JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Development and Validation of an Analytical Method for the Determination of *trans*- and *cis*-Resveratrol in Wine: Analysis of Its Contents in 186 Portuguese Red Wines

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ABSTRACT: A simple procedure based on solid-phase extraction and high performance liquid chromatography coupled to diode array detector has been developed and validated for the qualitative and quantitative analysis of *cis*- and *trans*-resveratrol in wines.

The method was linear from 0.025 (lower limit of quantitation, LLOQ) to 15 μg/mL for *trans*-resveratrol and from 0.023 (LLOQ) to 0.92 μg/mL for *cis*-resveratrol, with correlation coefficients higher than 0.99 for both isomers. Intra- and interday precision and accuracy were in conformity with the criteria normally accepted in method validation, that is, CVs inferior to 15% and mean relative errors within a ±14% interval. The extraction presented mean efficiencies close to 100% for both analytes.

The validated methodology was applied to 186 Portuguese red wines from different regions, grape varieties and vintage. The results obtained showed that the content of *trans*-resveratrol in red wines ranged from 0.05 to 10.9 μg/mL, while the concentrations of *cis*-resveratrol ranged from 0.04 to 8.71 μg/mL.

KEYWORDS: resveratrol, SPE, HPLC-DAD, red wine

INTRODUCTION

Portugal has a long tradition as a wine producing country, and there are several registered regions which produce quality wines with designation of origin (DO) recognized by the European Union. Resveratrol (3,4′,5-trihydroxystilbene) has been identified as the major active biological compound of the stilbene phytoalexins in wine.[1−3] It can be found in wines, grapes, legumes, berries, peanuts and pistachios.[4−8] Chemically, resveratrol can exist as two isomers, *trans*-resveratrol and *cis*-resveratrol. The concentrations of each isomer in different grape cultivars and the respective wines are extremely variable, depending also on geographical origin, winemaking processes, climate and fungal presence.[9] *trans*-Resveratrol isomerizes to *cis*-resveratrol when exposed to UV light[8] and this process causes changes in the respective concentrations in grapes, must and wine, despite the fact that is usually found at higher concentrations in wine.

Positive effects of resveratrol on health include cardiovascular protection and risk reduction concerning heart diseases, anticancer and anti-inflammatory properties and antioxidant and antibacterial activities.[10−16]

Since resveratrol is well-known as one of the most important compounds present in wine, numerous analytical procedures have been published for its determination. The most commonly used techniques are high performance thin-layer chromatography (HPTLC)[17] and high performance liquid chromatography (HPLC) coupled to UV,[18−21] fluorescence (FLD)[22,23] or electrochemical[24] detection. LC coupled to mass spectrometry (LC−MS) has also been successfully applied to analyze resveratrol in different foodstuffs.[25−27] Gas chromatography (GC) has also been used for this purpose, but this technique presents a drawback: a derivatization step is essential,[7,28] involving a long and tedious treatment prior to the analysis. In addition, the high temperatures achieved may cause the isomerization and degradation of the analyte.[14,23] Capillary electrophoresis (CE) has also been used for the analysis of these compounds.[29,30]

Wine samples can be analyzed by direct injection[21,23,31] or after pretreatment by means of either liquid−liquid extraction (LLE),[19,32−34] solid phase extraction (SPE)[35−38] or, more recently, solid-phase microextraction (SPME).[39]

This paper describes a novel procedure for the determination of *trans*- and *cis*-resveratrol in wine using solid phase extraction (SPE) and high performance liquid chromatography with diode array detector (HPLC-DAD). It should be noted that, to the best of our knowledge, this is the first time that a C_8 column with a new procedure for SPE is used with high extraction efficiencies and low limits of quantification (LLOQ).

The average levels of *trans*-resveratrol in red wines vary greatly from one region to another or between varieties or vintages.[9] An average red wine can be expected to contain 1.9 ± 1.7 μg/mL of *trans*-resveratrol, with nondetectable levels as the lower limit.[9,35,37]

Thus, the aim of this study was the development and validation of an analytical method based on SPE and HPLC with diode array detector for the determination of *trans*- and *cis*-resveratrol in different kinds of Portuguese red wines, 186 samples being analyzed. To our knowledge, this work is the first to produce a detailed analysis of the resveratrol contents in such a high number of wine samples.

Received: October 8, 2010
Accepted: January 24, 2011
Published: March 01, 2011

MATERIALS AND METHODS

Reagents and Standards. Acetonitrile, diethyl ether, methanol and dichromethane were obtained from Merck Co. (Darmstadt, Germany), all with 99.9% purity (HPLC grade). Ethyl acetate and *n*-hexane were obtained from JT Baker (Deverter, The Netherlands) both with 99.9% purity. Acetic acid (99.9% purity), isopropanol (99.8% purity) and ammonium (99.5% purity) were obtained from VWR (Fonteray-sous-Bois, France), José M. Vaz Pereira (Sintra, Portugal) and Fluka (Steinheim, Switerzland), respectively. Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). *trans*-Resveratrol (3,4,5-trihydroxystilbene) (99.9% purity) was purchased from Extrasynthése (Genay, France), and carbamazepine [internal standard (IS), 99,9% purity] was purchased from Sigma Aldrich Química (Sintra, Portugal). Stock solutions (1 mg/mL) of *trans*-resveratrol and the internal standard were prepared by dissolving each pure substance in methanol. Subsequent working solutions at 1, 10, and 100 μg/mL were also prepared by proper dilution in methanol. Solutions of *cis*-resveratrol, since its commercial standard is not available, were prepared from working standard solutions of *trans*-resveratrol as follows. A *trans*-resveratrol stock solution (4 μg/mL) was placed in a UV box (Vilber Loumart, Marne la Valleé, France) and was exposed at a distance of 20 cm to UV light (365 nm, potency equal to or higher than 8 W) for 30 min, obtaining an efficiency of 92%. All solutions were stored at 4 °C, avoiding exposure to direct light.

Wine Samples. Wine samples from different vintages, grape varieties, and from different Portuguese regions were kindly donated by winemakers.

Instrumentation. SPE was carried out on BondElut LRC certify C_8 cartridges (300 mg) (Varian B.V., Middelburg, The Netherlands). The HPLC system includes a quaternary pump with controller (model 600), a manual injector (Rheodyne 7725i), an in-line degasser (AF) and a diode array detector (DAD-2996) from Waters (Milford, MA, USA). Chromatographic separation was achieved using a 5 μm particle size XTerra MS C_{18} ODS reversed-phase analytical column (150 × 4.6 mm i.d.) from Waters (Milford, MA, USA). All the injections were made with a rheodyne valve, equipped with a 20 μL sample loop.

Gas chromatography−mass spectrometry (GC−MS) analysis was performed using an HP 6890N gas chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a model 5973 mass selective detector (Hewlett-Packard, Waldbronn, Germany). A capillary column (30 m × 0.25-mm i.d., 0.25 μm film thickness) with 5% phenylmethylsiloxane (HP-5 MS), supplied by J & W Scientific (Folsom, CA, USA), was used.

Chromatographic and Detection Conditions. The mobile phase consisted of a mixture of water, acetonitrile and acetic acid (66:33.9:0.1, v/v/v, pH 3.4) and was filtered through a 0.20 μm pore size membrane, degassed ultrasonically and pumped in isocratic mode through the chromatographic system at 0.5 mL/min. The eluate was monitored at three different wavelengths: 306, 284, and 211 nm, where *trans*- and *cis*-isomers of resveratrol and carbamazepine (internal standard, IS)[40] have maximum absorbance, respectively. The retention times were 7.1, 9.1, and 11.0 min for *trans*-resveratrol, *cis*-resveratrol and IS respectively, obtaining a good separation of all compounds. Carbamazepine was used as internal standard because the same extraction and chromatographic conditions, as well as detection wavelength, could be used. In addition, this compound is usually not found in wine samples.

