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Ultrasound-Assisted Extraction of Stilbenoids from Grape Stems

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ABSTRACT: A new method for fast determination of stilbenoids from grape stems was developed. Ultrasound-assisted extraction was applied prior to chromatographic determination of stilbenoids in the extracts, and the stability of stilbenoids under extraction conditions was checked. A fractional experimental design was developed to analyze the influence on the extraction process of seven different extraction variables: temperature, ultrasound amplitude, ultrasonic cycle duration, ultrasonic probe type, time, sample-solvent ratio, and solvent (mixtures of ethanol and water). The most important variables for the recovery of major stilbenoids were studied and the final conditions optimized. With this new method, the main stilbenoids found in grape stems can be extracted in 15 min, using 75 °C as the extraction temperature and 80% ethanol as the extraction solvent, and no cleaning step with organic solvent is needed. The optimized method allowed for the analysis of stilbenoid content from 22 grape stem samples, many of them analyzed for the first time.

KEYWORDS: *trans-resveratrol, ε-viniferin, vitisin-B, ultrasound-assisted extraction, HPLC, grape stems*

INTRODUCTION

Stilbenoids (stilbene-related compounds) are phenols derived from the phenylpropanoid and acetate−malonate pathway expressed in many plant families.[1] Stilbenes, mainly *trans*-resveratrol, have aroused increasing attention due to both their antifungal properties as phytoalexins[2] and their many health-promoting properties, including antioxidant,[3] anticarcinogenic,[4] anti-inflammatory,[5] cardioprotective,[6] and neuroprotective[7] activities, among others. They are naturally occurring compounds commonly found in plants from different botanical families,[8,9] many of them used in the food industry, such as hop, wheat, and rhubarb. However, their dietary sources are relatively limited to peanuts, pistachios, berries, dark-chocolate, tomatoes, grapes, and wine.

Vitis vinifera genus is a major dietary source,[10] mainly present in wine and grapes but also in other plant material including roots, stems, canes, flowers and leaves. However, biological studies from stilbenoids other than *trans*-resveratrol[11] are rather limited in the literature, probably due to a lack of commercial standards for many of them.

The grape and wine industry is at present a valuable part of the economy in several regions in the world, with a total forecast production of 69 million metric tons of grapes in 2011.[12] Up to 30−40% w/w solid byproducts are generated during winemaking,[13] such as grape stems, grape pomaces (skins and seeds), lees, as well as other solid wastes like trimmed vine shoots or grape canes discarded after the pruning season. Their possible utilization is gaining more attention because of their promising eventual applications and due to the environmental concerns. Indeed, they can be considered as potential sources of useful compounds for the pharmaceutical and food industries and support sustainable agricultural production.

Among these byproducts, grape stems, usually discarded at the beginning of the winemaking process, preserve their phytochemical composition almost completely and can therefore be recognized as an unexploited source of bioactive compounds such as stilbenoids.[14]

The analysis of plant stilbenoids is usually performed by extracting the sample with aqueous organic solvents (methanol or ethanol) and analyzing the extract by high performance liquid chromatography (HPLC) UV−vis or fluorescence detection.[15−17] However, these techniques often involve long extraction times and several extraction steps. New eco-friendly stilbene extraction techniques have been developed to increase yield, including supercritical fluid extraction of grape skins[18] or grape pomaces,[19] or ultrasound-assisted extraction (UAE) of grapes[20] and grape canes.[21] Among these, UAE is the cheapest technique and has the lowest instrumental requirements. Enhanced extraction efficiency of organic compounds by ultrasound is attributed to the cavitation phenomenon produced in the solvent by the passage of an ultrasonic wave.

In the present work, a simple method was developed for determining stilbenoids from grape stems by means of an UAE procedure prior to direct high-performance liquid chromatography analysis. Optimum extraction conditions based on UAE were determined and the final method designed without including several cleaning steps with organic solvents as recently described by Anastasiadi et al.[22] These authors only employed standard conditions for sonication (ultrasonic bath) and also included two additional cleaning steps (three extractions with petroleum ether and four with ethyl acetate) to the initial extraction of grape stems. The developed method is easier, saves time, and was optimized and successfully applied to stilbenoid content evaluation of 22 grape stems, many of them analyzed for the first time (Palomino fino, Vijiriega, Tempranillo, Garnacha, Tintilla de Rota, and Vitis silvestris), including compounds other than *trans*-resveratrol (the main

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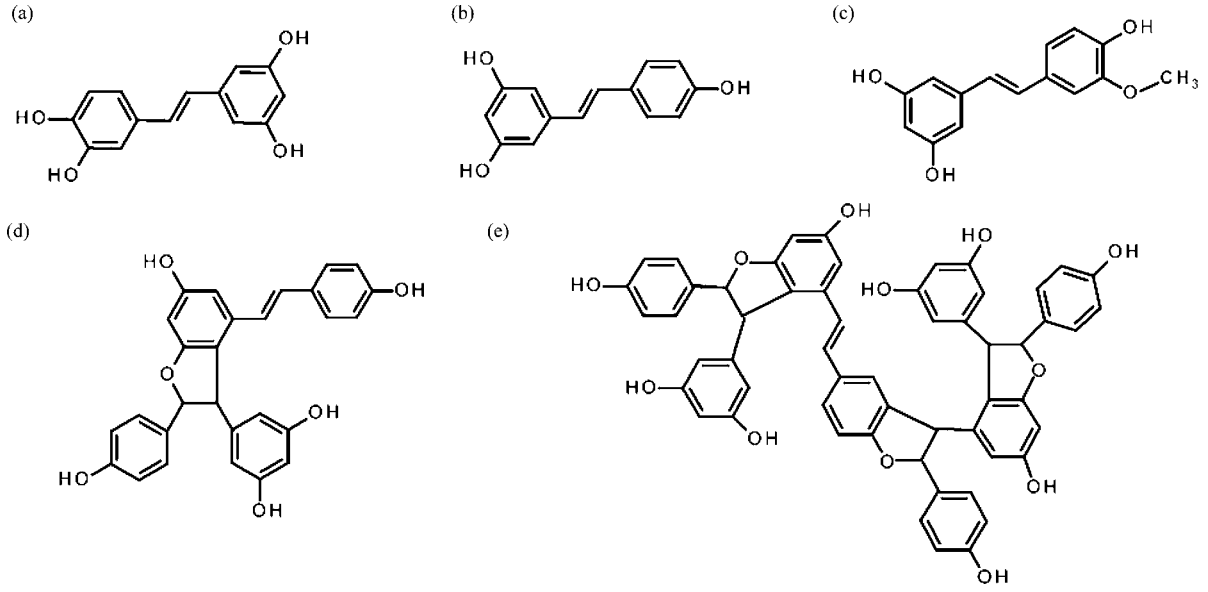

Figure 1. Chemical structures of piceatannol (a), *trans*-resveratrol (b), isorhapontigenin (c), ε-viniferin (d), and vitisin-B (e).

stilbene described) such as ε-viniferin, piceatannol, and vitisin B, which are widely described in grape canes but scarcely described in grape stems.[22−24]

MATERIALS AND METHODS

Chemicals and Reagents. Analytical grade methanol, acetic acid, diethyl ether, ethyl acetate, and ethanol were supplied by Panreac (Barcelona, Spain). *trans*-Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) and piceatannol (3,3′,4,5′-tetrahydroxystibene) were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water from a Mili-Q system (Millipore Corp., Bedford, MA) was used throughout this research.

