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Determination of *trans*-resveratrol in grapes by pressurised liquid extraction and fast high-performance liquid chromatography

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Abstract

A study has been made of the extraction of *trans*-resveratrol from grapes using pressurised liquids (PLE); for this, the first stage was to determine the stability of this compound during extractions at different temperatures (50, 100, 150 °C), with quantitative recoveries being obtained up to 150 °C. By employing solid-phase extraction (SPE) it was possible to retain this compound and separate it from other interfering substances present in the grape. The method developed comprises a sequential extraction of the sample adsorbed (0.5 g) on a polystyrene-divinylbenzene based sorbent in the extraction chamber, first with water at 40 °C and 40 atm of pressure (three cycles of 5 min), and then with methanol at 150 °C and 40 atm (three cycles of 5 min). The *trans*-resveratrol content of the methanolic extract is determined by means of liquid chromatography. A rapid (5 min) chromatographic method employing a monolithic column, with fluorescence detection, has been developed; for this, the conditions for detection of the compound were optimised (excitation at 310 nm and emission at 403 nm). The analytical parameters of the method of chromatographic analysis developed have been calculated: linear range (0.11–2.75 mg/L), detection limit (0.003 mg/L), quantification limit (0.004 mg/L). Using this method, three varieties of grape have been analysed and the concentration of *trans*-resveratrol in these has been determined.

Keywords: Resveratrol; Grape; Pressurised liquid extraction; Fast HPLC

1. Introduction

trans-Resveratrol (3,5,4′-trihydroxystilbene) is a natural compound present in many vegetables and in related foods; it is produced by plants in response to fungus infections (particularly of *Botrytis* cinerea) or to diverse abiotic factors, such as the presence of metallic ions, hydric stress, or exposure to UV light, acting therefore like phytoalexins [1].

The bibliography refers to studies, which demonstrate that this compound has beneficial effects on human health, which explains the interest in its determination that has arisen in recent years. Diverse biological properties have been attributed to it, such as protection against atherosclerosis, and against cardiovascular diseases [2], where it appears to be involved in the low mortality rates from these diseases that are observed in populations that regularly consume wine moderately, as well as anti carcinogenic properties, by inhibiting cellular processes related to tumor initiation, promotion and progression [3].

Various different techniques have been employed for the determination of *trans*-resveratrol in liquid samples: gas chromatography [4], CG–MS [5], capillary electrophoresis [6], the most commonly utilised being high-performance liquid chromatography (HPLC, normally in reverse phase), either with UV detection [7,8], electrochemical detection [9], fluorimetric detection [10] or with mass spectrometry [11]; this compound can be easily determined in wines and other drinks by means of direct injection [12].

Recently, columns of HPLC with monolithic supports have begun to be employed, which enable faster separations to be obtained [13]. This type of chromatographic column is currently used mainly for the determination of pharmaceutical compounds in different matrices [14–17], or of proteins and peptides [18]. In the bibliography there is a report of the development of a method for the determination of phenolic compounds in wines,

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among, which resveratrol is included, that requires 30 min for the elution of this compound [19].

In order to perform the analysis of *trans*-resveratrol in solid food matrices, a prior extraction stage is necessary. Generally, liquid–solid extraction has been utilized by maceration with organic solvents [20]. In some cases, solid-phase extraction (SPE) has also been employed [21] on the extracts obtained. These methods of maceration usually involve long periods of time for the extraction, which increases the possibility that degradations may occur [22]. This is especially problematic considering that *trans*-resveratrol is an easily oxidizable and photosensitive compound. For this reason, the extraction processes must be performed especially carefully, with the samples protected as much as possible from the air and light.

Extraction with pressurised liquids employs solvents at high temperatures to accelerate the extraction of compounds from solid samples. Since extractions can be performed protected from the light and under an inert atmosphere, it is a good alternative to the classic methods of extraction; degradations by oxidations or by light can be completely avoided; therefore this method is particularly recommended for extracting compounds that react readily with oxygen [23,24], as occurs with *trans*-resveratrol.

Given the complex nature of the matrix to which the extraction is to be applied, if this technique is combined with the solid-phase extraction, an efficient technique with high selectivity could be available. The sorbent selected is polystyrene-divinylbenzene, since it has previously been demonstrated that good results are obtained by applying classic SPE to samples of wine [25].

In the present article, a method is developed for determining *trans*-resveratrol in grapes by means of the coupling solid-phase extraction with pressurised liquid extraction (PLE), followed by the rapid determination by high-performance liquid chromatography with fluorimetric detection in the methanolic extract.