Gas chromatographic conditions were as follows: initial oven temperature was 90 °C for 2 min, which was increased by 20 °C min^{-1} to 300 °C and held for 3 min. The temperatures of the injection port and detector were set at 220 and 280 °C, respectively. The split injection mode was used (split ratio of 1:5), and helium with a flow rate of 0.8 mL min^{-1} was used as the carrier gas. The mass spectrometer was operated with a filament current of 300 μA and electron energy of 70 eV in the electron ionization (EI) mode.

Extraction Procedure. The extraction procedure was optimized previously (see below), and the final conditions were as follows. A 2 mL wine sample was diluted with 2 mL of water and 1 mL of phosphate buffer 0.1 M (pH 6.0) and spiked with 100 μL of the IS solution (100 μg/mL). The mixture was agitated in the roller mixer for 5 min. The mixture was loaded onto a BondElut LRC certify cartridge, previously conditioned with 1 mL of methanol and 1 mL of KH_2PO_4 0.1 M (pH 6).

After the sample had passed through, the column was washed sequentially with 3 mL of water, 1 mL of 0.1 M acetic acid and 2 mL of *n*-hexane, and dried under full vacuum for 10 min. The analytes were eluted with 3 mL of a mixture of dichloromethane:isopropanol:ammonium [78:20:2 (v/v/v)], which was afterward evaporated to dryness at 30 °C under a gentle stream of nitrogen. The dry extract was dissolved in 100 μL of mobile phase, and 20 μL was injected into the HPLC system. The whole extraction procedure was carried out in subdued light to avoid the light-induced isomerization of *trans*-resveratrol to the *cis*-isomer during sample handling.

RESULTS AND DISCUSSION

Preparation of the *cis*-Resveratrol Standard. Several ways of transforming a standard solution of *trans*-resveratrol into the *cis* form have been reported in the literature: exposure to daylight[18] and UV light at 360 and 254 nm.[8] The ideal conditions of irradiation to transform the *trans* into the *cis* form were established. The parameters that could influence the transformation of isomeric compound, such as UV radiation at 254 and 365 nm, time profiles (10, 20, and 30 min), the initial concentration of *trans*-resveratrol (4 μg/mL and 1 mg/mL), different solvents (methanol and 12% ethanol) and several distances between the lamp and the solution (10, 20, and 60 cm), were optimized. This optimization was performed employing a univariate approach, that is, each factor was evaluated while all other factors were kept constant. First, a 4 μg/mL solution of *trans*-resveratrol was used and the distance to the UV light was fixed at 20 cm; three exposition times were studied (10, 20, and 30 min). These experiments were performed at 254 and 365 nm. The maximum process efficiency was obtained for an exposition of 30 min at 365 nm (about 90%), while at 254 nm only 40% efficiency was obtained. Following these experiments, the wavelength and exposition time were fixed at 365 nm and 30 min respectively. Three distances to the UV source were evaluated (10, 20, and 60 cm). The best results were obtained for a distance of 20 cm (around 90%), while efficiencies of 68 and 26% were obtained at 10 and 60 cm respectively. Under these optimized conditions, further experiments were performed, in order to find the concentration of the *trans*-resveratrol solution which yielded the highest process efficiency (4 μg/mL versus 1 mg/mL). The best results were obtained for a 4 μg/mL solution. Finally it was tried to find the best solvent for this solution, and both methanol and a 12% ethanol solution (to simulate wine samples) were used. Higher process efficiencies were obtained when methanol was used (90 versus 45%), and therefore this solvent was used in this work.

The final optimized conditions for *trans*-resveratrol conversion were therefore as follows: a stock solution at 4 μg/mL in methanol was exposed at 20 cm to UV light (365 nm) for 30 min. Process efficiency was calculated by the difference between *trans*-resveratrol peak areas before and after the treatment with UV light (n = 6), and a value of 92.39 ± 0.27% was obtained. The quantity of *cis*-resveratrol was therefore considered to be 92% of

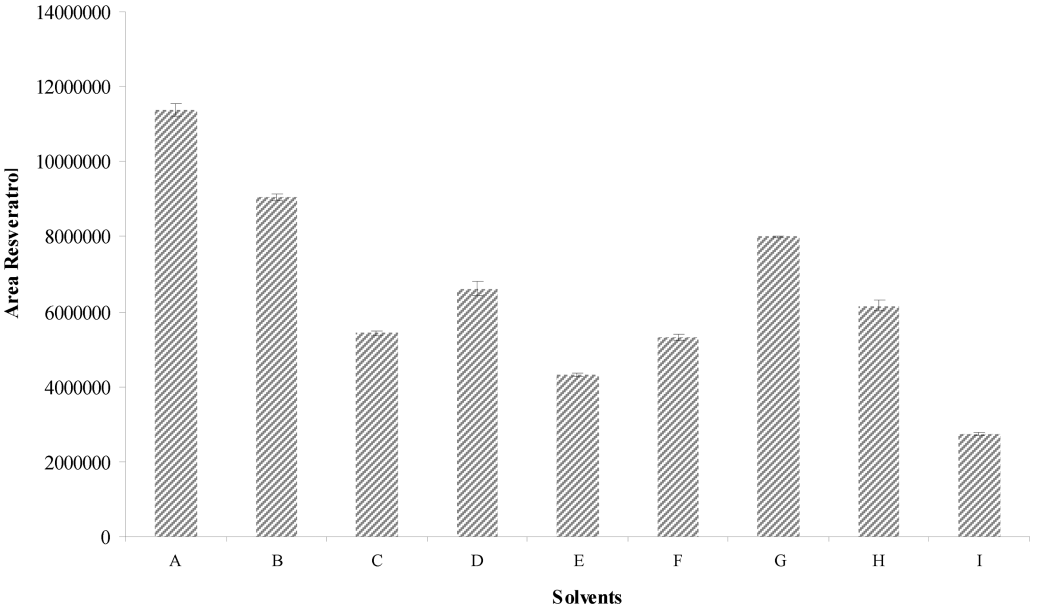

Figure 1. Influence of the extracting solvent on the peak area of *trans*-resveratrol: (A) diethyl ether, (B) ethyl acetate, (C) ethyl acetate:*n*-hexane (50:50), (D) ethyl acetate:*n*-hexane (90:10), (E) ethyl acetate:*n*-hexane (10:90), (F) dichloromethane:ethyl acetate (50:50), (G) dichloromethane:ethyl acetate (90:10), (H) dichloromethane:ethyl acetate (10:90), (I) acidic methanol:ethyl acetate (50:50).

the initial concentration of the *trans*-isomer. Those isomerization conditions are usually not described in detail in the literature, and variable recoveries have been reported. Indeed, Trela et al.[41] had conversion rates of 67% (exposure at 254 nm for 10 h), while Vian[18] and Romero-Perez[42] reported rates of 80−90% by exposing *trans*-resveratrol solutions to sunlight for 1 h[16] and for 10 min.[42] However, these latter methods present the disadvantage that sunlight is not constant throughout the day or on successive days, and therefore the conditions are difficult to reproduce adequately.

Optimization of Extraction Procedure. Red wines are highly complex and variable matrices, thus an extraction procedure is often deemed necessary to obtain adequate sample purification and to enhance the chromatographic column performance and lifetime.