Reference Extract Enriched in Stilbenoids. A mixture of 100 mL of several methanolic extracts was obtained from UV−C-irradiated grapes grown under an agro-ecological cultivation system with no chemical treatments. Batches of 5 kg of healthy grape clusters of each variety were selected and manually harvested. On the same day, these grape clusters were then irradiated according to the protocol under patent WO/2002/085137; ES 2177465. Subsequently, grape clusters were stored in a stainless steel vessel at 18 °C and 75% relative humidity for 7 days. During this period, 200 g of grapes were randomly and carefully sampled from different bunches, and peeled using a sharp knife. Grape skins were frozen at −20 °C until extraction with diethyl ether was performed according to Guerrero et al.[25]

All methanolic extracts were mixed, and the final volume was concentrated by a rotavapor (Heidolph rotavapor VV2001, Heidolph Instruments GmbH & Co., Germany) at a temperature below 40 °C until 30 mL (chemical structures in Figure 1): piceatannol (30.5 mg L^{-1}), *trans*-resveratrol (131.9 mg L^{-1}), isorhapontigenin (15.8 mg L^{-1}), ε-viniferin (20.9 mg L^{-1}), and vitisin-B (8.0 mg L^{-1}).

This enriched stilbenoid solution will be used as a standardized solution for method development (chromatogram is shown in Figure 2) and provide an advantage to the lack of commercial standards or specific equipment requirements for the isolation of many stilbenoids found in grape byproducts.[26]

Stilbenoids were previously identified by UPLC-DAD-TQD in our lab (conditions of MS/MS in ESI mode were as follows: capillary voltage, 2.50 kV; cone, 40.00 V; extractor, 3.00 V; RF, 0.1 V; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow (N2), 50 L h^{-1}; desolvation gas flow (N2), 650 L h^{-1}; and collision gas flow (Ar), 0.15 mL min^{-1}) according to Guerrero et al.[25] and were quantified by HPLC-DAD as *trans*-resveratrol. For identification purposes, standards of *trans*-resveratrol and piceatannol were used. Vitisin-B was obtained from a purified extract kindly provided by the GESVAB group from University of Bordeaux II, France. ε-Viniferin was identified by UPLC-DAD-ITQ as in previous works.[25]

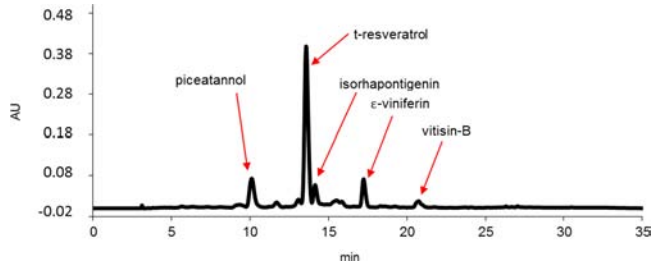

Figure 2. HPLC-DAD chromatogram corresponding to the stilbenoid enriched reference extract.

Plant Material. All of the used stem samples (corresponding to 14 tested varieties) were taken from grape clusters grown in the same place and harvested in the 2010, 2011, and 2012 vintages, at IFAPA-Rancho de la Merced Centre, in Jerez de la Frontera, Cádiz (SW Spain). The samples were obtained under different winemaking conditions from several grape varieties (Sauvignon blanc, Chardonnay, Vijiriega, Palomino fino, Tempranillo, Syrah, Garnacha, Tintilla de Rota, Vitis silvestris, Merlot, Cabernet Sauvignon, and Petit Verdot). Of these, only the Syrah variety was subjected to different elicitation treatments in two vintages to increase stilbenoid content: Syrah A 2010 and 2011 (UVC treated) and Syrah B (methyl jasmonate and UV−C-treated). The UV−C treatment was conducted according to the procedure described by the patent W0/2002/085137; ES 2177465 with some modifications as described by Guerrero et al.[25] Methyl jasmonate treatment was applied according to Fernandez-Marin et al.[27]

Grape stems were collected during the destemming process, and after grape remains removal, they were dried with paper and immediately lyophilized until reaching a constant weight (representing a loss ranging from 60 to 70% of the original weight); subsequently, they were crushed in a milling device and were kept at −20 °C until extraction.

Extraction Process. *Ultrasound-Assisted Extraction (UAE).* The extraction of stilbenoid compounds from grape stems by means of ultrasound was carried out under different extractions conditions according to the experimental design shown in Table 1. Each assay in the experimental design was run in duplicate. The studied variables were extraction temperature (5−65 °C), ultrasound amplitude (30−70%), ultrasonic cycle duration (0.2−0.7 s), ultrasonic probe tip (2−7

Table 1. Conditions and Results of the Extractions Based on the Fractional Factorial Experimental Design

