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A novel multicommuted fluorimetric optosensor for determination of resveratrol in beer

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ABSTRACT

Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin that plays a central role in the human diet because of its antioxidant, anticarcinogenic and antimutagenic properties. This paper shows the development of a multicommuted optosensing device for the determination of resveratrol in beer. The method is based on the measurement of the fluorescence (277/382 nm, $\lambda_{ex}/\lambda_{em}$) of the photoproduct on-line generated by UV-irradiation of resveratrol. The fluorescent photoproduct is monitored once it is retained on a solid support (Sephadex QAE A-25) in the detection area, which improves both sensitivity and selectivity. The sample was delipidated with toluene and cyclohexane and resveratrol was extracted by solid-phase extraction (SPE) on C_{18} cartridges, using methanol as eluent. This pre-treatment allowed recovering about an 82% resveratrol and removing its 3-*O*-β-D-glycoside (piceid) and other interfering substances present in beer. The method provides a detection limit (DL) of 1.0 ng mL^{-1} and a linear dynamic range (LDR) of 3.3–100 ng mL^{-1}. It was satisfactorily applied to the determination of resveratrol in top- and bottom-fermented beers by standard addition calibration. Resveratrol concentrations in the analysed samples varied from 4.1 to 14.1 ng mL^{-1}. This is the first proposed spectroscopic method for determination of resveratrol in beer.

1. Introduction

Resveratrol, which occurs in two isomeric forms, *trans* and *cis* [1], is a naturally occurring polyphenolic compound belonging to stilbenoids family. In the last decade, this compound has attracted the attention of researchers due to their wide range of biological activities. Although both forms are naturally occurring, most of the recorded health benefits are attributed to the *trans* form. Stilbenoids are secondary products of heartwood formation in trees that can act as phytoalexins. Resveratrol has been shown to inhibit multistage carcinogenesis [2], and collected evidence suggests that it may exert a protective effect in central nervous system under pathological conditions, and is associated with reduced risks of cardiovascular disease, diabetes, and Alzheimer's disease [3–5]. Resveratrol is highly enriched in a variety of sources, such as grapes [6], red wine [7], peanuts [8] and pistachios [9]. Hop, used almost exclusively by brewers for bitterness, flavor and antimicrobial protection, was also recently reported as an interesting source of resveratrol, highlighting the potential health-promoting effect of moderate beer consumption [10,11].

Various techniques have been employed for the determination of resveratrol in liquid samples, such as wine or plasma: capillary electrophoresis [12,13], gas chromatography (GC) [14] and GC–mass spectrometry (GC–MS) [15,16], with previous derivatization, the most commonly utilised technique being high-performance liquid chromatography (HPLC, normally in reverse phase), with either diode-array [17], electrochemical [18], chemiluminescent [19], fluorimetric detection [20–22] or with mass spectrometry [23,24]. In addition, one spectroscopic method, based on photochemically induced second-derivative fluorescence (PIF), has been reported for the resveratrol analysis, which was applied to its determination in wine [25].

The preparation techniques used in resveratrol analysis in liquid samples are usually liquid–liquid extraction [25,26], solid-phase extraction (SPE) [26–28], and solid-phase microextraction (SPME) [29]. In some cases, a preliminary removal of hydrophobic constituents is also necessary to improve extraction recovery [26].

On the other hand, to date, only two chromatographic methods (HPLC–MS/MS and GC–MS) have been proposed for resveratrol analysis in beer, being the concentrations found in real samples, however, just below the quantification limits [26]. Therefore, the determination of resveratrol in this matrix makes necessary the development of very sensitive analytical methods.

The present paper was focused to develop a sensitive automatic fluorimetric method as an alternative to the chromatographic determination of resveratrol in beer. With this purpose, a procedure based on the measurement of the fluorescence of the photoproduct generated from resveratrol, by on-line UV-irradiation in a flow-

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system, was developed. The measurement of the analytical signal of the target compound retained on Sephadex QAE A-25, packed in the flow-cell placed in the detection area, was adopted for improving both detection limit and selectivity. This methodology, called flow injection-solid phase spectroscopy (FI-SPS) or flow-through optosensor, combines advantages of FI with the analyte preconcentration on a small amount of a solid support, such as high sensitivity and selectivity, rapidity, and simplicity [30]. Multicommutation principles [31–33] were also exploited in the flow-system with the purpose of allowing independent handling of sample and carrier solutions, low sample and reagent consumption and the complete automation of the process. SPE with sequential elution was used to selectively extract resveratrol and separate it from other interfering substances present in the beer. To the authors' knowledge, this is the first spectroscopic determination of resveratrol in beer to be reported. This is also the first application of multicommutation continuous-flow methodology to the determination of this stilbene. The proposed method was applied to the analysis of this compound in different kinds of commercial beers.

