00 mL of diluted solutions of the analytes were placed in 25 mL beakers. In all cases, after retaining the analytes on the membranes, and dry them, the fluorescent signals were obtained using the device shown in Fig. 1. It was found that better reproducibility on fluorescence measurements was obtained when the membranes were immersed into the solutions, to carry out the retention of the analytes, despite the loss of sensitivity.

Once selected the way to carry out the retention of the analytes, studies were developed to select the appropriate solvent for the immersion of the membrane. Preliminary results [22,23] indicate

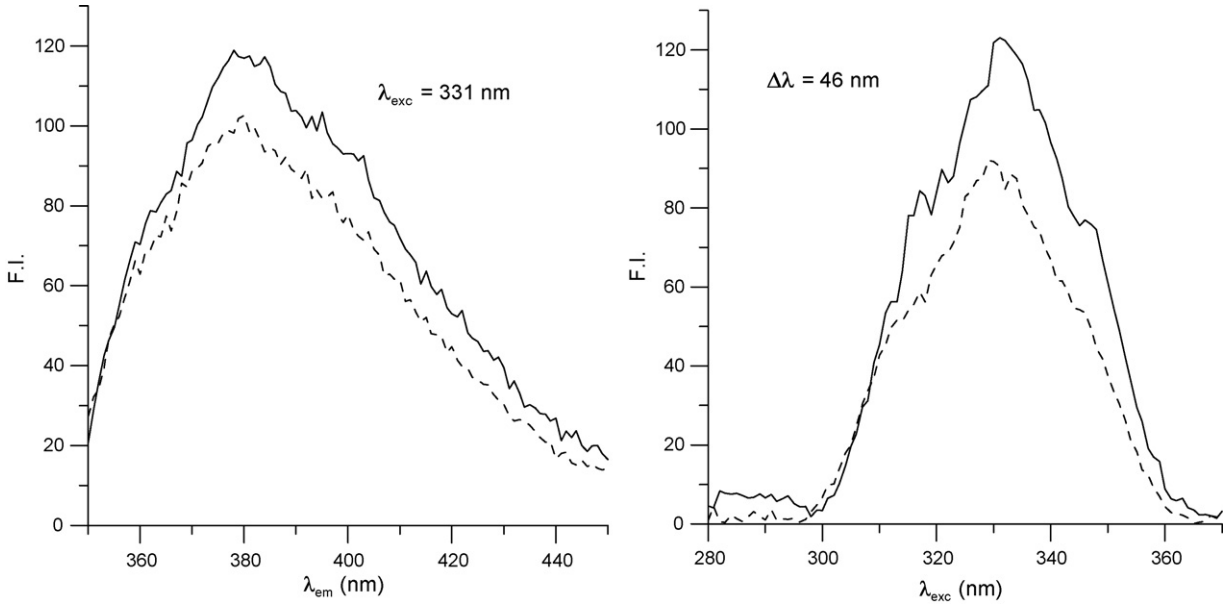

Fig. 5. Comparison of front-face emission (left) and synchronous (right) spectra corresponding to 0.096 mg L^{-1} *trans*-resveratrol (solid line) and *trans*-piceid (dashed line) retained on nylon membrane.

the convenience of performing a liquid–liquid extraction step in the procedure for the analysis of wine samples. Ethyl acetate is reported as the optimum extractant in the case of *trans*-piceid [23] whereas in the case of *trans*-resveratrol it was diethylether [22]. In this last case, after the wine extract is evaporated, the residue can be redissolved in another solvent less volatile and easier to manage for the immersion. Acetone, ethyl acetate and aqueous ethanol (10 to 40%v of ethanol) were assayed and it was proved that the interference from the wine matrix was diminished when ethanol:water 10:90 v:v was utilized. Therefore, ethanol:water 10:90 v:v was selected as optimum for the retention of the *trans*-resveratrol. As already mentioned, ethyl acetate is reported as the optimum solvent for the extraction of *trans*-piceid of wine [23], and then all the immersion experiments for *trans*-piceid were carried out in ethyl acetate so far. In Fig. 3 it is shown the influence of immersion time in the fluorescence intensity. The graph (A) corresponds to a solution in ethanol:water 10:90 v:v resulting of a wine sample fortified with 0.195 mg L^{-1} of *trans*-resveratrol and the graph (B) to a 0.307 mg L^{-1} solution of *trans*-piceid in ethyl acetate. It can be observed that 300 s and 600 s are the optimum immersion time of the *trans*-resveratrol and *trans*-piceid, respectively, since this time is sufficient to achieve, in each case, the distribution equilibrium of analyte on the membrane.