Before the application of the extraction technique to the wine samples, several experiments with a simulated wine ("blank" matrix) were carried out in order to select the optimum extraction process. For the optimization of the extraction procedure several kinds of simulated wine were tried (n = 3): an aqueous solution pH 3.4 with 1 M HCl and an aqueous solution containing ethanol (88:12 v/v) and tartaric acid (5 g/L) and bringing the apparent pH to 3.6 with 2 M NaOH.[36,43,44] The obtained results for *trans*-resveratrol peak areas using each of these solutions did not vary significantly, and therefore we have decided to use the one which could be more easily prepared. We could not use authentic wine, since resveratrol was always present. Due to the huge number of samples analyzed, to use the standard addition method would not have been practical.

The first approach to extract the compound was LLE as described elsewhere.[19,32−34] Several organic solvents and extraction times were tested. Although the most common extraction solvents are diethyl ether and ethyl acetate, in this study we also tested a mixture of ethyl acetate:*n*-hexane at different proportions (50:50, 90:10 and 10:90), dichloromethane with ethyl acetate (50:50, 90:10 and 10:90) and acidic methanol with ethyl acetate

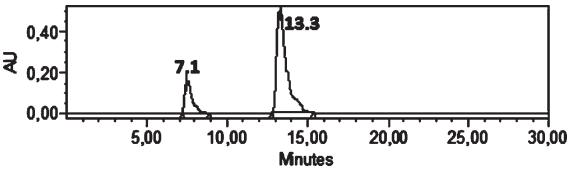

Figure 2. Chromatogram of a simulated wine sample after LLE with diethyl ether.

(50:50). The best results were obtained with diethyl ether (Figure 1). However, this solvent also extracted other monomeric phenols (e.g., catechins and gallic acid) from wines. Even when simulated wine was used, a new peak appeared (Figure 2). To try to identify this peak, the extracts were derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) containing 5% trimethylchlorosilane (TMCS)[7,28] and analyzed by GC−MS in the full scan mode (Figure 3A−C). This unknown compound seemed to be generated by reaction of *trans*-resveratrol with diethyl ether, and the formation of a resveratrol dimer may have occurred. So we tested solid-phase extraction (SPE) as an alternative to LLE. With SPE many of the problems associated with LLE can be prevented, such as incomplete phase separations, poor recoveries, and large solvent consumption. SPE is in general more efficient than LLE, as it yields a quantitative extraction that is easy to perform, and solvent use and laboratory time are reduced. Several types of cartridges were tested: Oasis HLB (60 mg) and MCX (60 mg), and BondElut C_8 (300 mg). Several extraction protocols were used, two for HLB and BondElut cartridges [first a standard protocol (PT1) and a second protocol promoting strong cation exchange (PT2)] and one to MCX cartridges.

Sample preparation was the same for the three protocols consisting of the following: a 2 mL wine sample was diluted with 2 mL of water and 1 mL of phosphate buffer 0.1 M (pH 6.0), and spiked with 100 μL of IS. The mixture was agitated in the roller mixer for 5 min.

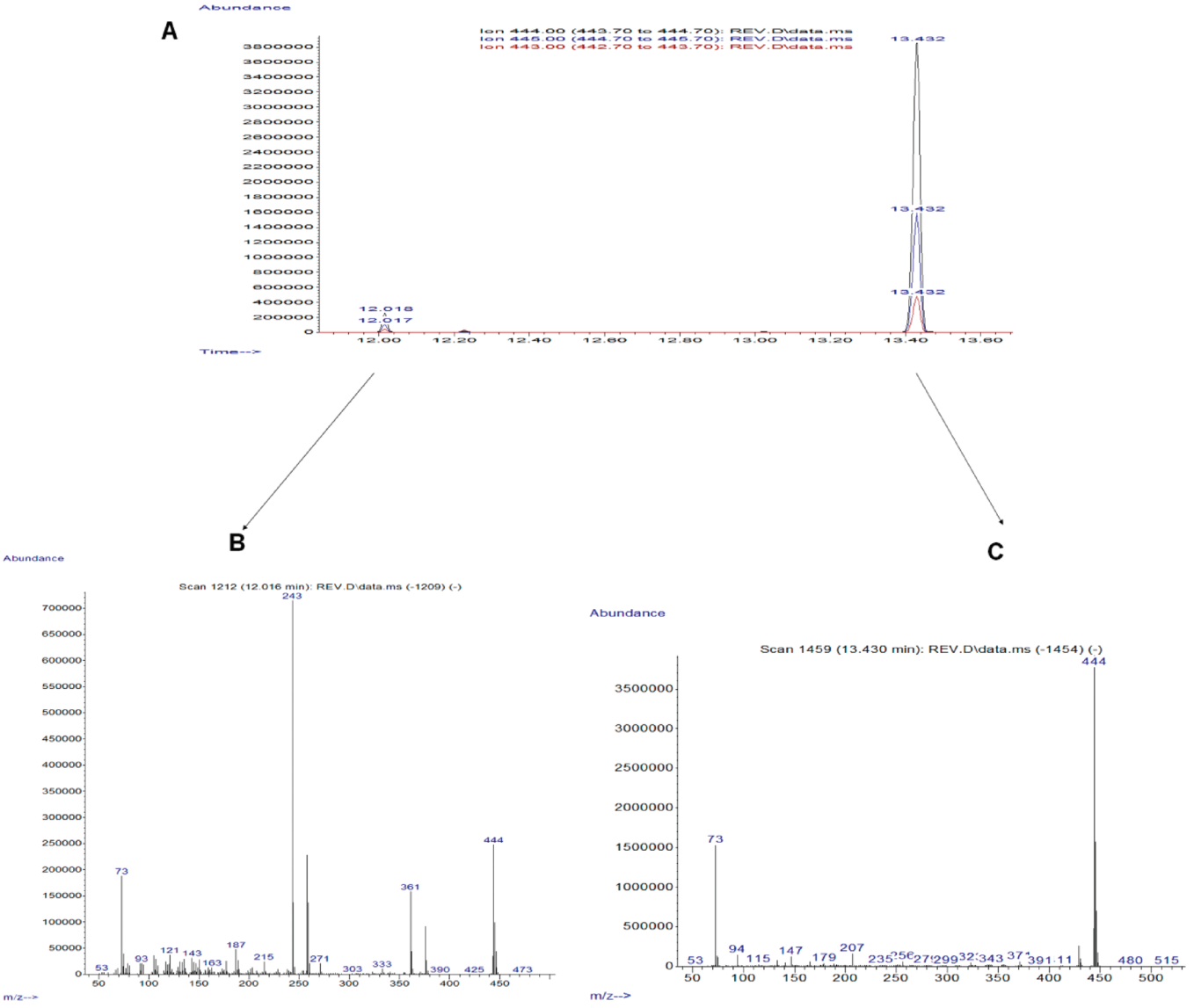

Figure 3. GC−MS chromatogram of a LLE of *trans*-resveratrol (5 μg/mL) with diethyl ether (A). Mass spectrum of the TMS-derivative of *trans*-resveratrol (B) and mass spectrum of the unknown compound (C).

Three different SPE protocols were tested, and in all of them the extracts were evaporated to dryness under a gentle nitrogen stream. All the experiments were performed in triplicate at three different concentrations.