2. Experimental

2.1. Reagents and solutions

All reagents were analytical reagent grade, and Milli-Q (Millipore, Bedford, MA) water was used throughout.

trans-Resveratrol (Sigma, St. Louis, MO, USA) stock solution, 200 mg L^{-1}, was prepared by dissolving the appropriate amount in ethanol (Panreac, Barcelona, Spain). It was kept away from light with an aluminium foil and stored at 4 °C in a refrigerator, remaining stable for at least four weeks. Working standard solutions were prepared daily by taking an aliquot of the stock solution and diluting with 0.01 mol L^{-1} $NaCH_3COO/HCH_3COO$ (NaAc/HAc) buffer solution, pH 6.0. The working solutions were stable for at least 8 h, when protected from light, which was enough to make the daily experiments.

The carrier solution (0.05 mol L^{-1} NaCl/0.001 mol L^{-1} HNO_3 (pH 3.0)) was prepared by dissolving the required weight of NaCl in water and setting the pH to 3.0 with HNO_3 solution (both obtained of Panreac) with the aid of a pH-meter.

Sephadex QAE A-25 (40–120 μm average particle size) (Sigma, Alcobendas, Madrid, Spain) was used as active solid support in the detection area. Other cation and anion-exchangers (Sephadex SP C-25, Sephadex CM C-25 and Sephadex DEAE A-25, all of them having 40–120 μm average particle size) (Sigma) and C_{18} bonded phase silica gel beads (55–105 μm average particle size) (Waters, Milford, MA, USA) were also tested for retention of the target compounds.

Methanol, ethanol, nitric acid, toluene and cyclohexane were obtained from Panreac. Octadecyl (C_{18}) Bakerbond SPE cartridges of 6 mL with 500 mg of packing material (J.T. Baker, Phillipsburg, NJ, USA) were also used.

2.2. Apparatus and instrumentation

The multicommuted flow system (Fig. 1) was built with a four channel Gilson Minipuls-3 (Villiers Le Bell, France) peristaltic pump fitted with a rate selector and pump tubing type Solvflex (Elkay Products, Shrewsbury, MA, USA), three 161T031 NResearch three-way solenoid valves (Neptune Research, MA, USA) and an electronic interface based on ULN 2803 integrate circuits. The valves were operated at an electric potential of 12 V and a direct current of 100 mA. PTFE tubing (0.8 mm i.d.) and methacrylate connections were also used. The software for controlling the system was developed in Visual Basic 6.0 by our research group. A 176.752-QS Hellma flow-cell (Müllheim, Baden, Germany) (inner volume, 25 μL; light path length, 1.5 mm) was used in the detection area. It was filled with an aqueous slurry of the solid support (Sephadex QAE A-25) just up to a height which enabled the light beam to pass completely through the solid phase. The outlet was locked with glass wool to avoid the beads movement and allow the continuous flow.

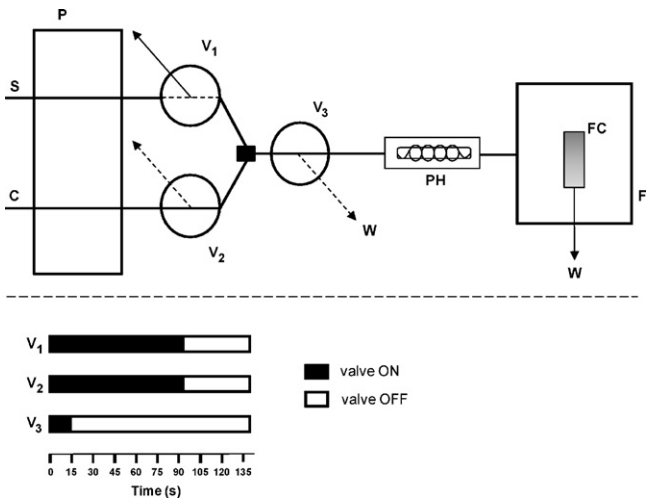

Fig. 1. Multicommuted flow-injection assembly. S, sample (buffered with 0.01 mol L^{-1} NaAc/HAc buffer solution, pH 6.0); C, carrier solution (0.05 M NaCl/0.001 M HNO_3, pH 3.0); P, peristaltic pump; V_1, V_2, and V_3, three-ways solenoid valves; PH, photoreactor (110 cm length); F, spectrofluorimeter; FC, flow-cell (packed with Sephadex QAE A-25); W, waste. For each solenoid valve, the solid and dotted lines refer to "OFF" and "ON" positions, respectively. Flow-rate: 2.2 mL min^{-1}. The scheme below manifold shows the valve time program (sampling time, 80 s).