Fig. 4 shows the photomicrographs of a 0.22 μm pore size nylon discs after its immersion in a mixture of ethanol:water 10:90 v:v (A) or ethyl acetate (C), as well as when *trans*-resveratrol (B) or *trans*-piceid (D) are retained on its surface. The concentrations of *trans*-resveratrol and *trans*-piceid in the solutions where the membranes had been immersed were 0.50 mg L^{-1} in both cases. The immersion time was 5 min for *trans*-resveratrol and 10 min for *trans*-piceid. In all cases, the membranes have been dried with a stream of nitrogen before taking the photographs. These microphotographs have been obtained using a magnification of ×2.0k. It can be observed that the pore size decreases when these compounds are retained on the surface, in the case of both analytes. This reduction of the pore size indicates the presence of other molecules over their internal surface. The membranes are made from nylon 6,6 which is a polymer of adipic acid and hexamethylene diamine. The primary chemical structure of nylon consists of amide groups separated by methylene sequences. The chains are oriented in such a way as to maximize hydrogen bonding between the amino and carbonyl groups [26]. t*rans*-Resveratrol or *trans*-piceid are retained into this molecular net, by the polar interaction of the hydroxyl groups of the polyphenol or the glucose residue, with the amide points. Thus, when *trans*-resveratrol or *trans*-piceid are retained inside nylon 6,6, extra hydrogen bonding can occur between the hydroxyl groups, which implies the observed reduction of the pore size.

3.2. Extraction conditions

Due to the complexity of the wine matrix it is usual to include a previous liquid–liquid extraction step for analysis of these compounds even when liquid chromatography is used [12,27]. In our case, preliminary studies showed that an important matrix signal occurs, if some cleaning stage is not included in the procedure [22,23], which does not allow the observation of the fluorescence signals corresponding to *trans*-resveratrol and *trans*-piceid. So, experiments were conducted in order to select the optimum solvent to carry out the extraction of *trans*-piceid and *trans*-resveratrol from red wine samples. Extraction recoveries under different conditions were monitored by means of UV–vis spectroscopy. We have taken into account the pK_a values of 8.2 and 9.7 for *trans*-resveratrol and 9.3 for *trans*-piceid, calculated by our research group based on photometric measurements [22,23]. The neutral polyphenol species (tri-ol for resveratrol and di-ol for piceid) are the predominant species present in acid and neutral media, and that is why the pH of the aqueous phase was buffered at 5.0 for the extraction of resveratrol or piceid [22,23]. The selection of a tartrate buffer to fix this pH value is justified since the tartrate ion is one of the most abundant naturally in wine. According to the literature [22] *trans*-resveratrol can be extracted with a yield close to 100% using diethylether as extracting solvent. A phase ratio of 2:1 (aqueous phase:organic phase) and a shaking time of 60 s were previously optimized by our research group. In the same way, ethyl acetate is the solvent mostly employed to carry out the extraction of *trans*-piceid from red wine samples, but isoamyl alcohol has been also tested by us and it resulted to yield extraction recoveries close to 100%. However, its high boiling point makes very difficult its evaporation to dryness. It has been proved that the extraction yield of *trans*-piceid increases with the proportion of isoamyl alcohol in the ethyl acetate/isoamyl alcohol mixture but, in all cases, after extraction, the isoamyl alcohol remained without evaporating to dryness. Finally, ethyl acetate was selected to extract *trans*-piceid of red wine, since it provided a yield close to 78% and its evaporation to dryness is possible in the rota-evaporator at a moderate temperature.

On the other hand, the recovery of extraction of *trans*-piceid in diethylether is practically zero, and this fact makes possible to determine both compounds separately in wine.