For Oasis HLB or BondElut Certify extraction cartridges, two different protocols were used, with differences in the conditioning, washing and elution steps. In the first protocol the mixture was loaded onto the cartridges, which had been previously conditioned with 2 mL of methanol and 2 mL of water. After the sample had passed through, the cartridge was rinsed with 2 mL of 5% of methanol, and dried for 10 min under vacuum. The analytes were then eluted with 2 mL of methanol. In the second protocol the mixture was loaded onto the cartridges, previously conditioned with 2 mL of methanol and 2 mL of 0.1 M KH_2PO_4. After the sample had passed through, the columns were washed sequentially with 2 mL of water, 2 mL of 0.1 M acetic acid and 2 mL of *n*-hexane, and dried under full vacuum for 10 min. The analytes were eluted with 2 mL of a mixture of dichloromethane:isopropanol:ammonium [78:20:2 (v/v/v)].

In the third protocol, mixed-mode (Oasis MCX) cartridges were used, which were previously conditioned with 2 mL of methanol and 2 mL of water. After the sample had passed through, the cartridge was then rinsed with 2 mL of HCl 0.1 M, 2 mL of methanol and 2 mL of *n*-hexane, and then dried for 10 min under vacuum. The analytes were then eluted with 2 mL of a mixture of dichloromethane:isopropanol:ammonium [78:20:2 (v/v/v)].

In all protocols the dry extract was dissolved in 100 μL of mobile phase, and 20 μL was injected into the HPLC system. The whole extraction procedure was carried out in subdued light to avoid the light-induced isomerization of *trans*-resveratrol to the *cis*-isomer during sample handling.

Figure 4 illustrates the obtained results, and as can be seen, the BondElut cartridge with the PT2 protocol originated better extraction yields. Therefore, it was chosen to perform the remaining experiments.

Method Validation. The analytical validation was performed according to the guiding principles of the FDA[45] and ICH.[46] The procedure was validated in terms of selectivity, linearity, limits of

detection and quantification, precision, accuracy, stability in processed samples and extraction efficiency.

Specificity. The method's specificity was checked by chromatographic analysis of other substances that might be present in an authentic sample [other polyphenols, such as catechin (peak resolution of 2.9), epicatechin (peak resolution of 2.6), rutin (peak resolution of 2.5) and gallic acid (peak resolution of 3.4), and vitamins, such as retinol (peak resolution of 2.6), thiamine (peak resolution of 3.6) and ascorbic acid (peak resolution of 3.0)]. Since it is not possible to test for all the possible interferences, only the most frequent were tested. It was found that any influence could be excluded due to different retention times and/or absorbance spectra, as no interfering peaks were observed at the retention times and selected wavelength for *trans*- and *cis*-resveratrol. In addition, in all the analyzed wine samples the UV spectra of the tested analytes were compared to those obtained in a quality control sample analyzed contemporaneously. A match quality higher than 90% was obtained for both compounds in all the tested wine samples, and therefore the method was considered specific.

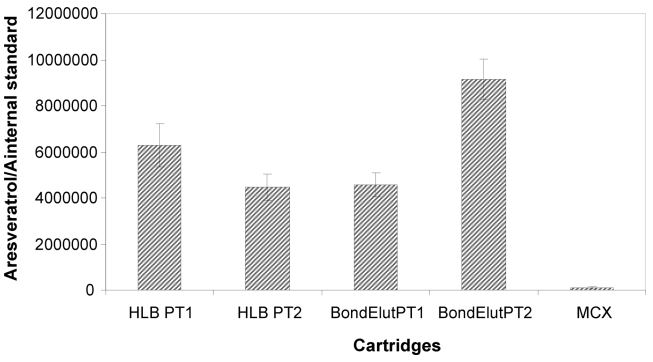

Figure 4. Comparison of different SPE cartridges with three distinct protocols. Vertical bars represent the standard deviation (n = 3).

Table 1. Linearity Parameters

		linearity		
compound	calibration range (μg/mL)	slope	intercept	R^2 values
trans-resveratrol	0.025−1.00	0.131	0.0008	0.9999
	1.00−15.00	0.129	0.008	0.9993
cis-resveratrol	0.023−0.92	0.057	0.0003	0.9999

Calibration Curves and Limits. Calibration curves for both resveratrol isomers were performed separately, in order to avoid the influence of the small percentage of *trans*-resveratrol present in the solution of *cis*-resveratrol standard.

For *trans*-resveratrol we determined the linearity of the method at concentrations ranging from 0.025 to 15.00 μg/mL. Sixteen calibrators were prepared in simulated wine and analyzed using the described procedure. It was necessary to divide the calibration range in two linear ranges, 0.025−1.00 μg/mL and 1.00−15.00 μg/mL. For *cis*-resveratrol the linearity range was shortened, from 0.023 to 0.92 μg/mL with eight calibrators. The calibration curves were obtained by plotting the peak-area ratio between each analyte and the IS versus analyte concentration (Table 1). Calibrator's accuracy [mean relative error (bias) between measured and spiked concentrations] was within ±15% of the true values for all concentration levels. The limit of quantitation (LLOQ), defined as the lowest concentration that could be measured reproducibly and accurately, was 0.025 μg/mL for *trans*-resveratrol and 0.023 for *cis*-resveratrol, and these were also the method's limits of detection, since at concentrations below the LLOQ the reliable identification of the analytes was not possible. These limits are comparable to those obtained by other authors; however, in those papers the limits were assessed mathematically,[35,37,47−49] while in our work the limits were determined by the analysis of samples with decreasing concentrations of the compounds, obtaining adequate precision (coefficient of variation of less than 20%) and accuracy (bias within a ±20% interval of the true concentration).[46]

Precision and Accuracy. Precision and accuracy were evaluated at three concentration levels (0.30, 4.50, and 12.50 μg/mL) for *trans*-resveratrol and at two concentration levels for *cis*-resveratrol (0.28 and 0.74 μg/mL), analyzing for six replicates for each concentration in the same day (intraday precision) and on different days over seven days (interday precision). The obtained CVs did not exceed 15% for all studied concentrations. These values for precision fell well within the criteria normally accepted in bioanalytical method validation.[45,46] The values obtained for accuracy (in terms of bias) were within a ±15% interval of the nominal concentration, and were considered acceptable (Table 2).

Table 2. Precision, Accuracy and Extraction Efficiency Results

			intraday precision					interday precision		
concn (μg/mL)	efficiency mean (%)	SD[a]	concn (μg/mL)	measd concn mean (μg/mL)	CV[b] (%)	bias[c] (%)	concn (μg/mL)	measd concn mean (μg/mL)	CV[b] (%)	bias[c] (%)
				trans-Resveratrol						
0.025	102.03	5.59	0.025	0.03	8.95	7.82	0.30	0.28	10.56	−4.78
1.00	100.02	9.47	1.00	1.03	7.07	3.41	4.50	4.60	8.84	1.96
15.00	99.27	6.62	15.00	14.31	3.84	−6.10	12.50	12.66	4.71	1.94
				cis-Resveratrol						
0.023	101.03	11.61	0.023	0.03	14.21	13.71	0.28	0.30	6.80	8.70
0.092	100.15	15.34	0.092	0.08	15.05	8.16				
0.92	99.98	8.34	0.92	0.97	9.82	5.01	0.74	0.79	8.50	7.60

[a] Standard deviation. [b] Coefficient of variation. [c] Bias: [(measured concentration) − (nominal concentration)]/(nominal concentration)] × 100.