	conditions assayed							mean area			
experiment	temperature (°C)	amplitude (%)	cycle (s)	probe tip (mm diameter)	time (min)	sample-solvent ratio	solvent (% ethanol in water)	piceatannol	*trans*-resveratrol	ε-viniferin	vitisin-B
1	5	70	0.2	7	15	1/50	100	n.d.[a]	63919 ± 6.9[b]	29657 ± 1.4	6434 ± 5.2
2	65	70	0.7	7	15	1/25	100	n.d.	529541 ± 7.2	174931 ± 4.3	41579 ± 1.9
3	5	30	0.7	2	15	1/25	100	n.d.	128315 ± 1.6	50661 ± 0.8	9938 ± 6.2
4	5	30	0.7	7	15	1/50	50:50	59980 ± 5.4	111517 ± 3.0	72139 ± 4.2	21201 ± 2.4
5	5	70	0.2	2	15	1/25	50:50	91400 ± 6.5	185194 ± 2.0	107691 ± 7.6	34137 ± 7.3
6	5	70	0.7	2	5	1/50	100	n.d.	67077 ± 3.0	30841 ± 6.6	6310 ± 4.9
7	5	30	0.2	7	5	1/25	100	n.d.	117784 ± 6.4	44353 ± 5.5	9671 ± 6.9
8	65	30	0.2	7	15	1/25	50:50	213455 ± 4.0	387845 ± 5.8	258330 ± 0.1	55110 ± 0.4
9	5	30	0.2	2	5	1/50	50:50	17460 ± 6.9	116484 ± 2.5	49317 ± 6.1	13405 ± 4.2
10	65	70	0.2	2	5	1/25	100	n.d.	406110 ± 4.3	148319 ± 0.5	33977 ± 4.3
11	65	70	0.7	2	15	1/50	50:50	103733 ± 6.7	252415 ± 0.9	156483 ± 4.7	33975 ± 3.8
12	65	30	0.7	2	5	1/25	50:50	193562 ± 2.5	364377 ± 2.2	252021 ± 6.0	60515 ± 6.9
13	65	30	0.2	2	15	1/50	100	n.d.	282791 ± 2.4	90335 ± 0.9	18373 ± 7.7
14	65	70	0.2	7	5	1/50	50:50	108386 ± 2.2	197777 ± 7.1	142076 ± 0.4	31389 ± 3.8
15	5	70	0.7	7	5	1/25	50:50	96243 ± 7.1	216940 ± 6.9	108096 ± 6.8	34746 ± 4.9
16	65	30	0.7	7	5	1/50	100	n.d.	223472 ± 0.7	80122 ± 0.5	16004 ± 2.7

[a]n.d.: not detected. [b]RSD (%).

mm diameter), extraction time (5−15 min), sample-solvent ratio (1:25−1:50), and solvent (50−100% v/v ethanol in water). The ultrasound extraction was carried out with a high intensity probe ultrasound generation system of 200 W and 24 kHz (model UP 200S, from dr.Hielscher GmbH, Teltow, Germany). Its amplitude controller allows the ultrasonic vibrations at the probe microtip to be set at any desired level in the 10−100% range of the nominal power. Also, the cycle controller allows the duration of the application of the ultrasound to be set, to a fraction of a second, in the 0.1−1.0 range.

A thermostatted water bath was used to control temperature during extraction. The obtained extracts were centrifuged at 4.000 rpm for 5 min in a Digicen 20-R centrifuge (Orto Alresa, Spain), paper filtered, finally filtered through a 0.22 μm filter (PVDF Teknokroma, Barcelona, Spain), and kept at −18 °C until analysis.

Solid−Liquid Extractions (SLE). Different solid−liquid extractions with magnetic stirring (60 min) were also checked to corroborate the effectiveness of ultrasound-assisted extraction. The checked solvents were ethyl acetate, diethyl ether, methanol acidified with 0.1% hydrochloric acid, and ethanol−water 80:20 (v/v).[20,28] Same solid−solvent ratio was applied. The obtained extract was later centrifuged at 4.000 rpm for 5 min in a Digicen 20-R centrifuge (Orto Alresa, Spain), the supernatant was removed, and the treated sample matrix was extracted twice under the same conditions. After centrifugation, all supernatants were combined, and the solvent was removed under vacuum at a temperature below 40 °C using a rotavapor (Heidolph rotavapor VV2001, Heidolph Instruments GmbH & Co., Germany). Dry samples were rediluted in 2 mL of methanol, HPLC grade, filtered through a 0.22 μm filter (PVDF Teknokroma, Barcelona, Spain), and kept at −18 °C until analysis.

Liquid Chromatography System. The chromatographic analysis was carried out in a Waters (Milford, MA, USA) high-performance liquid chromatographic system equipped with a 1525 pump model and a Waters 996 Photodiode Array Detector. Separations were performed on a Mediterranea Sea18 column (Tecknokroma, Barcelona, Spain) (RP-18, 25 × 0.46 cm; 5 μm particle size) and a guard column of the same material, at 30 °C. The mobile phases consisted of a water/methanol/acetic acid mixture, solvent A 88:10:2 and solvent B 8:90:2, at a flow rate of 1 mL min^{-1}. The elution program involved gradient elution from 35% B for 3 min to reach 50% B at 10 min, 70% B at 20 min, and 100% from 23 to 28 min.[29] Empower software used was supplied by Waters. Stilbenoids were quantified at 306 nm as *trans*-resveratrol (LOD = 0.01 ppm and LOQ = 0.04 ppm).

Statistical Software. Minitab v14.0 trial version (State College, PA, USA) software was used for the development and evaluation of the results of the experimental design. A fractional factorial design (2^{7-2}) was used, carrying out a total of 16 extractions in duplicate instead of the 128 possible combinations evaluated (extraction temperature, ultrasound amplitude, ultrasonic cycle duration, ultrasonic probe microtip, extraction time, sample−solvent ratio, and solvent).

This kind of experimental design has produced good results in the robustness evaluation of extraction methods previously developed for different compounds.[28,30] Table 1 shows the assayed conditions and the mean areas obtained. Graphic analysis of the principal effects and the interactions between the variables was used for interpretation of the results.

Significant differences between variables were assessed by analysis of variance (ANOVA) and Tukey's least significant difference test (LSD) using the Statistix, version 8.0, software (Tallahassee, FL, USA). The Statistica package, version 10.0 (StatSoft, Tulsa, OK, USA), was used for cluster analysis.

RESULTS AND DISCUSSION

Stilbenoid Stability. In order to evaluate the performance of different extraction conditions with accuracy, the stability of the enriched stilbenoid extract during the extraction was determined prior to the method development. Thus, a study was carried out to determine the stability of the stilbenoids at different working temperatures in the ultrasound system. Experiments were carried out with 1 mL of enriched stilbenoid extract placed in a volumetric flask (100 mL) filled up to 25 mL with 100% ethanol under standardized conditions for extraction (20 min extraction time, 50% amplitude, cycle 0.5, and 7 mm diameter tip) using different temperatures. This allowed for the selection of an adequate extraction temperature for an analytical method that improves extraction efficiency without affecting the stilbenoid profile of the sample (as they are labile compounds submitted to several isomerization and degradation reactions catalyzed by light and high temperatures). Values for individual stilbenoids after applying extraction conditions at different temperatures under extraction conditions is shown in Figure 3. These values are relative to the initial stilbenoid