3.3. Selection of the analytical signals

The main difference between front-face fluorescence and the classical methodology is the alteration of the incidence angle. This angle is normally around 30° in the front-face technique. The emission is measured at a 90° angle in relation to the excitation beam, as usual. Thus, the excitation of the sample and the measurement of the emitted radiation are carried out in the same cell-face, avoiding the exciting or emitted radiation to go through the bulk solution and eliminating the inner filter phenomenon. So, different angles close to 30° were utilized and finally a value of 34° was selected as optimum (Fig. 1). In that way, the reflected light, scattered radiation and depolarization phenomena are minimized, and further the value of the signal (fluorescence intensity) is higher.

Due to the large width of the obtained emission spectra when these analytes are retained on the nylon membrane (Fig. 2), the possibility of employing synchronous fluorescence was examined. In Fig. 5 it is shown the emission and synchronous spectra corresponding to membranes immersed in 0.096 mg L^{-1} *trans*-resveratrol and *trans*-piceid solutions for 300 s and 600 s, respectively. To obtain the synchronous spectra 46 nm was selected as $\Delta\lambda$, since this value is close to the Stokes shift of these compounds. The obtained emission bands are narrower and better defined by synchronous fluorescence, thus synchronous signals will be further employed. The synchronous spectra corresponding to both compounds are highly overlapped, being the maxima for both compounds centered at $\lambda_{exc}/\lambda_{em}$ 331/377 nm (Fig. 5). Finally, it is important to take into a count the decrease in the signals corresponding to these compounds retained on the nylon membrane when successive scans are made, as a result of the photo-degradation of them originated by the excitation radiation.

3.4. Behavior of cis-isomers in relation with the SS-RTF method

As mentioned in the introduction, resveratrol and piceid occur in wines in two different isomeric forms, *trans* and *cis*. Studies were conducted to clarify the effect of *cis*-isomers in the determination of *trans*-isomers based on SS-RTF. It is well known that when *trans*-isomers of resveratrol and piceid are irradiated with UV-radiation become highly fluorescent photoproducts [22,23,28,29]. The first stage of this transformation is the isomerization to *cis*-

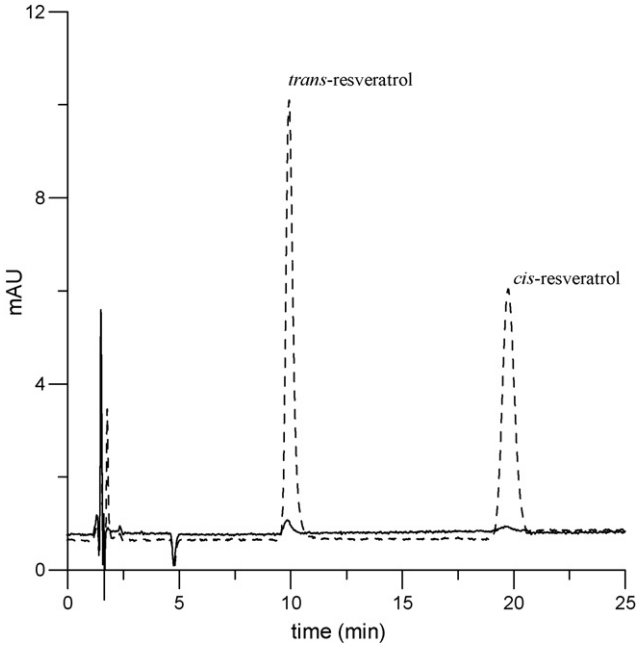

Fig. 6. Chromatograms corresponding to a solution of *trans* and *cis*-resveratrol being filtered (solid line) and without being filtered (dashed line) using a nylon membrane ($\lambda = 290$ nm).