Table 3. Resveratrol Levels (μg/mL) for 186 Red Wines from Portugal

				concn (μg/mL)	
wine sample	region	varieties	vintage	*trans*-resveratrol	*cis*-resveratrol
#1	Douro	blend	2008	2.89 ± 0.003	0.17 ± 0.001
#2	Douro	NA	NA	0.80 ± 0.004	<LLOQ
#3	Douro	NA	2001	1.33 ± 0.008	$0.45 \pm 3 \times 10^{-4}$
#4	Douro	NA	NA	0.05 ± 0.001	$0.19 \pm 2 \times 10^{-4}$
#5	Douro	NA	NA	1.22 ± 0.021	<LLOQ
#6	Douro	NA	NA	1.13 ± 0.021	0.21 ± 0.001
#7	Alentejo	blend	2003	1.12 ± 0.010	0.13 ± 0.001
#8	Douro	NA	1991	1.98 ± 0.004	0.15 ± 0.001
#9	Douro	blend	1991	$1.91 \pm 3 \times 10^{-4}$	0.81 ± 0.040
#10	Ribatejo	NA	2008	2.22 ± 0.011	0.73 ± 0.005
#11	Beira Interior	blend	2006	$0.50 \pm 3 \times 10^{-5}$	<LLOQ
#12	Península de Setúbal	blend	2005	1.69 ± 0.003	$0.33 \pm 6 \times 10^{-5}$
#13	Alentejo	NA	2008	0.95 ± 0.027	<LLOQ
#14	Península de Setúbal	blend	2006	3.09 ± 0.019	0.67 ± 0.005
#15	Douro	blend	2006	2.56 ± 0.018	1.06 ± 0.005
#16	Alentejo	blend	2008	2.04 ± 0.012	0.14 ± 0.001
#17	Beira Interior	blend	2005	0.68 ± 0.007	$0.09 \pm 2 \times 10^{-4}$
#18	Beira Interior	blend	NA	2.83 ± 0.003	0.12 ± 0.001
#19	Beira Interior	blend	2001	3.84 ± 0.009	$0.32 \pm 4 \times 10^{-4}$
#20	Beira Interior	blend	2005	1.21 ± 0.001	0.14 ± 0.001
#21	Beira Interior	blend	2005	0.70 ± 0.012	<LLOQ
#22	Beira Interior	monovarietal	2004	1.26 ± 0.013	<LLOQ
#23	Bairrada	blend	2006	3.30 ± 0.068	0.34 ± 0.004
#24	Alentejo	blend	2005	1.93 ± 0.007	$0.35 \pm 9 \times 10^{-5}$
#25	Beira Interior	blend	2006	1.60 ± 0.010	0.24 ± 0.003
#26	Beira Interior	blend	2007	3.44 ± 0.019	1.03 ± 0.004
#27	Dão	blend	1987	$0.23 \pm 5 \times 10^{-4}$	0.43 ± 0.002
#28	Alentejo	blend	2008	1.74 ± 0.013	0.69 ± 0.007
#29	Alentejo	blend	2008	2.80 ± 0.072	0.91 ± 0.010
#30	Beira Interior	blend	2008	3.38 ± 0.035	1.31 ± 0.003
#31	Ribatejo	blend	2008	0.61 ± 0.001	1.02 ± 0.003
#32	Ribatejo	blend	2007	1.27 ± 0.008	0.71 ± 0.001
#33	Ribatejo	blend	2007	2.14 ± 0.009	$4.86 \pm 2 \times 10^{-4}$
#34	Ribatejo	blend	2007	4.98 ± 0.086	2.39 ± 0.002
#35	Ribatejo	monovarietal	2007	1.60 ± 0.002	1.20 ± 0.001
#36	Ribatejo	blend	2008	2.63 ± 0.003	1.87 ± 0.003
#37	Ribatejo	monovarietal	2008	4.32 ± 0.007	1.53 ± 0.002
#38	Ribatejo	blend	2007	7.05 ± 0.004	5.27 ± 0.004
#39	Ribatejo	blend	2002	$0.47 \pm 4 \times 10^{-4}$	$0.44 \pm 4 \times 10^{-4}$
#40	Ribatejo	blend	2008	6.96 ± 0.005	8.91 ± 0.009
#41	Ribatejo	blend	2008	3.55 ± 0.001	1.93 ± 0.002
#42	Ribatejo	blend	2007	1.22 ± 0.006	$0.46 \pm 2 \times 10^{-4}$
#43	Ribatejo	blend	2007	3.19 ± 0.012	1.13 ± 0.002
#44	Ribatejo	blend	2007	$2.83 \pm 2 \times 10^{-4}$	2.04 ± 0.008
#45	Ribatejo	blend	2006	0.67 ± 0.003	$0.35 \pm 4 \times 10^{-4}$
#46	Ribatejo	blend	2008	1.87 ± 0.006	1.53 ± 0.003
#47	Ribatejo	blend	2007	1.65 ± 0.007	$0.88 \pm 1 \times 10^{-5}$
#48	Ribatejo	blend	2007	3.32 ± 0.012	1.86 ± 0.003
#49	Ribatejo	blend	2006	3.42 ± 0.005	1.07 ± 0.006
#50	Ribatejo	blend	2008	3.68 ± 0.013	1.48 ± 0.001
#51	Ribatejo	blend	2005	0.79 ± 0.004	0.65 ± 0.003
#52	Ribatejo	blend	2008	2.52 ± 0.006	4.45 ± 0.002
#53	Ribatejo	blend	2007	3.99 ± 0.043	2.82 ± 0.010
#54	Ribatejo	blend	2007	$1.19 \pm 3 \times 10^{-4}$	$0.68 \pm 3 \times 10^{-4}$
#55	Ribatejo	blend	2007	1.62 ± 0.009	0.40 ± 0.001
#56	Ribatejo	blend	2007	4.18 ± 0.012	$3.35 \pm 4 \times 10^{-4}$
#57	Ribatejo	blend	2008	2.16 ± 0.011	1.19 ± 0.006
#58	Ribatejo	blend	2009	1.42 ± 0.008	$0.04 \pm 5 \times 10^{-5}$
#59	Ribatejo	blend	2005	2.20 ± 0.001	1.14 ± 0.002
#60	Ribatejo	blend	2007	3.42 ± 0.021	2.43 ± 0.001
#61	Ribatejo	blend	2007	4.17 ± 0.008	2.15 ± 0.002
#62	Ribatejo	blend	2008	2.51 ± 0.006	1.82 ± 0.003