2. Experimental

2.1. Equipment

An extractor model ASE 200 of Dionex (Sunnyvale, CA, USA), incorporating extraction chambers of stainless steel of 11 mL and collection vials of 60 mL capacity was employed for the extractions. As usually, solvents used in the pressurised liquid extractions were sonicated to degas them before the process.

For the preservation of samples, an EZ-Dry model EZ 585 Q liophylizer by FTS Systems (Stone Ridge, NY, USA) was utilised.

The chromatographic analyses were performed in a Dionex chromatographic system (Sunnyvale, CA, USA), with a P680 pumping system, an ASI 100 automatic injector, a TCC-100 oven for columns, a PDA-100 UV–vis detector, an RF 2000 fluorescence detector, and Chromeleon 6.60 software, using a Chromolith Performance PR-18e monolithic column (Merck, Darmstadt, Germany) of 100 mm × 4.6 mm.

2.2. Materials

The *trans*-resveratrol standard was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). The sea sand utilised as support material was provided by Panreac (Barcelona, Spain). For the solid-phase extraction, a LiChrolut EN (40–120 μm) of Merck was employed. The solvents employed were Milli-Q water, and methanol (HPLC grade) from Scharlau Chemie (Barcelona, Spain).

Healthy grapes of the Viura (white) and Tempranillo (red) varieties cultivated in Jerez (Spain) and picked manually, and the Napoleon variety (red table grapes) acquired commercially were used as samples.

2.3. Pressurised liquid extraction (PLE)–solid phase extraction (SPE)

2.3.1. Preparation of the standard solution of trans-resveratrol

The *trans*-resveratrol was dissolved in methanol (27.5 mg/L) and the solution was conserved at −20 °C protected from the light. For the stability assays, 0.5 mL of the solution was added directly onto the sand that had been placed in the extraction chamber. In the case of the coupled PLE–SPE system, the 0.5 mL were added onto the sand that was inserted on top of the sorbent previously placed in the chamber.

2.3.2. Preparation of the samples

The grapes were lyophilised until reaching a constant weight (representing a loss of approximately 80% of the original weight); then they were triturated in a milling device and were kept at −20 °C until the extraction. In the analysis, a quantity of around 0.5 g of sample was employed.

2.3.3. Preparation of the extraction chamber for the PLE–SPE

First 2.5 g of the adsorbent LiChrolut EN was placed in the chamber. After the adsorbent, a cellulose paper (Dionex) was inserted to separate it from the sample to be extracted, which was added next. Lastly the remaining space was filled with sand until the extraction chamber was completely full.

2.3.4. Extraction conditions

The extractions were performed following the method previously developed [26]. Briefly, an extraction phase was performed employing water as solvent, in three cycles for 5 min, at 40 °C and 40 atm of pressure. Then an extraction/elution stage was performed employing methanol at 150 °C and 40 atm, in this case with three cycles of 5 min duration. The methanolic extract was made up to 25 mL and an aliquot was taken for chromatographic analysis.

Experiments by direct lixiviation of the same powder used in the pressurized liquid extraction were also done. Using a magnetic stirring system the powder (0.5 g) was extracted with water (25 mL, 20 min) and methanol (25 mL, 20 min) at 65 °C.

2.4. Chromatographic analysis

The extracts were filtered through nylon filters of 0.45 μm pore size. Two solvents were used: solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol). The elution was performed in isocratic mode at 5 mL/min with 30% of solvent B and 70% of solvent A; the *trans*-resveratrol peak appeared after an elution time of 2.0 min.

3. Results and discussion

3.1. Chromatographic analysis

With the columns customarily employed in liquid chromatography, the retention times are frequently too high in respect of the characteristics of the compound to be analysed; therefore the possibility of employing a chromatographic column of the monolithic type was considered. By using this type, a higher elution flow was utilised working at the same pressure. Using this type of column combined with a high amount of methanol in the eluting solvent a faster elution of the *trans*-resveratrol was obtained in comparison with others methods found in the bibliography, where the elution time is 50 min according to Domínguez et al. [11], 30 min according to Castellari et al. [19], or 12 min according to Careri et al. [27].

A fluorescence detector was utilised as the system of detection in the chromatographic analysis, given that the levels of concentration of *trans*-resveratrol at which this compound is found in grapes make it necessary to employ a more sensitive detection system than UV–vis spectroscopy. To optimise the conditions of detection, the excitation and emission spectra of *trans*-resveratrol were recorded; the absorption maxima were reached with excitation at 310 nm and emission at 403 nm, as can be seen in Fig. 1. These conditions are similar to those found in the bibliography [28].

Several analyses were performed employing an elution under constant conditions of composition of the mobile phase, employing percentages of solvent A between 90 and 60%; the best separation results of the peak corresponding to *trans*-resveratrol were obtained under the conditions of 70% of solvent A and 30% of solvent B.