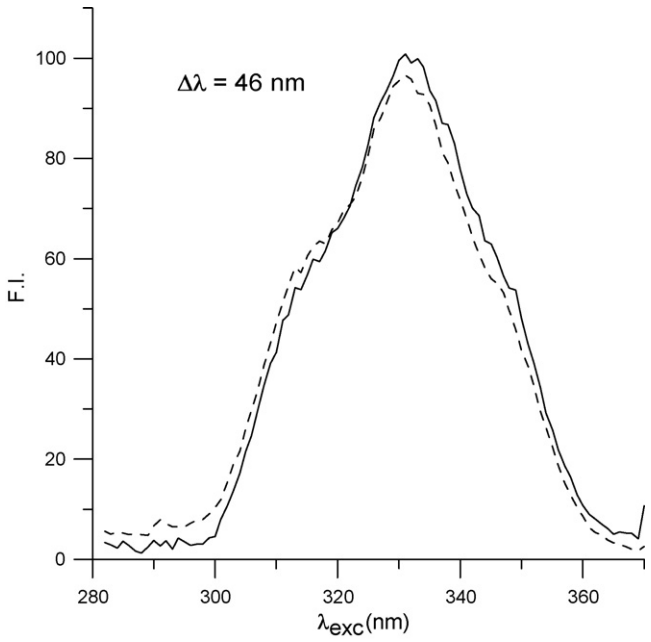

Fig. 7. Synchronous fluorescence spectrum of a 0.06 mg L^{-1} standard solution of *trans*-resveratrol (solid line) and a 0.24 mg L^{-1} solution of *trans*-resveratrol after the exposure to the sunlight during 60 s (dashed line).

isomers which lead to these photoproducts. Thus, it is possible to obtain the *cis*-isomers directly by exposition of *trans* to the sunlight.

It has been tested, by means of LC experiments, that both *trans* and *cis*-isomers of resveratrol are extracted from the wine matrix when diethylether is used as an extracting solvent with a phase ratio of 2:1 (aqueous phase:organic phase) and a shaking time of 60 s. In the same way, both *cis* and *trans* remained together after the cleaned stage with C18 cartridges (see procedure in Section 2.5) and it has been also tested that the two isomers are retained on the surface on the nylon membranes. For instance, this behavior can be observed in the Fig. 6, which shows the chromatograms of a standard solution of *trans* and *cis*-resveratrol injected in the LC system with or without previous filtration through nylon membranes and detected as the same compounds in the DAD detector. The *cis*-isomer is obtained allowing the sunlight incises in the solution of *trans*-resveratrol, for a time around 60 s. The disappearance of the peaks of both *trans*- and *cis*-isomers when the solution is filtered using a nylon membrane is proved. These studies were carried out using the LC system described in Section 2.2 but, in this case only data of photometric detector were examined.

Taking into account the results described above, the possibility of the *cis*-isomer to contribute to the fluorescence signal on the nylon membrane was examined. Synchronous spectra were registered using $\Delta\lambda = 46$ nm to carry out the measurements using solid support room temperature synchronous fluorescence. SS-RTF measurements and, at the same time chromatographic analysis, were carried out with a 0.24 mg L^{-1} solution of *trans*-resveratrol, in ethanol:water 10:90 v:v, freshly prepared and also with a solution exposed to sunlight for about 60 s. It was found that the chromatographic peak of *trans*-resveratrol decreased in the last, being this decrease accompanied with the appearance of the *cis* peak. From the comparison with the chromatogram corresponding to the fresh *trans*-resveratrol solution, an estimation of remaining concentration of *trans*-resveratrol in the irradiated solution was made, being 0.06 mg L^{-1}. In parallel, SS-RTF signals of both solutions were obtained, as well as the signal of a new *trans*-resveratrol 0.06 mg L^{-1} solution. It was proved that the signal of this last is almost the same of the signal obtained for the solution exposed to sunlight (Fig. 7) and therefore containing *cis*-resveratrol.

From these results it can be deducted that *cis*-resveratrol does not contribute to the analysis of *trans*-resveratrol since, in the selected conditions for *trans*-resveratrol analysis, does not provide any signal when it is retained on the nylon membrane, and despite it is extracted from the wine and retained on the nylon membrane. *cis*-Piceid also shows this behavior, that is to say, it has been found that it does not present signal, in the conditions for the *trans*-piceid measurement, when it is retained on the surface of the nylon membrane. Therefore, the analysis of the *trans*-isomers can be carried out without the interference of the *cis*-isomers.

3.5. Determination of trans-resveratrol by means of RT-SSF. Application to red wine samples

The relationship between the synchronous fluorescence intensity at $\lambda_{exc}/\lambda_{em} = 331/377$ nm with the concentration of *trans*-resveratrol was studied and a linear relationship was found for concentrations of *trans*-resveratrol between 0.040 and 0.242 mg L^{-1} under the conditions previously optimized. The calibration plots were established by the external standard calibration method. The analytical figures of merit are summarized in Table 1. The repeatability of the method was investigated by repeating seven times the analytical procedure: nylon membranes were submerged in independent 0.207 mg L^{-1} *trans*-resveratrol solutions in ethanol:water 10:90 v:v and then, after the drying the membranes, the synchronous spectra were registered in front-face mode, in the optimum conditions. It is worthy to highlight that the obtained value of % RSD is low enough as to guarantee the reproducibility of the proposed methodology. It is the first time that reproducibility results are reported for a RT-SSF methodology.