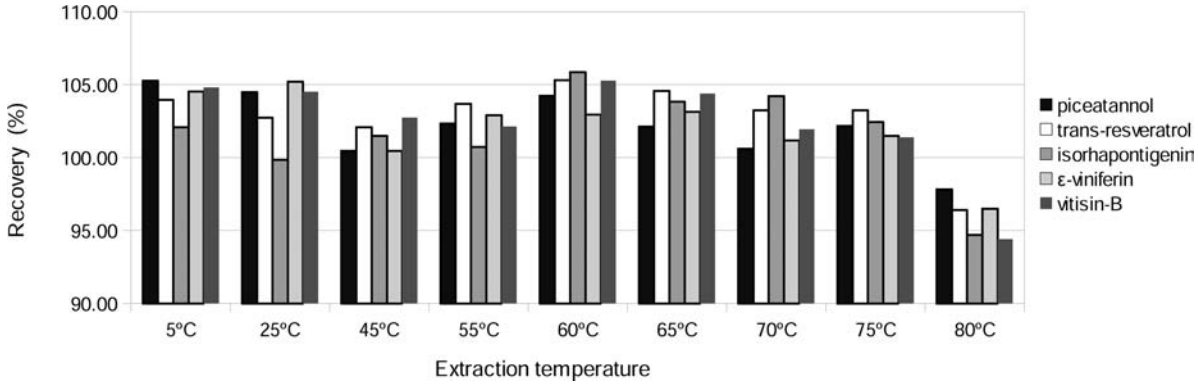

Figure 3. Stability of stilbenoids during extraction at different temperatures.

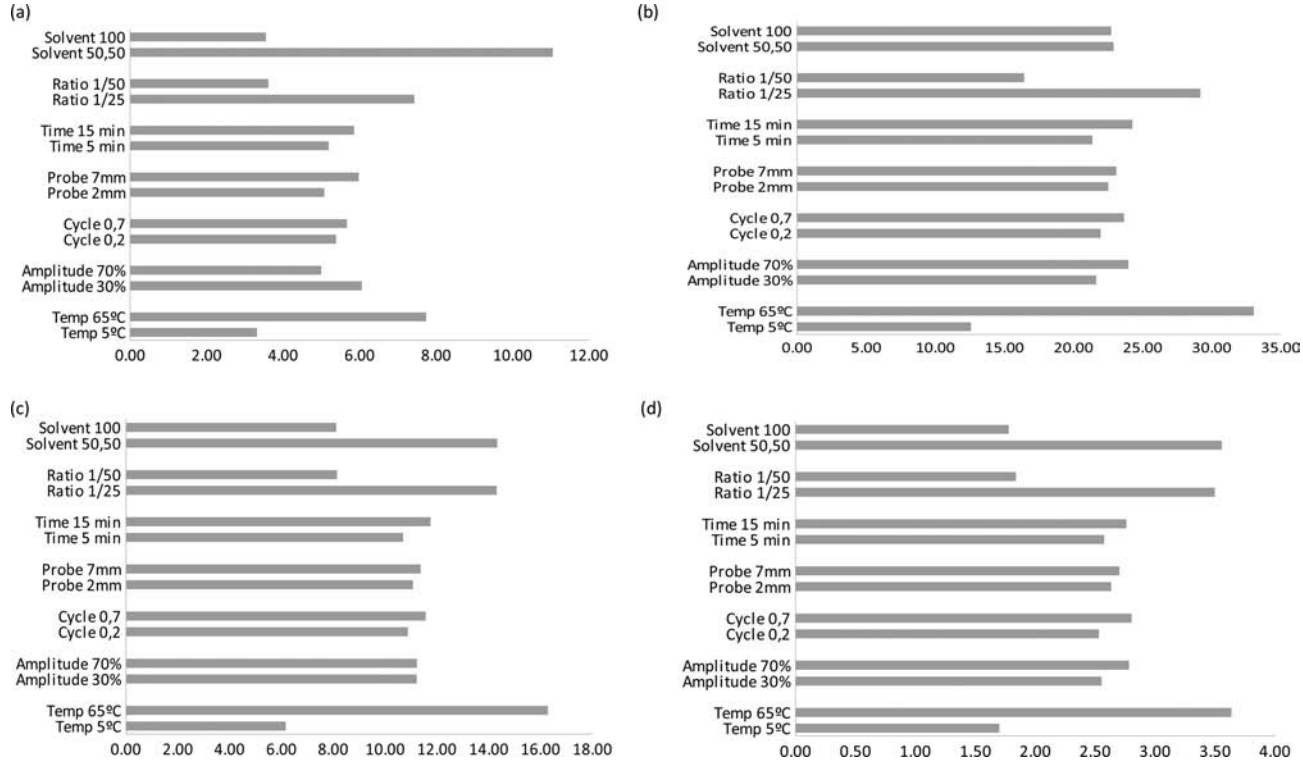

Figure 4. Main effects plot on the mean recovery (area 10.000^{-1}) for piceatannol (a), *trans*-resveratrol (b), *ε*-viniferin (c), and vitisin-B (d).

concentration in the standardized extract (100%) considering the same dilution factor. Each extraction was repeated twice, and all of these assays were carried out in darkness (flasks were covered with aluminum foil) to prevent degradation and/or isomerization by light.

As Figure 3 shows, the average recovery for all of the extracted compounds was over 100%, with the exception of extractions performed at 80 °C, where recoveries began to decrease. The latter was the maximum temperature assayed for practically being the boiling point for ethanol−water azeotrope (78.2 °C).[31] When extracting real samples, it could be advantageous to perform the extraction at the highest temperature where target compounds remain stable in order to get a faster extraction. Additional experiments were also conducted to check the stability at 75 °C when increasing time (data not shown), and the extract remained stable for up to 35 min of continuous extraction. Therefore, 75 °C is the maximum temperature to be used in developing the UAE method.

Development of the Method. After setting the maximum extraction temperature, the next step was to study the influence of the factors which can affect the process on the recovery. For this goal, a fractional factorial experimental design was selected instead of the full factorial design in order to reduce the total number of experiences needed. A similar design has been used for the determinations of anthocyanins in grapes[32] or hydroxytyrosol in wines.[33] Syrah 2010 samples were used throughout all of the experimental design and method optimization assays. Preliminary studies were conducted to establish the magnitude of sample−solvent ratio with a good chromatographic signal. Thus, 1 g, 2 g, and 3 g of sample in 25 mL of 80% ethanol in water were checked. A 1 g:25 mL ratio was selected (data not shown) as the starting point for experimental design. This way, using the fractional factorial design, the effects of seven different extraction conditions were evaluated. A total of 16 experiences instead of the 128 (2^7) available combinations for the tested experimental variables were run. Table 1 shows the values for each of these variables in

the experiments carried out and the relative recovery of each of the analyzed compounds. All injections were run in duplicate.

Figure 4 shows the graphic analysis of the results obtained for the mean peak areas for the four analyzed stilbenoids (piceatannol, *trans*-resveratrol, ε-viniferin, and vitisin-B).