Table 1
Mean values ($n = 3$) of the peak for resveratrol found on performing the chromatographic elution at three different temperatures

	10 °C	25 °C	40 °C
Peak width	n.a.	0.18	0.24
Resolution	n.a.	1.30	0.83

Sample: Tempranillo grape variety. n.a.: not available, peaks not resolved.

Table 2
Repeatability of the chromatographic method for the peak of *trans*-resveratrol in the sample of the Tempranillo red grape variety ($n = 6$)

	RSD (%) $n = 6$
Time	1.04
Concentration	1.94

The effect of different column temperatures (10, 25 and 40 °C) on the resolution and width of the peak of *trans*-resveratrol was also evaluated. In function of the results presented in Table 1, the following were selected as optimum conditions for the elution: temperature of 25 °C, in isocratic mode of 30% of solvent B and 70% of solvent A, with a flow rate of 5 mL/min. Under these conditions we achieved the elution of *trans*-resveratrol in a time of 2.0 min.

At the same time the repeatability of the chromatographic method was evaluated. The results employing a sample resulting from an extract of the Tempranillo grape variety are shown in Table 2. The corresponding chromatogram is presented in Fig. 2. It can be observed that, both in time and in concentration, the results show a high repeatability (RSD < 2%).

Table 3 gives the analytical characteristics of the calibration curves prepared employing detection by absorption at 310 nm against detection by fluorescence. As can be seen, the analytical sensitivity of this latter method is much higher than that recorded employing detection by absorption at 310 nm. This table also shows the limits of quantification (LOQ) and detection (LOD) calculated employing the Alamin program [29].

The procedure described therefore provides a rapid method for the chromatographic determination of *trans*-resveratrol in methanolic extracts of grapes.

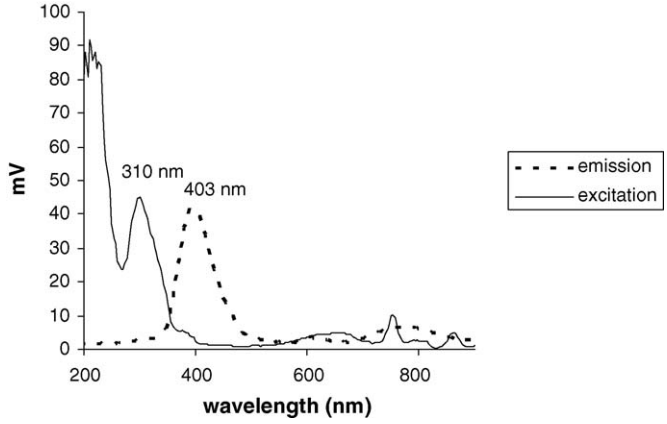

Fig. 1. Fluorescence excitation and emission spectra of *trans*-resveratrol.

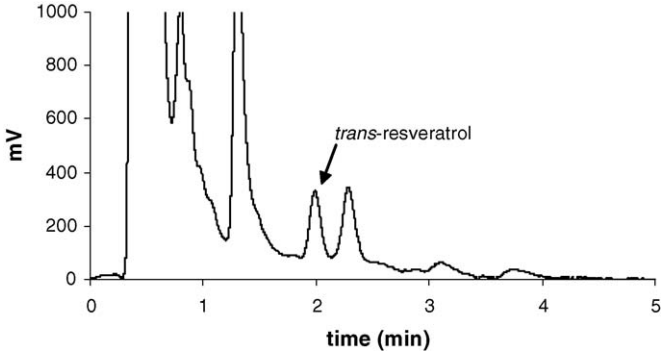

Fig. 2. Chromatogram of a Tempranillo grape extract (fluorescence signal, emission 403 nm).

Table 3
Calibration curves of *trans*-resveratrol

Detection	Intercept	Slope	Coefficient of regression	Interval (mg/L)	LOD (mg/L)	LOQ (mg/L)
310 nm	−4060.1	135372	0.9999	0.11–2.75	0.017	0.058
Fluorescence	−188091	42113985	0.9998	0.11–2.75	0.003	0.004

3.2. Extraction with pressurised liquids

For the application of PLE, it is first necessary to study the stability of this compound when subjected to the action of the high temperatures employed in PLE, since work has been published indicating that this compound degrades during extraction processes [22].

For this study, extractions were made of a standard solution of *trans*-resveratrol (27.5 mg/L) supported on an inert substance: sand. Since there is no interaction between the sand and the analyte, the recoveries obtained against the references prepared daily represent the stability of the compound under the extraction conditions. The assays performed at 50, 100 and 150 °C were performed in triplicate, and the following are the mean recoveries and the standard deviations obtained: 104.2% ± 4.5 (50 °C), 106.6% ± 4.3 (100 °C) and 99.5% ± 5.7 (150 °C).