When the developed method was applied to the analysis of red wine an important matrix effect was appreciated. This was not completely eliminated by liquid–liquid extraction and subsequently, according previous results of our group [30] another cleaning stage using solid-phase extraction with C18 was included, as described in the proposed procedure. Finally, despite of this process it was

Table 1
Analytical and statistical parameters for the Solid-Surface Room Temperature Front-Face Synchronous Fluorescence determination of *trans*-resveratrol and *trans*-piceid.

Parameters	*trans*-Resveratrol	*trans*-Piceid
Analytical signal	F.I. ($\lambda_{exc}/\lambda_{em}$ = 331/377 nm)	F.I. ($\lambda_{exc}/\lambda_{em}$ = 331/377 nm)
Linear range (mg L^{-1})	0.040–0.242	0.0096–0.288
Intercept (a) ± s_a	10 ± 10	1 ± 2
Slope (b) ± s_b (L mg^{-1})	1793 ± 72	819 ± 16
Determination coefficient (r^2)	0.992	0.991
% Linearity	96.0	98.0
Analytical sensitivity (γ^{-1}) (μg L^{-1})	8.5	8.7
LOD[a] (μg L^{-1})	18	6.6
LOD[b] ($\alpha = \beta = 0.05$) (μg L^{-1})	30	1.9
RSD (%)[c]	3.5 (0.207 mg L^{-1})	7.5 (0.196 mg L^{-1})

[a] Long and Winefordner method [31].
[b] Clayton et al. method [32].
[c] Relative standard deviation (n = 7).

not possible to eliminate the matrix effect so the standard addition method was employed to carry out the calibration. Different aliquots of a wine sample spiked with increasing levels of *trans*-resveratrol were independently subjected to the whole analytical procedure previously optimized. A linear trend was observed between the added concentration of *trans*-resveratrol and the analytical signal, and the slope of this plot was 2543 L mg^{-1}. The ratio between the slopes for the standard addition and the external standard calibration plots is 1.42, which indicates an important and positive matrix effect. The standard addition procedure was also applied on the hydroethanolic extract obtained after the cleaning-up stages, in an effort to locate the origin of the matrix effect. Thus, this extract was divided into different aliquots and fortified at increasing levels with *trans*-resveratrol prior to the immersion of the membranes. Similar standard addition plots were obtained when applying the standard addition method at these two different points, which indicates that the matrix effect is due to the presence of wine interferents in the final extracts, which could be themselves adsorbed in the membranes, instead of an incomplete recovery after the cleaning procedure. This finding implies an important advantage in terms of simplicity and rapidness for further analysis, since the cleaning-up treatment on the wine sample has to be carried out just once, and then the standard addition can be directly applied on the obtained clean extract.

A pool consisting in equal volumes of six wine samples found in the market was prepared and analysed, in order to take into account the majority of the common interferences for further analysis. The concentration of *trans*-resveratrol found in this pool is listed in Table 2, in comparison with the concentration found by LC (fluorimetric detection at $\lambda_{exc}/_{em}$ 260/364 nm) in the validation stage, according to the method previously published by Durán-Merás et al. [15], which allows the separate determination of *trans*- and *cis*-isomers. In the LC method, calibration was done from the fluorescence data, as we had this kind of detector integrated in our LC system, and it is known to be more selective and sensitive than a photometric one.

3.6. Determination of trans-piceid by means of RT-SSF. Application to red wine samples

A linear relationship was found between the analytical signal and concentrations of *trans*-piceid between 0.009 and 0.288 mg L^{-1}, under the conditions previously optimized. The calibration curves were established by the external standard calibration method and the analytical and statistical parameters are summarized in Table 1. The repeatability of the method was investigated by measuring seven identical solutions under similar conditions: nylon membranes were submerged in independent 0.196 mg L^{-1} *trans*-piceid solutions in ethyl acetate and then, after the drying the membranes, the synchronous spectra were registered in front-face mode, under the previously optimized conditions. Again, as in the case of *trans*-resveratrol, the obtained value for % RSD, supports the quality of the here developed analytical methodology.