Main differences were found for three tested experimental conditions (temperature, ratio, and solvent). *trans*-Resveratrol, however, showed no significant differences for the solvent but presented the same behavior for temperature and ratio. Piceatannol showed a dramatic difference when using 50:50 ethanol/water instead of 100% ethanol, as it is the most polar compound in the sample. When using 50:50 ethanol/water, higher recoveries were also found for ε-viniferin and vitisin-B. Minimal differences were registered in individual compounds for cycle, amplitude, and probe tip (data not shown). Surprisingly, extraction time showed no significant variation, although it showed a rising trend with increasing time for all assayed compounds.

In view of the significant differences in the influences of the different variables, the most significant ones, the composition of the solvent and the sample−solvent ratio were optimized, while the other variables were fixed at optimal conditions based on the principal effects shown in Figure 4. With regard to the extraction temperature, the higher the temperature, the higher is the recovery; thus, the temperature was fixed at 75 °C according to stability results. Therefore, final extraction conditions were 0.7 s cycle, 70% amplitude, 7 mm diameter probe tip, and 75 °C as extraction temperature. With regard to the extraction time, it is clear for all cases that this variable favors the increase of extraction. In any case, this variable must be optimized at a later point in order to guarantee quantitative recoveries of the compounds.

Optimization of the Main Extraction Variables. Assays were carried out using mixtures of ethanol in water between 80% and 30%. The total recoveries of extracted compounds are shown in Table 2. As can be observed, the conditions which produced the best recovery for all of the compounds were 80% ethanol in water, according to bibliography.[20] On the basis of these results, the percentage of ethanol in water was fixed at 80%.

Table 2. Effects of Different Ethanol Concentrations on Total Stilbenoid Extraction[a]

	mean relative recovery of major stilbenoids[b] (%)		
solvent (% ethanol in water)	*trans*-resveratrol	ε-viniferin	total stilbenoids[b] (mg kg^{-1} d.w.)
80:20	100.0 a	100.0 a	192.7 a
60:40	96.8 a	95.5 a	191.7 a
50:50	80.7 b	91.9 a	176.5 b
40:60	63.3 c	82.3 a	156.3 c
30:70	39.9 d	61.1 b	120.4 d
significance level	***	**	**

[a]Values followed by distinct letters are significantly different at significance levels. *P < 0.05, **P < 0.01, and ***P < 0.001; NSD, no significant difference. [b]Extraction conditions: 15 min time, 0.7 s cycle, 70% amplitude, 7 mm diameter probe tip, and 75 °C temperature.

Different sample−solvent ratios were also checked between 1:30 and 1:15 interval (Table 3), as can be seen, the ratio 1:30 leads to higher total recovery for all assayed compounds.

Table 3. Effects of Different Sample-Solvent Ratios on Total Stilbenoid Extraction[a]

	mean relative recovery of major stilbenoids[b] (%)		
sample-solvent ratio	*trans*-resveratrol	ε-viniferin	total stilbenoids[b] (mg kg^{-1} d.w.)
1:30	100.0 a	98.9 a	221.9 a
1:25	97.9 a	99.8 a	216.0 a
1:20	89.9 b	97.9 a	199.7 b
1:15	80.0 c	89.6 b	179.1 c
significance level	***	*	***

[a]Values followed by distinct letters are significantly different at significance levels. *P < 0.05, **P < 0.01, and ***P < 0.001; NSD, no significant difference. [b]Extraction conditions: 15 min time, 80% ethanol in water as solvent, 0.7 s cycle, 70% amplitude, 7 mm diameter probe tip, and 75 °C temperature.

With regard to the determination of the time necessary for extraction, the recovery of the four compounds was studied using times between 15 and 35 min. The results are shown in Table 4. As shown, there were no significant differences found between any of them, and therefore, the shortest period, 15 min, was used as the extraction time. This result proves the effect on speed of the ultrasound in the extraction process.

Table 4. Effects of Different Extraction Times on Stilbenoid Extraction[a]

	mean relative recovery of major stilbenoids[b] (%)		
extraction time (min)	*trans*-resveratrol	ε-viniferin	total stilbenoids[b] (mg kg^{-1} d.w.)
15	96.4	93.0	223.3
20	92.7	96.5	210.5
25	96.4	98.8	218.4
30	99.8	99.9	217.8
35	100.0	98.3	221.3
significance level	NSD	NSD	NSD

[a]*P < 0.05, **P < 0.01, and ***P < 0.001; NSD, no significant difference. [b]Extraction conditions: sample-solvent ratio 1:30, 80% ethanol in water as solvent, 0.7 s cycle, 70% amplitude, 7 mm diameter probe tip, and 75 °C temperature.

In summary, to determine all assayed stilbenoids from grape stems, the optimum extraction conditions and final designed method were as follows: a single extraction cycle with 75 °C as extracting temperature, 1:30 sample−solvent ratio, 80% ethanol in water as extracting liquid, 7 mm diameter probe tip, 70% amplitude, 0.7 s cycle time, and 15 min extraction time.

To verify quantitative extraction, the volume of extracting solvent needed for a total recovery was checked. Thus, two successive extraction steps with fresh solvent were performed, including a rinsing step of the solid between the first and second extraction. For this, after the first extraction with ethanol 80%, the sample was vacuum filtered, the extract was collected and filled up to 25 mL, the solid rinsed with 10 mL of fresh solvent (collected separately), and dried for 5 min. Afterward, the sample was re-extracted with fresh solvent following the same procedure, collecting the third fraction. All fractions were analyzed separately. Recoveries for the assayed stilbenoids in each fraction are shown in Table 5. As can be seen, all compounds are almost totally recovered in the first fraction; however, all of them are also detected in the rinsing

Table 5. Effect of the Re-extraction Step on Stilbenoid Recoveries

step	mean relative recovery of major stilbenoids (%)		total stilbenoids (mg kg^{-1} d.w.)
	trans-resveratrol	ε-viniferin	
extraction	78.8	75.1	210.1
rinsing step (10 mL)	17.9	17.6	25.1
re-extraction	3.3	7.3	34.7

fraction. Therefore, a rinsing step is needed to obtain quantitative recoveries (higher than 90%) for all of the assayed compounds.

Analytical Characteristics of the Method. *Precision.* The precision of the method was studied as intra- and interday assays (n = 5) for each compound. This variation was assessed by analyzing replicates of the same grape stem sample (Table 6). The method was found to be precise with relative standard deviation of the concentration (mg kg^{-1} d.w.) of extracts for intraday analyses within 0.9% for piceatannol and 6.6% for *trans*-resveratrol. For interday analyses, the relative standard deviation ranged from 1.8% (vitisin-B) to 10.0% (piceatannol).