For the on-line photochemical conversion of resveratrol, a home-made photoreactor was prepared by loosely coiling 110 cm of PTFE tubing (0.8 mm i.d., 1.6 mm o.d.) around a low-pressure mercury lamp, Philips TUV 15W FAM (15 W, 254 nm). The UV lamp was wrapped in aluminium paper and introduced into an aluminium box for maximum light reflection and heat dissipation. The photoreactor was placed just between V_3 and the detection area. All the experiments were carried out at room temperature.

Luminescence measurements and spectra recordings were performed with a Cary-Eclipse Luminescence Spectrometer (Varian Inc., Mulgrave, Australia), controlled by a computer equipped with a Cary-Eclipse (Varian) software package for data collection and treatment. Instrument excitation and emission slit widths were set at 20 and 20 nm, respectively, and the detector voltage was 590 V. The excitation and emission wavelengths were established at 277/382 nm, respectively.

UV–vis spectra were recorded with a Varian Cary 50 Spectrophotometer (Madrid, Spain) controlled by means of a PC fitted with the Varian computerized spectroscopy software, WIN-UV. A 100-QS Hellma cell with a light path length of 10 mm was employed for absorbance measurements.

Other apparatus consisted of a vacuum system 12-port Visiprep SPE Vacuum Manifold (Supelco, Bellefonte, PA), a Crison Model 2002 pH-meter with a glass/saturated calomel combination electrode (Crison, Barcelona, Spain) and a Selecta Ultrasons ultrasonic bath (Barcelona, Spain).

2.3. Sample preparation

The beer samples were purchased from local markets. Along all the process, the sample was kept away from the daylight, as far as possible, with the aid of aluminium foil.

Pre-cleaning to remove hydrophobic compounds. Aliquots of 25 mL of the samples were spiked with known amounts of *trans*-

resveratrol and then, they were treated under gentle stirring, in consecutive 3 min steps, first with 25 mL of toluene (three times) and then with 25 mL of cyclohexane (three times). At the end of each step, the mixture was centrifuged for 5 min at 4200 × *g*, and the supernatant was discarded. Finally, cleaned beer was dried under vacuum (30 °C) to get rid of residual solvent.

Extraction of resveratrol. A C_{18} cartridge was conditioned by passing consecutively 3 mL of methanol and 6 mL of water. Subsequently, cleaned beer was passed through the cartridge at a flow-rate of 3–4 mL min^{-1} under vacuum. Then, the cartridge was washed firstly with 6 mL of water and secondly with 6 mL of 40% methanol:water (40:60, v/v). The elution of resveratrol retained in the cartridge was carried out with 3 × 2 mL of methanol and the eluate was concentrated to dryness under a gentle stream of nitrogen. The final residue was solubilised in 10 mL of 0.01 mol L^{-1} NaAc/HAc buffer solution, pH 6.0.

2.4. Procedure

The schematic manifold is shown in Fig. 1. All experiments were carried out in triplicate and the results were expressed as peak-height mean values. The determination of resveratrol was carried out by following the next steps:

- In the initial status, being all the valves deactivated (solid lines), the peristaltic pump (P) and the UV lamp of the photoreactor (PH) were turned on to condition the flow-system until a stable baseline was recorded. Thus, the carrier solution (C) flowed through the system and the sample solution (S) was recycled to its vessel.
- Next, valves V_1, V_2 and V_3 were activated (dashed lines) for 95, 95 and 15 s, respectively. Thus, the sample solution (S) circulated through the system, whereas the carrier solution (C) was recycled to its recipient. For the first 15 s of this step, the sample plug was directed towards the waste through V_3, so cleaning the tubing between V_1 and V_3 with the new sample solution. Over the next 80 s, as V_3 was deactivated (solid line), the sample plug was pumped towards the detection area, passing through the photoreactor (PH). This originated the photochemical conversion of resveratrol into a fluorescent photoproduct.
- From 95 s, all the valves were deactivated and so, the sample solution (S) was recycled to its recipient, whereas the carrier solution (C) circulated again through the system, thus propelling the sample plug to the detector. When the fluorescent photoproduct of resveratrol reached the detection area, it was temporarily retained on the solid support and monitored (277/382 nm, $\lambda_{ex}/\lambda_{em}$). The solid support was regenerated by the carrier solution (C) itself and the system was prepared for the next insertion of sample.