The optimized methodology for the analysis of *trans*-piceid in red wine samples includes the previous separation of *trans*-resveratrol by extracting it in diethylether as reflected in the proposed procedure. The fluorescent behavior of *trans*-resveratrol retained on the nylon membrane is exactly the same as *trans*-piceid. This makes *trans*-resveratrol the main interfering compound for the analysis of *trans*-piceid and viceversa. When analysing *trans*-resveratrol, the interference of *trans*-piceid is eliminated in the first extraction with diethylether, since the extraction recovery of *trans*-piceid in diethylether is 0%. On the other hand, the extraction recovery of *trans*-resveratrol in ethyl acetate is near 100%, and therefore removing *trans*-resveratrol from the aqueous phase prior to the extraction of *trans*-piceid is a must. It is achieved by extracting the aqueous phase with diethylether before extracting *trans*-piceid with ethyl acetate.

The standard addition method was applied in order to evaluate the existence of matrix effect. In this case, taking into account the incomplete extraction of *trans*-piceid from wine with ethyl acetate, increasing volumes of an ethanolic stock solution of *trans*-piceid were added to different aliquots of a diluted wine sample before applying the whole analytical procedure on it. Other option, in pro of the simplicity of the method, would have been obtaining a single extract, dividing it into aliquots and carrying out the additions on it before immersing the membranes, but in this case the obtained concentration of *trans*-piceid should have been corrected according to the recovery percentage. The ratio between the slopes of the standard addition and the external standard lines was 0.65. This assesses the existence of a negative matrix effect that could be due to the competitive adsorption of non-fluorescent interferences to the active places in the membrane, making them un-accessible for *trans*-piceid.

A pool consisting in equal volumes of six wine samples found in the market was prepared and analysed, in order to take into account the majority of the common interferences for further analysis. The concentration of *trans*-piceid found in this pool is listed in Table 2, in comparison with the concentration found by LC (fluorimetric detection at $\lambda_{exc}/_{em}$ 260/364 nm) in the validation stage, according to the method previously published by Durán-Merás et al. [15]. In the LC method, calibration was done from the fluorescence data, as

Table 2
Results obtained in the analysis of a pool of commercial red wines.

Method	[*trans*-Resveratrol] (mg L^{-1})	[*trans*-Piceid] (mg L^{-1})
This paper	1.08 (±0.21)	1.49 (±0.36)
LC (peak area)	1.26 (±0.27)	1.55 (±0.89)
LC (peak height)	1.01 (±0.16)	1.43 (±0.68)

we had this kind of detector integrated in our LC system, and it is known to be more selective and sensitive than a photometric one.

4. Conclusions

The present studies demonstrate the feasibility of using a nylon membrane as a support for the induction of *trans*-resveratrol and *trans*-piceid fluorescence. These analytes are very weakly fluorescent in solution but the emission is significantly improved when they are retained on the surface of the nylon membrane. Thus, it has been developed a method which use the native fluorescence of *trans*-resveratrol and *trans*-piceid. As far as we know, this is the first time that the native fluorescence of *trans*-resveratrol and *trans*-piceid is used for its analysis.

The more appropriate mode to get the retention of each analyte on the nylon membrane was found to be the immersion in solutions of these in appropriate solvents.

All fluorescence measurements were performed in front-face mode and the emission spectra on the solid surface were very broad and poorly defined, whereas the synchronous spectra, that are narrower than emission spectra, showed better selectivity and were selected as analytical signals.

The developed methods were applied to the determination of *trans*-resveratrol and *trans*-piceid in red wines. A previous liquid–liquid extraction to separate these analytes of the wine matrix is required in both cases. Diethyleter and ethyl acetate were selected as optimum extractant solvents, for *trans*-resveratrol and *trans*-piceid respectively. In the case of *trans*-resveratrol it is also necessary a cleaning stage with C18 cartridges after de liquid–liquid extraction. In both cases the linear range was established and the analytical and statistical parameters were obtained. It is worthy to highlight that it is the first time that reproducibility results are reported for a RT-SSF methodology being the obtained values of % RSD low enough as to guarantee the reproducibility of the proposed methodologies. Finally, both analyses were carried out using the standard addition method, due to the existence of matrix effect from the wine.

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