Table 6. Intra- and Interday Precision of Analyzed Stilbenoids in Syrah Grape Stem Samples

	piceatannol	*trans*-resveratrol	ε-viniferin	vitisin-B
Intraday (n = 5)				
mean (mg kg^{-1} d.w.)	19.8	150.7	60.5	20.7
RSD (%)	0.9	6.6	4.2	4.1
Interday (n = 5)				
mean (mg kg^{-1} d.w.)	20.0	138.0	63.7	13.0
RSD (%)	10.0	3.5	4.1	1.8

Accuracy. The accuracy of the method was established by determining the recovery of stilbenoids spiked to the sample, running in triplicate, according to the proposed method. One milliliter of the stilbenoid-enriched extract was added to the sample before being submitted to extraction conditions. Mean recovery for analyzed samples ranged from 90.3% to 109.1% (n = 3) for *trans*-resveratrol, piceatannol, and ε-viniferin, but only 75% (n = 3) for vitisin-B. The RSD for all samples and compounds was below 7.5%.

Comparison of the Method with Classical Solid–Liquid Extraction. The resulting recoveries using the developed UAE method were compared with those obtained using different solid–liquid methods previously described[34,35] (n = 2), employing four different solvents. Significant differences ($p < 0.05$) were found for the analyzed stilbenoids, all of them having higher UAE recoveries (data not shown), e.g., *trans*-resveratrol concentration in the UAE extract was 116.9 mg kg dry weight^{-1} versus 29.7, 46.2, 41.5, and 94.7 mg kg dry weight^{-1} achieved with ethyl acetate, diethyl ether, methanol acidified with 0.1% hydrochloric acid, and ethanol–water 80%, respectively. Additionally, it has to be noted that using the new method, up to 12 samples can be processed sequentially spending the same time as that with the best SLE method assayed (ethanol/water).

Application to Real Samples. The optimized procedure was successfully applied to the determination of stilbenoid levels in 22 grape stem samples, and the results are shown in Table 7. Among them, Palomino fino, Vijiriega, Tempranillo, Garnacha, Tintilla de Rota, and several Vitis silvestris cultivars were analyzed for the first time.

As expected,[25,36] among the analyzed red grape stem varieties, the Syrah B sample for vintage 2011 (methyl

Table 7. Stilbenoid Concentrations in 22 Grape Stem Samples (n = 3)

cultivar	stem sample	vintage	piceatannol[a]	*trans*-resveratrol[a]	ε-viniferin[a]	vitisin-B[a]	total stilbenoids[a]
white	Sauvignon blanc	2010	nd[b]	nd	147.1 ± 6.5	61.1 ± 2.7	208.2 ± 9.2
	Chardonnay	2010	nd	nd	60.6 ± 0.1	7.8 ± 0.1	68.3 ± 0.2
	Vijiriega	2010	nd	traces	48.0 ± 1.1	9.7 ± 0.2	57.7 ± 1.2
	Palomino fino	2010	nd	traces	24.7 ± 0.2	9.6 ± 0.2	34.3 ± 0.4
	Chardonnay	2012	nd	42.2 ± 5.6	25.7 ± 0.2	6.8 ± 0.1	74.7 ± 5.7
	Palomino fino	2012	nd	nd	14.3 ± 0.2	nd	14.3 ± 0.2
red	Tempranillo 1	2010	nd	79.8 ± 0.2	60.5 ± 1.3	14.6 ± 0.3	154.8 ± 1,7
	Tempranillo 2	2010	nd	87.8 ± 3.6	80.6 ± 2.8	21.3 ± 0.4	189.7 ± 6.7
	Syrah CT	2010	16.6 ± 0.1	122.5 ± 0.8	71.1 ± 0.2	22.2 ± 0.1	232.4 ± 1.1
	Syrah A[c]	2010	17.8 ± 0.1	135.4 ± 1.8	52.0 ± 0.3	18.2 ± 0.1	223.5 ± 2.2
	Garnacha	2010	nd	traces	29.4 ± 0.1	10.1 ± 0.4	39.6 ± 0.4
	Tintilla de Rota	2010	nd	118.9 ± 4.1	91.6 ± 0.1	15.2 ± 0.1	225.8 ± 4.3
	Vitis silvestris 1	2010	nd	49.9 ± 1.8	59.0 ± 1.1	13.5 ± 0.1	122.4 ± 3.1
	Vitis silvestris 2	2010	nd	traces	38.7 ± 0.8	8.6 ± 0.3	47.3 ± 1.1
	Vitis silvestris 3	2010	nd	33.0 ± 2.2	74.8 ± 1.8	12.7 ± 0.3	120.5 ± 4.3
	Syrah A[c]	2011	nd	64.0 ± 1.7	41.7 ± 1.4	12.9 ± 0.3	118.6 ± 3.4
	Syrah B[d]	2011	21.1 ± 0.1	139.1 ± 0.8	65.1 ± 1.1	13.0 ± 0.1	238.3 ± 1.9
	Tempranillo	2012	nd	nd	28.3 ± 0.4	7.2 ± 0.1	35.5 ± 0.4
	Tintilla de Rota	2012	nd	traces	39.2 ± 0.9	9.0 ± 0.3	48.2 ± 1.2
	Merlot	2012	nd	nd	30.1 ± 0.3	10.0 ± 0.2	40.1 ± 0.4
	Cabernet sauvignon	2012	nd	nd	17.6 ± 0.3	9.1 ± 0.1	26.7 ± 0.4
	Petit verdot	2012	nd	nd	20.5 ± 0.2	8.3 ± 0.1	28.8 ± 0.3

[a]mg kg dry weight^{-1} ± SD. [b]nd: not detected. [c]UV–C irradiated (postharvest). [d]Metil jasmonate added (preharvest) and UV–C irradiated (postharvest).

jasmonate added preharvest + UV−C-irradiated) and Syrah A 2010 (UV−C-irradiated) contained the highest amount of *trans*-resveratrol in their respective vintages and the highest ε-viniferin in vintage 2011 since they were stimulated for stilbene increase (see Materials and Methods). Tintilla de Rota reached the highest concentration of ε-viniferin, whereas Syrah 2010 (CT, A) and Tempranillo 2 2010 achieved the maximum vitisin-B content (Table 7). Furthermore, only Syrah 2010 (CT, A) and Syrah B 2011 showed piceatannol content, whereas ε-viniferin and vitisin-B were the stilbenoids found in all analyzed red grape stem samples.

As for white grape stem samples, Chardonnay 2012 was the only one with *trans*-resveratrol content and Sauvignon blanc 2010 was the only sample in which isorhapontigenin was detected (data not shown, 19.8 mg kg dry weight^{-1}). This white variety also showed the highest content of ε-viniferin (147.1 mg kg dry weight^{-1}) and vitisin-B (61.1 mg kg dry weight^{-1}). Palomino fino 2011 was the only sample where vitisin-B was not detected.