Table 3. Continued

wine sample	region	varieties	vintage	concn (μg/mL)	
				trans-resveratrol	*cis*-resveratrol
#63	Ribatejo	blend	2008	3.28 ± 0.007	0.97 ± 0.005
#64	Ribatejo	blend	2007	4.56 ± 0.007	3.42 ± 0.003
#65	Ribatejo	blend	2007	0.98 ± 0.002	0.69 ± 0.002
#66	Ribatejo	blend	2001	0.91 ± 0.003	1.05 ± 0.001
#67	Ribatejo	blend	2006	$0.34 \pm 1 \times 10^{-5}$	0.34 ± 0.001
#68	Beira Interior	monovarietal	2008	6.46 ± 0.027	4.33 ± 0.006
#69	Beira Interior	blend	2008	6.14 ± 0.032	2.22 ± 0.002
#70	Beira Interior	blend	2008	8.09 ± 0.006	4.59 ± 0.005
#71	Beira Interior	blend	2008	5.94 ± 0.019	0.85 ± 0.005
#72	Beira Interior	blend	2008	3.40 ± 0.013	0.71 ± 0.004
#73	Beira Interior	blend	2008	3.68 ± 0.010	1.35 ± 0.001
#74	Beira Interior	monovarietal	2004	3.46 ± 0.024	2.38 ± 0.008
#75	Beira Interior	monovarietal	2004	4.15 ± 0.019	2.87 ± 0.002
#76	Beira Interior	blend	2003	1.15 ± 0.018	0.56 ± 0.002
#77	Beira Interior	blend	2005	4.54 ± 0.028	4.78 ± 0.016
#78	Beira Interior	NA	2005	0.55 ± 0.004	$0.23 \pm 2 \times 10^{-4}$
#79	Beira Interior	blend	2006	2.36 ± 0.005	1.68 ± 0.020
#80	Beira Interior	blend	2007	3.55 ± 0.023	1.56 ± 0.013
#81	Beira Interior	blend	2006	1.89 ± 0.003	1.46 ± 0.002
#82	Beira Interior	blend	2006	1.06 ± 0.012	0.29 ± 0.001
#83	Beira Interior	blend	2006	2.05 ± 0.019	$0.58 \pm 4 \times 10^{-4}$
#84	Beira Interior	blend	2006	1.64 ± 0.004	0.57 ± 0.002
#85	Beira Interior	blend	2006	3.46 ± 0.006	0.56 ± 0.002
#86	Alentejo	blend	1999	0.77 ± 0.009	0.11 ± 0.001
#87	Douro	blend	2007	4.11 ± 0.008	0.33 ± 0.002
#88	Douro	blend	2002	0.85 ± 0.014	$0.14 \pm 2 \times 10^{-4}$
#89	Beira Interior	blend	2008	8.69 ± 0.066	0.65 ± 0.003
#90	Beira Interior	monovarietal	2007	10.46 ± 0.058	0.07 ± 0.001
#91	Ribatejo	monovarietal	2006	1.31 ± 0.004	0.51 ± 0.004
#92	Beira Interior	blend	2007	8.61 ± 0.079	3.78 ± 0.036
#93	Beira Interior	blend	2007	7.29 ± 0.095	5.43 ± 0.040
#94	Beira Interior	blend	2008	$0.62 \pm 3 \times 10^{-5}$	0.95 ± 0.001
#95	Beira Interior	blend	2008	1.13 ± 0.006	0.49 ± 0.002
#96	Beira Interior	blend	2008	8.12 ± 0.120	6.39 ± 0.047
#97	Beira Interior	blend	2008	6.16 ± 0.012	4.87 ± 0.010
#98	Beira Interior	blend	2005	0.80 ± 0.003	0.07 ± 0.001
#99	Beira Interior	blend	2007	3.73 ± 0.002	1.35 ± 0.002
#100	Beira Interior	blend	2005	0.23 ± 0.003	0.08 ± 0.001
#101	Beira Interior	blend	2007	6.66 ± 0.009	3.18 ± 0.004
#102	Beira Interior	blend	2007	4.60 ± 0.023	1.81 ± 0.003
#103	Beira Interior	blend	2007	1.48 ± 0.011	3.33 ± 0.001
#104	Beira Interior	blend	2007	0.83 ± 0.004	1.95 ± 0.008
#105	Beira Interior	blend	2007	2.01 ± 0.008	$3.00 \pm 2 \times 10^{-4}$
#106	Beira Interior	monovarietal	2008	5.17 ± 0.067	5.25 ± 0.026
#107	Beira Interior	blend	2008	3.77 ± 0.005	3.26 ± 0.001
#108	Beira Interior	blend	2008	0.99 ± 0.001	0.73 ± 0.001
#109	Beira Interior	blend	2007	4.87 ± 0.078	3.91 ± 0.015
#110	Beira Interior	monovarietal	2007	2.86 ± 0.001	8.71 ± 0.012
#111	Beira Interior	blend	2007	1.44 ± 0.017	1.43 ± 0.004
#112	Beira Interior	monovarietal	2007	1.23 ± 0.002	5.18 ± 0.339
#113	Beira Interior	blend	2008	1.97 ± 0.009	1.73 ± 0.003
#114	Beira Interior	blend	2008	1.63 ± 0.004	0.92 ± 0.004
#115	Beira Interior	monovarietal	2007	$5.26 \pm 3 \times 10^{-4}$	2.53 ± 0.001
#116	Beira Interior	blend	2007	5.97 ± 0.006	4.60 ± 0.002
#117	Beira Interior	blend	2008	1.68 ± 0.005	1.27 ± 0.004
#118	Beira Interior	blend	2007	5.08 ± 0.006	3.22 ± 0.004
#119	Beira Interior	blend	2007	8.80 ± 0.032	4.86 ± 0.003
#120	Beira Interior	blend	2007	6.68 ± 0.048	$5.92 \pm 3 \times 10^{-4}$
#121	Beira Interior	blend	2007	5.41 ± 0.017	3.22 ± 0.001
#122	Beira Interior	blend	2005	0.63 ± 0.011	0.31 ± 0.003
#123	Beira Interior	monovarietal	2005	0.45 ± 0.009	$0.16 \pm 1 \times 10^{-4}$
#124	Beira Interior	NA	NA	0.87 ± 0.007	$0.46 \pm 4 \times 10^{-4}$
#125	Beira Interior	blend	2005	1.90 ± 0.020	0.50 ± 0.001