3. Results and discussion

3.1. Optimization of solid-phase extraction

In order to extract resveratrol from beer, a new extraction procedure was developed. The fluorescent photodegradation product generated from resveratrol is sorbed on the anion-exchanger Sephadex QAE A-25, thus excluding a lot of organic species from the matrix. However, a previous clean-up step was necessary to eliminate other species which could be retained on the solid-phase and compete with the photodegradation product for the active sites. As we could check, the most serious interfering species was resveratrol-3-*O*-β-D-glycoside (piceid), which is also present in beer [26] and shows a very similar behaviour to that of resveratrol in the flow-system. Under UV irradiation, piceid also originated a fluorescent photoproduct, which was retained on Sephadex QAE A-25 (257/382 nm, $\lambda_{ex}/\lambda_{em}$) and showed a complete spectral overlapping with the photoproduct of resveratrol. Caffeic and gallic acids interfered with the determination of resveratrol when their amounts were over 5 and 80-fold (w/w) that of resveratrol. Nevertheless, no interference was observed for the amounts of catechin and quercetin usually found in beer.

Table 1
Recovery percentages of *trans*-resveratrol according to the solvent assayed for extraction.

Solvent	Recovery percentage (%)	
	Resveratrol	Piceid
Methanol:water (20:80, v/v)	0	0
Methanol:water (35:65, v/v)	0	35
Methanol:water (40:60, v/v)	0	81
Methanol:water (50:50, v/v)	10	80
Methanol:water (80:20, v/v)	75	81
Methanol	82	83

According to the literature [10], a preliminary removal of hop resins and lipids is most likely necessary to recover high amounts of polyphenols. Although diethyl ether is often used for this previous cleaning, the significant solubility of resveratrol in this solvent makes impossible its use in this case. The removal of hydrophobic compounds was carried out with toluene and cyclohexane by following a procedure previously described [10], by introducing slight modifications.

Next, liquid–liquid extraction was tested for the selective extraction of resveratrol from beer. Diethyl ether and ethyl acetate are the solvents commonly used for the extraction of resveratrol and piceid from liquid samples. In the case of beer, diethyl ether provided an extraction recovery for resveratrol close to 80%, but piceid was also significantly extracted (5%). The use of a more polar solvent such as ethyl acetate even provided a better extraction percentage for piceid (10%). Consequently, the liquid–liquid extraction was discarded for the extraction of resveratrol and SPE was chosen for this purpose.

In order to optimize the SPE procedure, different solvents for the rinsing and elution steps were utilised: water, ethyl acetate, diethyl ether and methanol. With this purpose, the washout fractions were monitored with the UV–vis spectrophotometer from 250 to 425 nm. A first rinsing step with water allowed the elimination of most polar compounds from the matrix. The optimum water volume was studied in the range 2–8 mL and found to be 6 mL. In a second rinsing step, aqueous solutions containing different methanol percentages (20, 35, 40 and 50%) were assayed for the removal of piceid and other possible polar interferences of beer, without eluting resveratrol (Table 1). 6 mL of a 40% methanol solution proved to be the optimal one for this purpose.

Ethyl acetate, diethyl ether and methanol (80 and 100%) were tested for the elution of resveratrol and, finally, pure methanol was selected because it gave the best recovery (82%).

3.2. Preliminary tests of sorption

Resveratrol is weakly fluorescent and extremely photosensitive. When protected from light the *trans* form is stable for months, unless in high-pH buffer, whereas the *cis* form is stable only at neutral pH when completely protected from light [34]. Intense UV irradiation of solutions of *trans*-resveratrol originates first its conversion into *cis*-resveratrol and then leads to the formation of a highly fluorescent compound [25,35]. This photoconversion allows the total (*trans*-isomer plus *cis*-isomer) resveratrol determination in a sample. On the basis of literature data, it can be assumed that the fluorescent photoproduct gener-