Stilbenoid concentration ranged from n.d. or traces to 139.1 mg kg dry weight^{-1} for *trans*-resveratrol; 14.3 to 147.1 mg kg dry weight^{-1} for ε-viniferin; n.d. to 61.1 mg kg dry weight^{-1}; and n.d. to 21.1 mg kg dry weight^{-1} for vitisin-B and piceatannol.

These *trans*-resveratrol concentrations were in the range described by other authors for different varieties such us Merlot, Cabernet Sauvignon or Pinot Noir: 7−15 mg kg dry weight^{-1},[23] Cabernet franc, Gewurztraminer, Marzemino, Merlot, Moscato, Pinot gris, Sauvignon, Tocai friulano, approximate average content 50 mg kg dry weight^{-1};[24] for different Greek cultivars, 74−266 mg kg dry weight^{-1} [22] but considerably lower than those described by Cho et al.[20] for Gerborg and Campbell grapes cultivated in Korea, even when using similar UAE methodology (up to 484 mg kg dry weight^{-1}). These publications also show the high *trans*-resveratrol content variability between different cultivars inside the same growing region, e.g., ranging between 7 and 440 mg kg dry weight^{-1} for Cabernet sauvignon and Tintet varieties, respectively, in a Moravian vineyard[23] or 74 and 266 mg kg dry weight^{-1} for native Aidani and Mandelaria Greek varieties in Santorini.[22]

For ε-viniferin content, the analyzed grape stem varieties were also similar to those described in the literature (ranging between 167 and 499 mg kg dry weight^{-1}).[22]

Finally, an increase in piceatannol and *trans*-resveratrol content in grape stems from UV−C-treated versus untreated grapes was observed, according to Guerrero et al.[25] Besides, the combination of vineyard preharvest treatment (methyl jasmonate) with UV−C postharvest treatment showed an increase in piceatannol, *trans*-resveratrol, and ε-viniferin concentration versus UV−C grape stems. Thus, the combination treatment (pre- and postharvest) proved an interesting alternative for stilbenoid-enriched grape cluster (grapes and stems) production, according to Fernandez-Marin et al.[27]

On the basis of these observations, our findings were not unexpected since a number of abiotic or biotic stress factors such as UV radiation, heavy metal ions, or infection by fungi are known to affect stilbenoid biosynthesis during grapevine growth. Cluster analysis of total stilbenoid content for all samples shows no clear clustering according to variety or vintage (data not shown), despite being grown in the same place, with good sanitary conditions, and submitted to similar environmental factors. Thus, the grape cultivar seems to influence stilbenoids levels, in addition to microclimate and sanitary growing conditions.[25,36] In any case, as a rule, stilbenoid phytochemical behavior is usually expressed in grapes and leaves.

In conclusion, a simple, rapid, and reliable UAE method was validated for stilbenoid analysis in grape stem samples for the first time. The proposed method allowed for the determination of piceatannol, *trans*-resveratrol, ε-viniferin, and vitisin-B in wine after 15 min of UAE extraction. This method only involved a single extraction cycle and avoided an additional rinsing step with organic solvents, which are additional advantages versus those of other reported procedures. It also allowed for the analysis of stilbenoids other than *trans*-resveratrol, the main compound determined in the scarce literature related to grape stem analysis. Furthermore, the findings of this study provide a deeper knowledge of the content of these biologically active phenolic compounds in different grape stem varieties.

This content provides useful information for exploring the stilbenoid content of different byproducts and characterizing them on the basis of the abundance of these potentially beneficial compounds.

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Notes

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ABBREVIATIONS USED

UAE, ultrasound-assisted extraction; SLE, solid−liquid extraction; SD, standard deviation; RSD, relative standard deviation; d.w., dry weight

REFERENCES

(1) Shen, T.; Wang, X.-N.; Lou, H.-X. Natural stilbenes: an overview. *Nat. Prod. Rep.* **2009**, *26*, 916−935.

(2) Kuc, J. Phytoalexins, stress metabolism, and disease resistance in plants. *Annu. Rev. Phytopathol.* **1995**, *33*, 275−297.

(3) Gulcin, I. Antioxidant properties of resveratrol: A structure−activity insight. *Innovative Food Sci. Emerging Technol.* **2010**, *11*, 210−218.

(4) Asensi, M.; Medina, I.; Ortega, A.; Carretero, J.; Bano, M. C.; Obrador, E.; Estrela, J. M. Inhibitory effects of *trans*-resveratrol analogs molecules on the proliferation and the cell cycle progression of human colon tumoral cells. *Mol. Nutr. Food Res.* **2008**, *52*, 538−548.

(5) Wang, K.-T.; Chen, L.-G.; Tseng, S.-H.; Huang, J.-S.; Hsieh, M.-S.; Wang, C.-C. Anti-inflammatory effects of resveratrol and oligostilbenes from Vitis thunbergii var. taiwaniana against lipopolysaccharide-induced arthritis. *J. Agric. Food Chem.* **2011**, *59*, 3649−3656.

(6) Hao, H. D.; He, L. R. Mechanisms of cardiovascular protection by resveratrol. *J. Med. Food* **2004**, *7*, 290−298.