dx.doi.org/10.1021/jf105004y |J. Agric. Food Chem. 2011, 59, 2157–2168

Table 3. Continued

				concn (μg/mL)	
wine sample	region	varieties	vintage	*trans*-resveratrol	*cis*-resveratrol
#126	Beira Interior	blend	2005	1.41 ± 0.027	0.33 ± 0.002
#127	Beira Interior	monovarietal	2007	3.02 ± 0.003	2.78 ± 0.002
#128	Beira Interior	monovarietal	2007	3.80 ± 0.015	3.08 ± 0.004
#129	Beira Interior	monovarietal	2007	10.87 ± 0.051	7.53 ± 0.004
#130	Beira Interior	blend	2006	0.54 ± 0.015	0.87 ± 0.009
#131	Beira Interior	blend	2006	2.76 ± 0.009	0.65 ± 0.002
#132	Beira Interior	blend	2006	3.37 ± 0.003	1.84 ± 0.001
#133	Beira Interior	blend	2005	3.21 ± 0.048	0.32 ± 0.004
#134	Beira Interior	monovarietal	2006	9.77 ± 0.014	2.70 ± 0.001
#135	Beira Interior	monovarietal	2006	0.80 ± 0.014	0.35 ± 0.003
#136	Beira Interior	monovarietal	2006	2.70 ± 0.005	$0.24 \pm 5 \times 10^{-4}$
#137	Beira Interior	blend	2006	9.42 ± 0.051	2.04 ± 0.003
#138	Beira Interior	blend	2005	2.85 ± 0.005	1.02 ± 0.001
#139	Beira Interior	blend	2008	4.74 ± 0.019	4.43 ± 0.005
#140	Douro	blend	2007	4.93 ± 0.027	2.10 ± 0.007
#141	Dão	blend	2006	3.71 ± 0.022	2.65 ± 0.005
#142	Beira Interior	blend	2008	5.25 ± 0.005	3.06 ± 0.008
#143	Douro	NA	2008	4.37 ± 0.027	2.53 ± 0.004
#144	Beira Interior	NA	1995	1.36 ± 0.010	<LLOQ
#145	Beira Interior	blend	2003	1.50 ± 0.003	$0.30 \pm 7 \times 10^{-4}$
#146	Beira Interior	blend	2004	0.80 ± 0.026	0.30 ± 0.001
#147	Beira Interior	blend	2008	5.51 ± 0.009	1.48 ± 0.002
#148	Vinhos Verdes	monovarietal	2009	$0.20 \pm 3 \times 10^{-6}$	4.40 ± 0.001
#149	Vinhos Verdes	blend	NA	1.98 ± 0.004	$1.21 \pm 4 \times 10^{-4}$
#150	Vinhos Verdes	blend	2009	2.88 ± 0.014	$3.20 \pm 2 \times 10^{-4}$
#151	Vinhos Verdes	monovarietal	2009	1.17 ± 0.001	$0.92 \pm 1 \times 10^{-4}$
#152	Vinhos Verdes	monovarietal	2009	1.48 ± 0.002	3.72 ± 0.005
#153	Vinhos Verdes	blend	2009	1.33 ± 0.009	0.82 ± 0.001
#154	Vinhos Verdes	monovarietal	2009	0.71 ± 0.004	3.76 ± 0.003
#155	Vinhos Verdes	blend	2009	1.81 ± 0.011	3.95 ± 0.013
#156	Vinhos Verdes	blend	2008	3.88 ± 0.005	1.77 ± 0.001
#157	Vinhos Verdes	blend	2009	4.84 ± 0.040	6.45 ± 0.005
#158	Vinhos Verdes	blend	2009	0.50 ± 0.013	4.62 ± 0.015
#159	Vinhos Verdes	monovarietal	2009	3.77 ± 0.018	1.99 ± 0.004
#160	Vinhos Verdes	monovarietal	2009	0.58 ± 0.010	3.78 ± 0.001
#161	Vinhos Verdes	monovarietal	2009	0.49 ± 0.007	2.09 ± 0.010
#162	Vinhos Verdes	blend	2009	4.36 ± 0.011	5.10 ± 0.006
#163	Vinhos Verdes	monovarietal	2009	3.69 ± 0.001	2.28 ± 0.006
#164	Vinhos Verdes	blend	2009	8.59 ± 0.006	4.88 ± 0.005
#165	Vinhos Verdes	blend	2009	6.76 ± 0.014	3.42 ± 0.005
#166	Vinhos Verdes	monovarietal	2009	3.12 ± 0.007	1.20 ± 0.002
#167	Vinhos Verdes	blend	2009	4.92 ± 0.011	1.65 ± 0.005
#168	Vinhos Verdes	blend	NA	0.43 ± 0.003	3.09 ± 0.004
#169	Vinhos Verdes	monovarietal	2009	2.85 ± 0.004	1.98 ± 0.001
#170	Vinhos Verdes	blend	NA	1.93 ± 0.016	1.36 ± 0.005
#171	Dão	blend	2007	4.32 ± 0.017	2.07 ± 0.002
#172	Dão	blend	2000	3.90 ± 0.070	2.03 ± 0.011
#173	Dão	NA	NA	5.92 ± 0.031	6.71 ± 0.005
#174	Península de Setúbal	blend	NA	2.26 ± 0.006	1.31 ± 0.001
#175	Península de Setúbal	monovarietal	2008	6.03 ± 0.001	2.04 ± 0.023
#176	Bairrada	blend	2007	6.20 ± 0.006	$2.30 \pm 5 \times 10^{-4}$
#177	Bairrada	blend	2006	4.58 ± 0.021	1.21 ± 0.001
#178	Dão	blend	2008	2.26 ± 0.001	0.74 ± 0.001
#179	Península de Setúbal	NA	NA	6.67 ± 0.032	1.26 ± 0.001
#180	Dão	blend	2005	4.57 ± 0.002	3.28 ± 0.001
#181	Península de Setúbal	blend	2008	2.56 ± 0.008	1.62 ± 0.005
#182	Península de Setúbal	monovarietal	2009	1.55 ± 0.003	1.10 ± 0.002
#183	Bairrada	blend	2005	2.98 ± 0.008	0.80 ± 0.001
#184	Bairrada	blend	2006	2.81 ± 0.014	0.77 ± 0.002
#185	Bairrada	blend	2005	3.08 ± 0.002	0.74 ± 0.002
#186	Bairrada	blend	2006	3.09 ± 0.001	1.35 ± 0.005

NA - not available; Concerning the samples presenting values higher than the upper limit of quantitation (ULOQ), those were diluted and reanalyzed.

The method's precision using authentic samples was not systematically evaluated, but all wine samples were analyzed in duplicate, presenting good precision.

Stability. In order to study stability in processed samples at three concentration levels, simulated wine was spiked with 0.025, 1.00, and 15.00 μg/mL of *trans*-resveratrol and 0.023, 0.092, and 0.92 for *cis*-resveratrol, and extracted using the above-mentioned procedure ($n = 3$). After extraction, the samples were evaporated and resuspended on 100 μL of mobile phase and left at 4 °C for 18 h. These samples were injected with another set of samples, which had been freshly prepared. The measured concentrations of both sets of samples did not deviate more than 15% from the nominal concentration. Stability was evaluated in authentic wine samples as well, wine extracts being reanalyzed after 18 h at 4 °C. The obtained results were consistent with those which had been previously obtained.

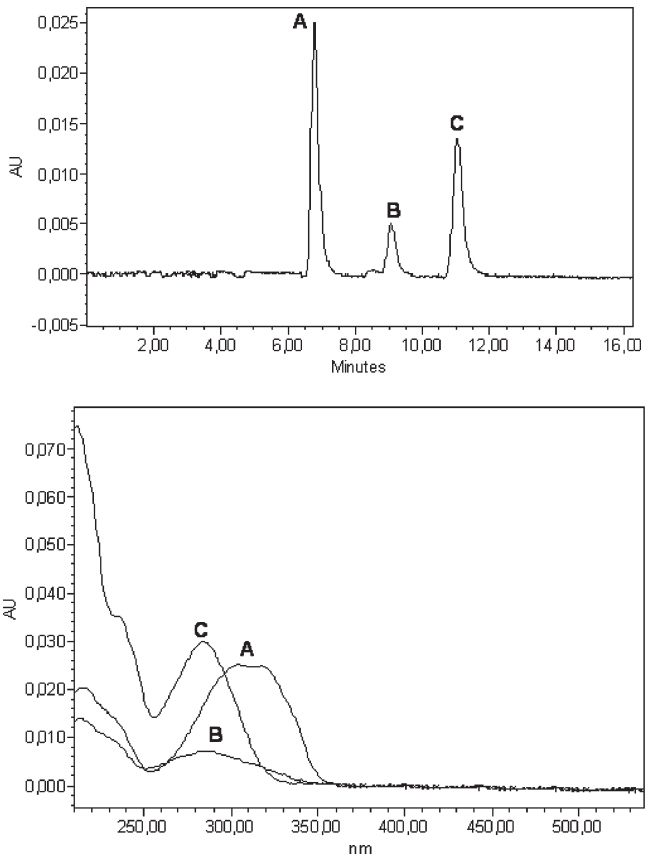

Figure 5. (top) Chromatogram of a standard mixture of (A) *trans*-resveratrol (retention time 7.1 min), (B) *cis*-resveratrol (retention time 9.1 min) and (C) carbamazepine (retention time 11 min) at 306 nm and (bottom) respective absorption spectra obtained by photodiode array detection.