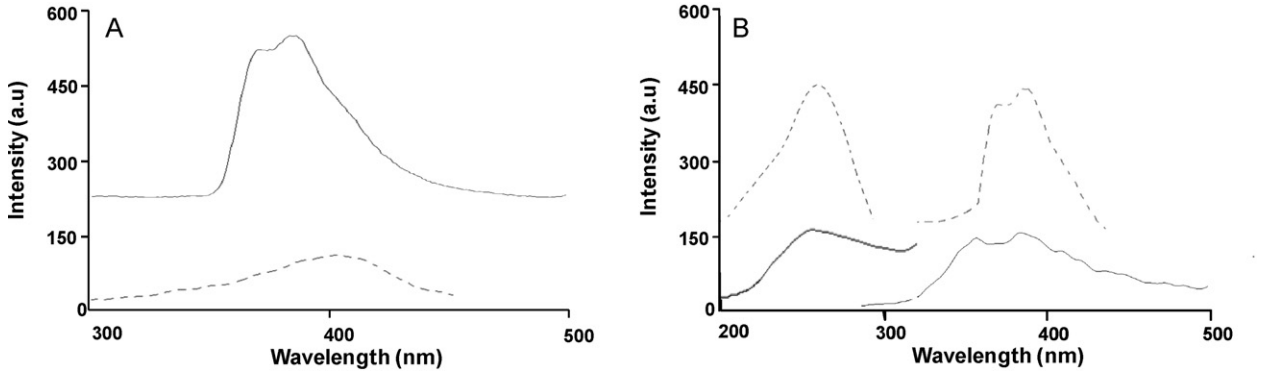

Fig. 2. Fluorescence spectra of *trans*-resveratrol and its photoproduct. (A) Emission spectra on Sephadex QAE A-25: *trans*-resveratrol (dashed line), photoproduct of *trans*-resveratrol (continuous line), 100 ng mL^{-1} *trans*-resveratrol. (B) Excitation and emission spectra of the photoproduct of *trans*-resveratrol: in homogeneous solution (continuous line), on Sephadex QAE A-25 (dashed line), 2.5 mg L^{-1} *trans*-resveratrol (in solution); 90 ng mL^{-1} *trans*-resveratrol (in solid phase). pH 6.0; 15 s irradiation time.

ated from *trans*- or *cis*-resveratrol is a phenanthrene derivative, 2,4,6-trihydroxyphenanthrene [36].

In order to improve sensitivity and selectivity of the determination of resveratrol by measuring the fluorescence of its photochemically generated photoproduct, anion-exchangers (Sephadex QAE A-25 and Sephadex DEAE-25), cation-exchangers (Sephadex SP C-25 and Sephadex CM C-25) and a non-ionic support (C_{18} silica gel) were tested, at different pH values (below 7.0), to retain this compound. These tests proved that C_{18} silica gel and both anion-exchangers allowed the retention of both, *trans*-resveratrol and its photoproduct. Finally, Sephadex QAE A-25 was selected for retention purposes since, at weakly acidic pH value, it provided the maximum analytical signal. In addition, the anionic character of this solid-support could provide a higher selectivity in the determination of resveratrol.

No spectral changes were observed for *trans*-resveratrol spectra retained on Sephadex QAE A-25, in the range of pH studied (2.0–7.0), showing an excitation maximum at 326 nm and an emission maximum at 406 nm. In the same pH range, the photoproduct of *trans*-resveratrol presented excitation and emission maxima at 277 and 382 nm, respectively. The fluorescence signal was greatly improved by the sorption of *trans*-resveratrol or its photoproduct on the solid-support and their consequent preconcentration on this latter. Finally, the measurement of the fluorescence of the photoproduct was chosen since it provided the highest sensitivity (Fig. 2A). In addition, taking into account the complexity of the matrix, we considered that this choice could allow a better selectivity in the method to be developed. This way, the improvement in the analytical signal was 60-fold, when comparing to that obtained in homogeneous solution under the same working conditions (Fig. 2B).

The instrument excitation and emission slits were optimized at 20 and 20 nm, respectively, since these values provided the best ratio fluorescence signal–background noise. In the same way, the voltage of the photomultiplier tube was set at 590 V. The research was performed at room temperature, observing no difference in the analytical signal between 15 and 30 °C.

3.3. Nature and pH of sample and carrier solution

The pH showed to be a key variable since the analytical signal changed significantly with the pH of the sample and the carrier solution. It is necessary to point out that the optimum working pH value will be that providing the maximum conversion of *trans*-resveratrol in the fluorescent photodegradation product, the maximum fluorescence of the photoproduct generated and the maximum sorption of this latter on the solid support in the detection area.

The study of the influence of pH of the sample was performed by varying it between 2.0 and 10.0, using HNO_3 or NaOH solutions. A maximum and constant fluorescence was obtained in the pH range 5.5–7.0 (Fig. 3). Higher or lower pH values caused a drastic decrease in the fluorescence signal. A pH value of 6.0 was selected for the next experiments. Several buffer solutions at pH 6.0 were tested in order to adjust the pH of the sample solution (citric acid/sodium hydroxide; sodium dihydrogen phosphate/sodium hydroxide; sodium acetate/acetic acid) and the best results were achieved with NaAc/HAc buffer solution. The influence of its concentration on the analytical response was assayed in the range 0.005–0.05 mol L^{-1} and finally, a 0.01 mol L^{-1} concentration was chosen for buffering the sample, since it provided the best analytical signal. Higher concentrations caused a lower fluorescence signal, probably due to the competition between buffer ions and the photodegradation product for the actives sites in the ionic solid-support. Lower concentrations originated a lower signal too, since the sorption of the photodegradation product on the solid-support took place just above the light beam in the detection area.