(7) Richard, T.; Paulus, A. D.; Iglesias, M. L.; Pedrot, E.; Waffo-Téguo, P.; Mérillon, J. M.; Monti, J. P. Neuroprotective properties of resveratrol and derivatives. *Ann. N.Y. Acad. Sci.* **2011**, *1*, 103−108.
(8) Harborne, J. B. The comparative biochemistry of phytoalexins induction in plants. *Biochem. Syst. Ecol.* **1999**, *27*, 335−367.
(9) Chong, J.; Poutaraud, A.; Hugueney, P. Metabolism and roles of stilbenes in plants. *Plant Sci.* **2009**, *177*, 143−155.
(10) Pawlus, A. D.; Waffo-Téguo, P.; Mérillon, J. M. Stilbenoid chemistry from wine and the genus *Vitis*, a review. *J. Int. Sci. Vigne Vin* **2012**, *46*−*2*, 1−55.
(11) Smoliga, J. M.; Baur, J. A.; Hausenblas, H. A. Resveratrol and health − A comprehensive review of human clinical trials. *Mol. Nutr. Food Res.* **2011**, *55*, 1−13.
(12) Statistical Report on World Vitiviniculture 2012; International Organization of World Vitiviniculture (OIV). http://www.oiv.int/oiv/files/0%20-%20Actualites/EN/Report.pdf.
(13) Makris, D. P.; Boskou, G.; Andrikopoulos, N. K. Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. *J. Food Compos. Anal.* **2007**, *20*, 125−132.
(14) Makris, D. P.; Boskou, G.; Andrikopoulos, N. K.; Kefalas, P. Characterisation of certain major polyphenolic antioxidants in grape (Vitis vinifera cv. Roditis) stems by liquid chromatography-mass spectrometry. *Eur. Food Res. Technol.* **2008**, *226*, 1075−1079.
(15) Careri, M.; Corradini, C.; Elviri, L.; Nicoletti, E.; Zagnoni, I. Direct HPLC Analysis of quercetin and *trans*-resveratrol in red wine, grape, and winemaking byproducts. *J. Agric. Food Chem.* **2003**, *51*, 5226−5231.
(16) Moreno, A.; Castro, M.; Falqué, E. Evolution of *trans*- and cis-resveratrol content in red grapes (Vitis vinifera L. cv Mencía, Albarello and Merenzao) during ripening. *Eur. Food Res. Technol.* **2008**, *227*, 667−674.
(17) Anastasiadi, M.; Pratsinis, H.; Kletsas, D.; Skaltsounis, A.-L.; Haroutounian, S. A. Bioactive non-coloured polyphenols content of grapes, wines and vinification by-products: Evaluation of the antioxidant activities of their extracts. *Food Res. Int.* **2010**, *43*, 805−813.
(18) Pascual-Martí, M. C.; Salvador, A.; Chafer, A.; Berna, A. Supercritical fluid extraction of resveratrol from grape skin of Vitis vinifera and determination by HPLC. *Talanta* **2001**, *54*, 735−740.
(19) Casas, L.; Mantell, C.; Rodríguez, M.; Martínez de la Ossa, E. J.; Roldán, A.; De Ory, I.; Caro, I.; Blandino, A. Extraction of resveratrol from the pomace of Palomino fino grapes by supercritical carbon dioxide. *J. Food Eng.* **2010**, *96*, 304−308.
(20) Cho, Y.-J.; Hong, J.-Y.; Chun, H. S.; Lee, S. K.; Min, H.-Y. Ultrasonication-assisted extraction of resveratrol from grapes. *J. Food Eng.* **2006**, *77*, 725−730.
(21) Palma, M.; Barroso, C. G. Ultrasound-assisted extraction and determination of tartaric and malic acids from grapes and winemaking by-products. *Anal. Chim. Acta* **2002**, *458*, 119−130.
(22) Anastasiadi, M.; Pratsinis, H.; Kletsas, D.; Skaltsounis, A.-L.; Haroutounian, S. A. Grape stem extracts: Polyphenolic content and assessment of their in vitro antioxidant properties. *Food Sci. Technol.* **2012**, *48*, 316−322.
(23) Melzoch, K.; Hanzlikova, I.; Filip, V.; Buckiova, D.; Smidrkal, J. Resveratrol in parts of vine and wine originating from Bohemian and Moravian vineyard regions. *Agric. Conspectus Sci.* **2001**, *66*, 53−57.
(24) Bavaresco, L.; Cantu, E.; Fregoni, M.; Trevisan, M. Constitutive stilbene contents of grapevine cluster stems as potential sources of resveratrol in wine. *Vitis* **1997**, *36*, 115−118.
(25) Guerrero, R. F.; Puertas, B.; Fernández, M. I.; Palma, M.; Cantos, E. Induction of stilbenes in grapes by UV-C: Comparison of behaviour of different subspecies of Vitis. *Innovative Food Sci. Emerging Technol.* **2010**, *11*, 231−238.
(26) Zga, N.; Papastomoulis, Y.; Toribio, A.; Richard, T.; Delaunay, J. C.; Jeandet, P.; Renault, H.; Monti, P.; Mérillon, J. M.; Waffo-Téguo, P. Preparative purification of antiamyloidogenic stilbenoids from Vitis vinifera (Chardonnay) stems by centrifugal partition chromatography. *J. Chromatogr., B* **2009**, *877*, 1000−1004.
(27) Fernandez-Marin, M.; Guerrero, R.; Puertas, B.; Garcia-Parrilla, M.; Collado, I. G.; Cantos-Villar, E. Impact of preharvest and postharvest treatment combinations on increase stilbene content in grape. *J. Int. Vigne Vin* **2013**, *47*, 203−212.
(28) Liazid, A.; Barbero, G. F.; Palma, M.; Brigui, J.; Barroso, C. G. Optimization of a new extraction technique for analysis of verbenone and cis-Verbenol in Pine seeds. *Chromatographia* **2007**, *66*, 571−575.
(29) Jiménez-Sánchez, J. B.; Crespo-Corral, E.; Santos-Delgado, M. J.; Orea, J. M.; Gonzalez-Urena, A. Analysis of *trans*-resveratrol by laser ionization mass spectrometry and HPLC with fluorescence detection. *J. Chromaotgr., A* **2005**, *1074*, 133−138.
(30) Nabli, R.; Achour, S.; Jourdes, M.; Teissedre, P. L.; Helal, A. N.; Ezzil, B. Anthocyanin composition and extraction from grenache noir (vitis vinifera l.) vine leaf using an experimental design I- by ethanol or sulfur dioxide. *J. Int. Sci. Vigne Vin* **2012**, *46*, 295−304.
(31) Rousseau, R. W. In *Handbook of Separation Process Technology*; Rousseau, R. W., Ed.; Wiley-Interscience: Hoboken, NJ, 1987; pp 261−262.
(32) Liazid, A.; Guerrero, R. F.; Cantos, E.; Palma, M.; Barroso, C. G. Microwave assisted extraction of anthocyanins from grape skins. *Food Chem.* **2011**, *124*, 1238−1243.
(33) Pineiro, Z.; Cantos-Villar, E.; Palma, M.; Puertas, B. Direct liquid chromatography method for the simultaneous quantification of hydroxytyrosol and tyrosol in red wines. *J. Agric. Food Chem.* **2011**, *59*, 11683−11689.
(34) Vergara, C.; von Baer, D.; Mardones, C.; Wilkens, A.; Wernekinck, K.; Damm, A.; Macke, S.; Gorena, T.; Winterhalter, P. Stilbene levels in grape cane of different cultivars in Southern Chile: determination by HPLC-DAD-MS/MS method. *J. Agric. Food Chem.* **2012**, *60*, 929−933.
(35) Sun, B.; Ribes, A. M.; Leandro, M. C.; Belchior, A. P.; Spranger, M. I. Stilbenes: quantitative extraction from grape skins, contribution of grape solids to wine and variation during wine maturation. *Anal. Chim. Acta* **2006**, *563*, 382−390.
(36) Fernández-Marín, M. I.; Guerrero, R.; García-Parrilla, M. C.; Puertas, B.; Ramírez, P.; Cantos-Villar, E. Terroir and variety: two key factors for obtaining stilbene-enriched grapes. *J. Food Comp. & Anal.* **2013**, *31*, 191−198.