Extraction Efficiency. This parameter was determined by replicate analysis ($n = 6$) of simulated wine samples spiked at 0.025, 1, and 15 μg/mL for *trans*-resveratrol and at 0.023, 0.092, and 0.920 μg/mL for *cis*-resveratrol; a second set of simulated wine samples (nonspiked samples) was also prepared and analyzed. After SPE, the internal standard was added to both sets of samples, while the second set of samples was further spiked with the same amounts of resveratrol isomers (100% efficiency). The obtained peak area ratios were compared, and extraction efficiency was thus calculated.

The mean calculated values were 100.44% for *trans*-resveratrol and 100.39% for *cis*-resveratrol (Table 2). The obtained efficiency values were higher than those normally seen in SPE methods,[35−38] in LLE[19,32−34] or when direct injection is used.[21,23,31]

Application of the Method to Wine Samples. To demonstrate the applicability of this method, 186 commercially available red wines from different geographical regions, grape varieties and vintage were analyzed in duplicate and the obtained results are presented in Table 3. *trans*- and *cis*-resveratrol were identified by their retention times and by the wavelength corresponding to the maximum absorbance of each compound (Figure 5). A typical chromatogram of a red wine sample [#129] is shown in Figure 6. The values found are higher than those reported in the literature for most of the Portuguese red wines,[33,34,37,47,48,50−52] which could be due to the different geographical origins of the wine or grape variety, since the content of these compounds depends on the climate and on the temperature.[9]

The obtained results follow a normal distribution, and homogeneity of variance between each group of data was tested with the *F* test. The comparison between grouped data was performed using Student's two-tailed *t* test assuming nonhomogeneous variance between the compared sets ($p \leq 0.05$). The lowest

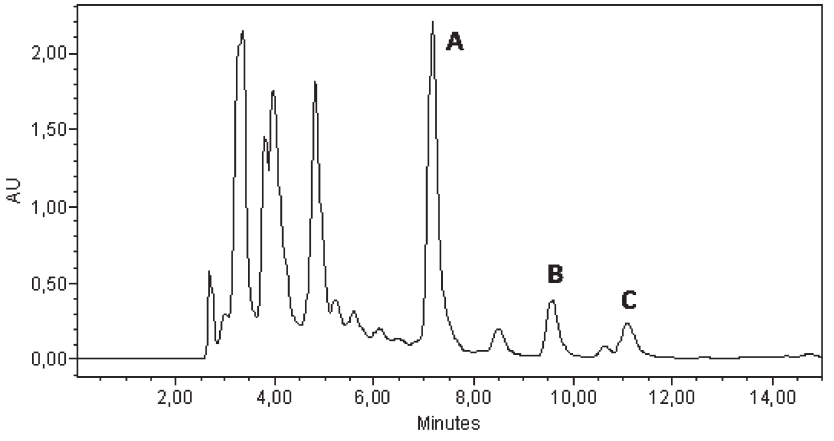

Figure 6. Chromatogram obtained by injection of a processed wine sample [#129], 306 nm: (A) *trans*-resveratrol, (B) *cis*-resveratrol and (C) internal standard.

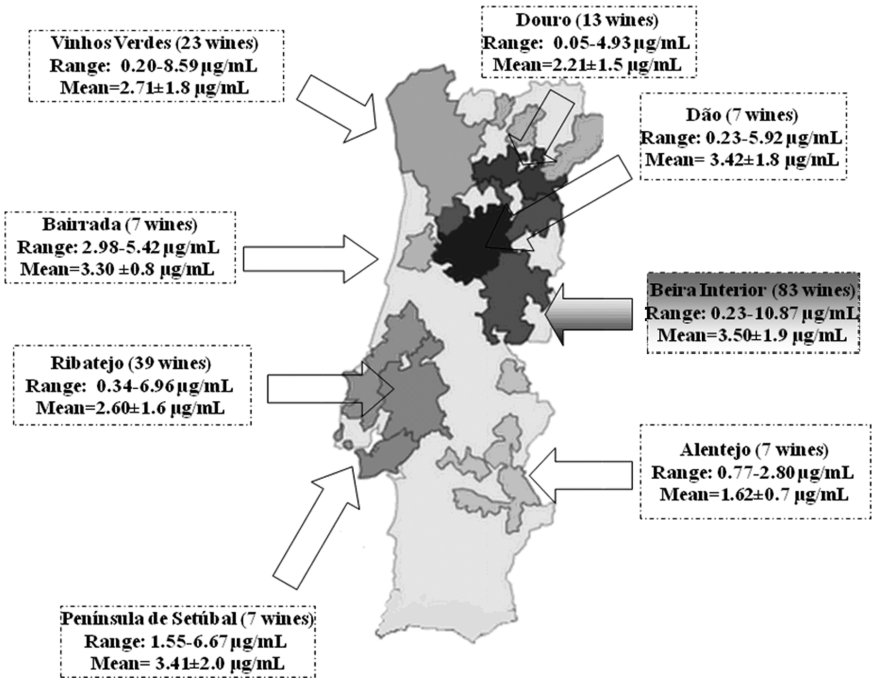

Figure 7. Map of Portugal mainland with average of *trans*-resveratrol levels in different wine regions (concentration ranges, mean values ± standard deviations).

average level of *trans*-resveratrol was found in wines from the Alentejo region (1.62 ± 0.7 μg/mL), while Beira Interior presented the highest level (3.50 ± 1.9 μg/mL). Although some regions were found to be significantly different from other regions, such as, e.g., Alentejo compared to Beira Interior, Dão and Vinhos Verdes, there are situations in which resveratrol contents in wine do not differ significantly between regions, such as in the comparison of wines from the Península de Setúbal region to the remaining regions. These data are presented in Figure 7.

According to the literature Canada produced red wines with the highest average level of *trans*-resveratrol of 3.2 ± 1.5 μg/mL with Greece and Japan at the other hand with 1.0 ± 0.5 μg/mL and 1.0 ± 0.6 μg/mL, respectively.[9] Furthermore, the highest *trans*-resveratrol levels reported in the literature were 11.9 μg/mL in a 1997 Swiss wine made from the Pinot Noir grape[53] and 14.3 μg/mL in a Hungarian, 2002 Merlot.[54] According to the literature, levels of *cis*-resveratrol in red wines follow the same trend as seen for *trans*-resveratrol. The highest average level of *cis*-resveratrol has been found in wines from Canada 1.9 ± 1.1 μg/mL.[9] In the present work the highest *trans*-resveratrol value was 10.9 μg/mL in a 2007 Beira Interior denomination of origin made from the Touriga Nacional grape, while the highest value obtained for the *cis*-isomer was observed in a wine from the Beira Interior region (8.71 μg/mL). By using this method, the presence of *trans*- and *cis*-resveratrol was confirmed in all the red wine samples analyzed.

In conclusion, we have developed a simple and rapid method for the quantification of *trans*- and *cis*-resveratrol in wine samples. The procedure is sensitive and specific, presenting low limits of detection and quantification. The sample pretreatment procedure, based on SPE with C_8 cartridges, has granted excellent extraction efficiencies for both isomers. The method has been completely validated, including stability tests, showing excellent results for all the studied parameters. The present work contributes definitely to a better scientific knowledge of Portuguese wines.

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Funding Sources

The authors acknowledge the Portuguese Foundation for Science and Technology (FCT) for supporting this work in the form of a doctoral fellowship to L. Paulo (SFRH/BD/28168/2006) and a postdoctoral fellowship to E. Gallardo (SFRH/BPD/34733/2007).

ACKNOWLEDGMENT

We express our gratitude to Comissão Vitivinícola Regional da Beira Interior, Comissão Vitivinicola Regional dos Vinhos Verdes and Comissão Vitivinícola Regional do Tejo, for providing wine samples from their production regions.

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