The effect of pH of the carrier solution was studied by inserting a 100 ng mL^{-1} resveratrol solution (pH 6.0) into different aqueous solutions adjusted at pH values ranging from 1.0 to 7.0 with HNO_3 (Fig. 3). The maximum fluorescence signal was obtained at pH 3.0. For pH values higher than 3.0 a drastic decay in the fluorescence signal was obtained, keeping this latter constant and negligible from 4.0 to 7.0. The fluorescence signal also decreased for pH values lower than 3.0 and, in addition, overpressure problems were observed due to the compaction of the solid-support. Finally, a pH of 3.0 for the carrier solution was chosen as optimum. None of the

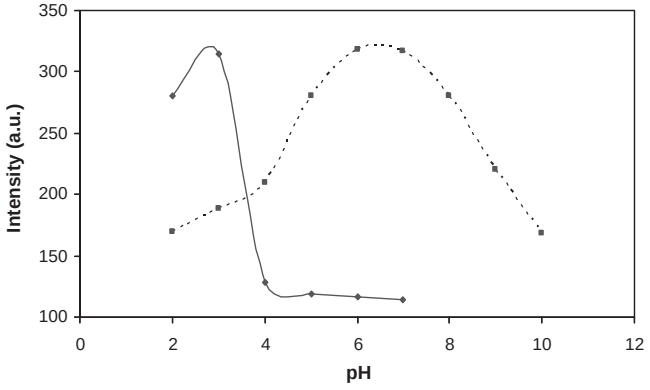

Fig. 3. Influence of pH of the sample (dashed line) and carrier (continuous line) solutions. 100 ng mL^{-1} *trans*-resveratrol; carrier solution, 0.001 mol L^{-1} HNO_3; irradiation time, 20 s.

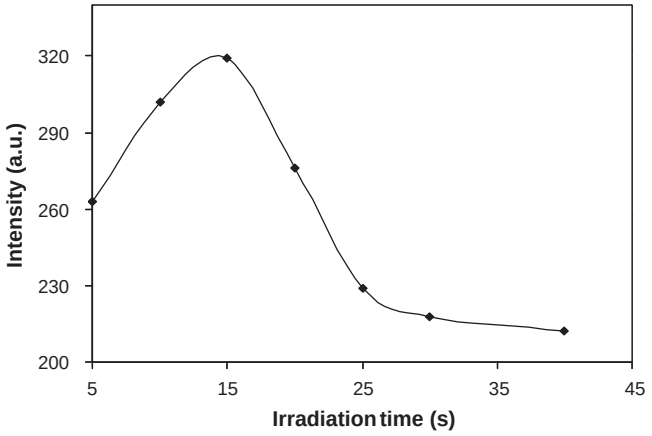

Fig. 4. Influence of irradiation time. 100 ng mL^{-1} *trans*-resveratrol; sampling time, 40 s; flow-rate, 1.9 mL min^{-1}; photoreactor length, 250 cm.

tested aqueous solutions provided the elution of the photoproduct of *trans*-resveratrol in a reasonable time.

With the purpose of achieving the quick and complete elution of the fluorescent photoproduct from the solid-support, thus increasing the sampling frequency, different electrolytes (NaCl, KCl, $NaNO_3$, Na_2CO_3) were added to the aqueous carrier solution (pH 3.0), in concentrations ranging from 0.01 to 0.1 mol L^{-1}. The best results were obtained for a 0.06 mol L^{-1} NaCl concentration. This solution provided the highest analytical signal and a rapid and complete elution of the species monitored from the solid-support, which was completely regenerated. Consequently, the use of an additional eluting solution was not necessary. Although NaCl concentrations higher than 0.06 mol L^{-1} provided a quicker elution of the photoproduct, they also originated a significant decrease in the analytical signal. Therefore, that NaCl concentration was selected as a compromise between analytical signal and peak width. Lower NaCl concentrations resulted in analytical signals similar to that obtained with 0.06 mol L^{-1} NaCl, but wider peaks were also obtained, thus decreasing the throughput. The assay of different buffer solutions to adjust the pH of the carrier solution at 3.0 did not provide a significant improvement in the analytical signal or in the time of elution of the photoproduct. Therefore, the carrier solution consisted of 0.06 mol L^{-1} NaCl/0.001 mol L^{-1} HNO_3 (pH 3.0).

3.4. Irradiation time

Another key variable for the generation of a fluorescent photodegradation product from *trans*-resveratrol is the residence time of the sample plug inside the photoreactor under UV irradiation. To optimize this variable, a 100 ng mL^{-1} *trans*-resveratrol solution was inserted in the system (sampling time, 40 s; flow-rate, 1.9 mL min^{-1}), the flow was stopped just when the whole plug of sample was within the photoreactor (250 cm) and the sample was irradiated for increasing periods of time. The results showed that the kinetic of photo-degradation of *trans*-resveratrol in the working conditions is very quick. The fluorescence signal increased with the irradiation time to reach a maximum value for 15 s and thereafter it decreased (Fig. 4). The shape of the curve obtained suggests a two-step photolysis mechanism, consisting of the formation of a strongly fluorescent photoproduct and the posterior photo degradation of this compound into non-fluorescent product(s). The length of the transport system between the photochemical reactor and the flow-cell was the minimum allowing both units to be connected.