Resveratrol was completely recovered even at the highest assayed temperature (150 °C). It means that this compound in not degraded at all using the PLE. Most likely, the inert atmosphere employed and the absence of light are responsible for the *trans*-reveratrol to be stable under the extraction conditions.

Next the same standard solution was utilised to optimise the extraction method when a sorbent is employed in the extraction chamber. This design of the extraction method was based on studies previously undertaken on PLE–SPE for the extraction of other phenolic compounds [26]. When water is employed as extractant, *trans*-resveratrol is not eluted from the sorbent, but it is possible that the sugars present in the sample may pass over. Hence, by eluting the *trans*-resveratrol with methanol, we are able to obtain an extract free from interfering sugars, and enriched with the analyte of interest.

In summary, the method of extraction to apply to the real samples comprises two stages. The first stage consists of three cycles of 5 min with water as solvent at 40 °C and 40 atm; this is the extraction/retention process, by this way the polar compounds are collected in the extract. The second stage consists of three cycles of 5 min with methanol as solvent at 150 °C and 40 atm; this is the extraction/rinsing process, in this case and additional extraction occurs; *trans*-resveratrol is not retained or rinsed from the sorbent and then collected in the extract. When extracting real samples, it could be advantageous to perform the extraction at the highest temperature in order to get a faster the extraction; this is the reason for using the highest available temperature in the second stage of the extraction method.

3.3. Application to real samples

Once the extraction method had been developed, it was applied to three varieties of grape (two varieties for vinification, white and red, and one variety of red table grape) in order to quantify *trans*-resveratrol in grapes. Three samples of each variety were analysed. The extracts obtained were diluted up to the closest exact volume, and were analysed by liquid chromatography after filtration, using a filter of 0.45 μm; the resulting chromatogram is shown in Fig. 2.

Table 4
Repeatability of the complete method for determination of resveratrol

Extract	Amount of resveratrol (mg/kg)
1	2.09
2	2.23
3	2.33
4	2.14
5	2.06
6	2.25
Mean	2.18
SD	0.104
RSD	4.75

Sample: Grapes of the Viura variety.

In the case of the aqueous extracts obtained in the first stage, *trans*-resveratrol was not detected in these (LOD 4 ppb), in any of the samples analysed. A re-extraction of the sample was performed using the same conditions. Again *trans*-resveratrol was not detected in the re-extracts.

At the same time, the repeatability of the method of analysis was evaluated when applied to real samples; it was able to confirm that the RSD is below 5% ($n = 6$), the data corresponding to the measurement of the repeatability of the complete method for determining resveratrol (PLE + HPLC) are given in Table 4.

Samples were analyzed in triplicate. The results obtained were: 0.11 (±0.006) mg/kg for the Napoleon table grape variety; 2.78 (±0.141) mg/kg for the Viura grape variety; and 3.42 (±0.174) mg/kg for the Tempranillo grape variety.

The same samples were subjected to an extraction using a magnetic stirring system. The grape powder was extracted for 40 min using 25 mL of water (20 min) and 25 mL of methanol (20 min) at 65 °C. Both extracts were joined and the resveratrol amount was determined by HPLC. The average resulting amounts of resveratrol were: 0.091 (±0.005) mg/kg for the Napoleon variety; 2.45 (±0.136) mg/kg for the Viura variety; and 2.92 (±0.133) mg/kg for the Tempranillo variety. It means that recoveries using the developed PLE–SPE method were higher than using a magnetic stirring assisted maceration extraction with the same solvents.

4. Conclusions

The coupling of solid-phase extraction with extraction using pressurised liquid is a possible alternative for the extraction at

high temperatures of *trans*-resveratrol in grapes, without the necessity of a cleaning stage, additional to the routine filtration, for its subsequent analysis by HPLC.

With the method developed, the time required for the extraction stage is reduced to some 40 min (representing a considerable reduction when compared with the traditional methods of maceration of solid samples); it also enables the quantity of *trans*-resveratrol existing in grapes to be determined, rather than in the peels only (as in the customary methods).

The most notable features of the chromatographic method developed are that it enables *trans*-resveratrol to be determined extremely fast (in around 2 min) and simply, and that, with the method of detection by fluorescence developed, an increase has been achieved in the sensitivity of the method of analysis by a factor of 250 times, compared with detection by UV–vis spectroscopy.

The complete method developed enables resveratrol to be determined at a rate of 45 min per sample, and the RSD of the results is below 5%.

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