The residence time of *trans*-resveratrol in the photochemical reactor and, consequently, the irradiation time can be controlled by the flow-rate of the carrier solution and/or the length of the tubing around the UV lamp. Firstly, the optimum irradiation time (15 s) was established by using a 2.2 mL min^{-1} flow-rate combined with a 110 cm long photoreactor. This flow-rate value was chosen since it provided the highest throughput and higher flow-rates originated overpressure problems in the system. After this, by keeping constant the sample volume inserted in the system, which implied varying the sampling time, different combinations of photoreactor length and flow-rate (0.8–2.0 mL min^{-1}) were assayed. The use of shorter photoreactor lengths combined with flow-rates lower than 2.2 mL min^{-1} provided lower peak heights and throughputs. Consequently, the optimum irradiation time was established by combining a 110 cm photoreactor and a 2.2 mL min^{-1} flow-rate.

Table 2
Analytical parameters.

Parameter	
Linear dynamic range (ng mL^{-1})	3.3–100
Calibration graph	
Intercept	−10.162
Slope (mL ng^{-1})	3.284
Correlation coefficient	0.9998
Detection limit (DL) (ng mL^{-1})	1.0
Quantification limit (QL) (ng mL^{-1})	3.3
RSD (%) (n = 10)	1.8[a]
Throughput (h^{-1})	12

[a] *trans*-Resveratrol concentration, 40 ng mL^{-1}.

3.5. Sampling time

As known in FI-SPS systems, the insertion of increasing sample volumes in the system originates an improvement in sensitivity (for a constant concentration of analyte). This is a consequence of the sorption of increasing amounts of the species monitored on a constant amount of solid-support. In addition, the improvement in sensitivity allows the reduction of matrix effects by means of a previous dilution of the sample, which is carried out before its insertion in the system. In the case of multicommuted systems, the sample volume can be easily controlled by varying the sampling time [37].

The influence of this variable was tested by inserting in the flow-system a 50 ng mL^{-1} *trans*-resveratrol solution for sampling times ranging from 10 to 90 s. This is easily achieved by varying the time during which the valve V_1 is activated (Fig. 1). The fluorescence signal increased linearly until a value of 80 s. Sampling times above this value did no suppose a significant increment in the analytical signal. Taking into account that the flow-rate and sampling time were established at 2.2 mL min^{-1} and 80 s, respectively, it corresponded with a sample volume of 2900 μL, which was finally selected.

3.6. Analytical parameters

Taking into account the above optimized working conditions, the analytical parameters of the system were studied (Table 2). Calibration curve was constructed by following the procedure above described after injecting sample solutions in triplicate, containing increasing concentrations of *trans*-resveratrol. Quantification was carried out by using peak height as analytical signal. The data were fitted by standard least-squares treatment. The proposed methodology responds linearly in the concentration range 3.3–100 ng mL^{-1}. The detection and quantification limits were estimated as the concentration of analyte which produced an analytical signal equal to three and ten times, respectively, the standard deviation of the background fluorescence [38,39].

The intra-day repeatability was established for ten independent analyses of sample solutions containing 40 ng mL^{-1} of *trans*-

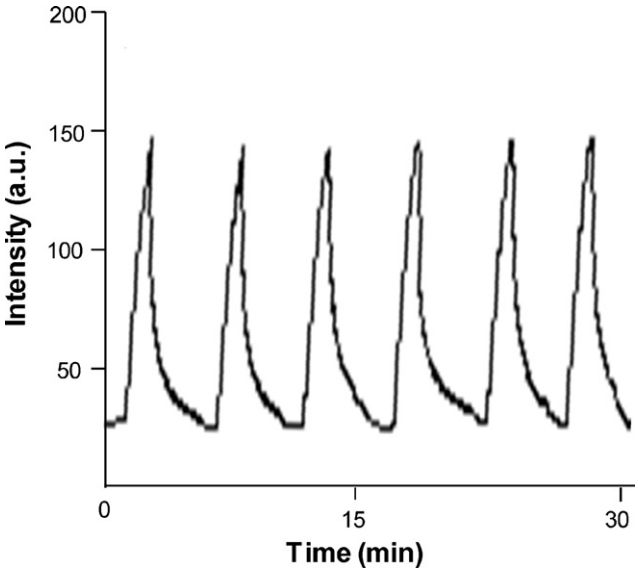

Fig. 5. Typical analytical signal registered for the photoproduct of *trans*-resveratrol. 40 ng mL^{-1} *trans*-resveratrol, $n = 6$.

resveratrol, RSD being 1.8%. Fig. 5 shows the typical registered analytical signal. The inter-day repeatability was also performed for ten consecutive days obtaining RSD 3.2%.

3.7. Analytical applications

When the method was applied to the analysis of beer samples an important matrix effect was observed, which was not completely eliminated by the pre-treatment procedure developed. Therefore, the standard addition method was utilised for calibration of the method. The slope of the calibration curve obtained by spiking the final extracts with *trans*-resveratrol was different to that obtained by spiking the original samples. In addition, in both cases the slopes were lower than that obtained by external calibration. Therefore, the matrix effect is due to the presence in the final extracts of interfering species and the incomplete recovery of resveratrol after the pre-treatment. This latter is probably caused by the presence in beer of supramolecular structures involving hop constituents. Consequently, the analysis was carried out by spiking different aliquots of a beer sample with increasing amounts of *trans*-resveratrol, before subjecting them to the whole analytical procedure. The matrix effect was evaluated by comparing the slopes of aqueous standards and standard addition calibration graphs for different beer samples ($m_{\text{standard}}/m_{\text{standard addition}} \approx 1.1$).

The results obtained are shown in Table 3. The resveratrol content found in three of the samples analysed was lower than quantification limit (QL) of the method proposed. Consequently, a recovery study was performed on these samples at two concentration levels. In all the cases good recoveries, ranging from 99.5 to 101.5%, were achieved.

The applicability of the proposed method to the analysis of resveratrol in beer was demonstrated by comparison with a reference HPLC–MS/MS method [26]. In all the cases the results obtained were in good agreement with those obtained by the reference method.

4. Conclusions

For the first time an automatic spectroscopic method for the analysis of resveratrol in beer is reported. The method makes use of the on-line photodegradation of resveratrol in a multicommuted flow-system and the posterior monitoring of the generated highly fluorescent photoproduct (2,4,6-trihydroxyphenanthrene), once it is retained on an appropriate solid-support (Sephadex QAE A-25). The extraction of resveratrol from beer by SPE with methanol improves significantly selectivity, since it allows removing its glycoside (piceid), which is a serious interfering species. The measurement of the fluorescence of the photoproduct of resveratrol in solid-phase also contributes to improve selectivity, allowing the exclusion of a large amount of organic species of the matrix.

The method developed shows characteristics such as: (a) high sensitivity, because of the sorption of the photoproduct of resveratrol on a small amount of the solid support; (b) low reagent consumption; (c) easy to handle; (d) rapidity and simplicity and (e) low cost. It can be considered as an interesting alternative to the chromatographic determination of resveratrol in beer, since it provides higher throughputs and lower detection limits and cost of instrumentation.

Although the amounts of resveratrol found in some of the samples analysed are below the QL of the proposed method, a DL lower than those previously reported (HPLC–MS/MS and GC–MS) has been reached, using a simple and rapid optosensing device, with low-cost instrumentation. Therefore, in our opinion the proposed method constitutes an interesting contribution to the analysis of resveratrol, and in general of stilbenes, in beer. In spite of the low concentrations of resveratrol found in beer, taking into account the important health benefits of resveratrol, we think that it would be very interesting to carry out complementary studies in order to produce resveratrol-enriched hop extracts, in a similar way to studies currently carried out to enhance resveratrol content in wines.

Table 3
Determination of resveratrol in commercial beers.

Sample	Proposed method		Reference method[a]
	Added (ng mL^{-1})	Found $\pm \sigma$[b] (ng mL^{-1})	Found $\pm \sigma$[b] (ng mL^{-1})
Top-fermented			
Ale 1	–	6.2 ± 0.5	<QL
Ale 2	–	<DL	<DL
	20	20.3 ± 0.5	20.8 ± 0.6
	40	40.6 ± 0.7	41.0 ± 1.2
Bitter	–	<DL	<DL
	60	60.5 ± 1.1	59.6 ± 1.3
	80	80.8 ± 1.3	79.2 ± 1.4
Stout	–	6.3 ± 0.3	<QL
Bottom-fermented			
Lager 1	–	<DL	<DL
	20	19.9 ± 0.5	19.5 ± 0.5
	40	40.3 ± 1.0	39.8 ± 0.7
Lager 2	–	4.1 ± 0.2	<DL
Pilsen 1	–	14.1 ± 0.5	<QL
Pilsen 2	–	12.2 ± 0.5	<QL

[a] Ref. [26]. QL, 15 ng mL^{-1}; DL, 5 ng mL^{-1}.
[b] Three replicates.

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