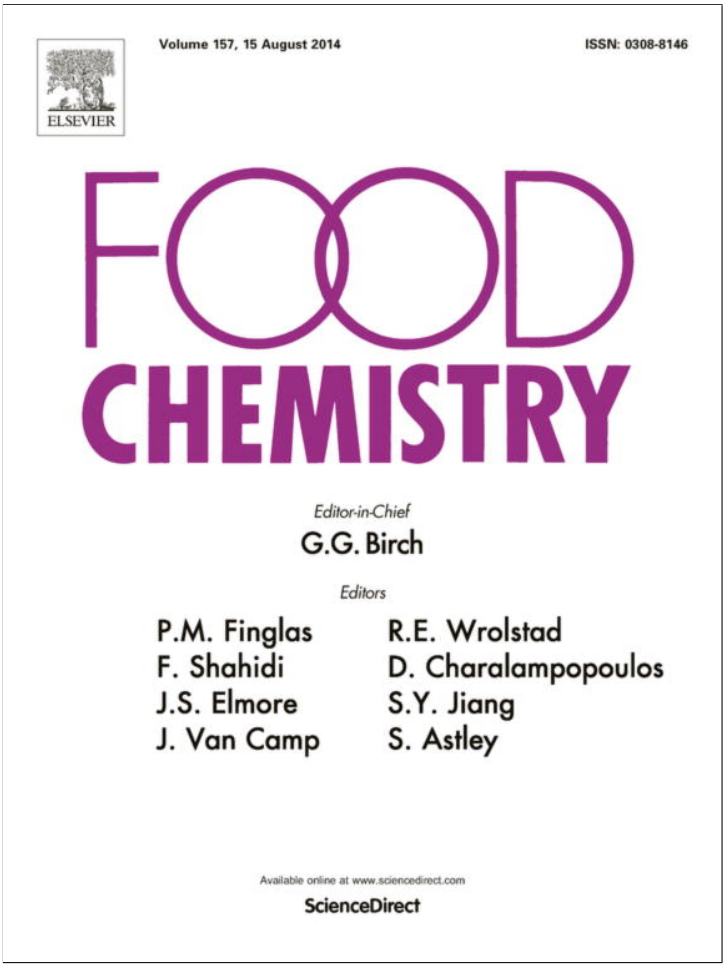

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Analytical Methods

Effect of experimental parameters in the pressurized solvent extraction of polyphenolic compounds from white grape marc

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ABSTRACT

A method based on pressurized solvent extraction (PSE) to determine main polyphenolic compounds in the grape marc obtained as a byproduct of the white winemaking process has been developed. As response variables in the optimisation process include main individual polyphenols, as well as spectrophotometric indexes. The optimised PSE procedure implies the use of 1 g of sample, without preliminary clean-up step, sea sand as dispersant, temperature of 105 °C, methanol (63%) in water as solvent, and 5 min of extraction time (2 static cycles). The performance of the proposed method has been assessed in terms of recovery (91–105%), linearity ($R^2 > 0.995$) and precision. The applicability of the method was demonstrated by the analysis of bagasse samples collected from 12 wineries located in Galicia (NW Spain). Data of the *in vitro* antioxidant activities of the PSE extracts are also discussed.

1. Introduction

Plant polyphenols exhibit a remarkably diverse range of biophysicochemical properties that make them rather unique natural products, implicated in diverse functional roles (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011). Solid byproducts from wine industry are a very rich source of such bioactive phytochemicals, compared with other agri-food solid wastes on the basis of their content in phenolics, and therefore grape marc can be exploited as a source of added-value products (Makris, Boskou, & Andrikopoulos, 2007). Research on the extraction of polyphenols from their different sources has been very active in recent decades; solvent extraction, microwave assisted extraction (MAE), solid phase extraction (SPE) and supercritical fluid extraction (SFE) have been applied to get polyphenolic compounds, but there is no single extraction protocol which can be considered optimal for all type of samples (Escribano-Bailon & Santos-Buelga, 2003).

In particular, the extraction of polyphenols from grape byproducts has been mainly focused on soaking with organic solvents (Kammerer, Claus, Carle, & Schieber, 2004; Karacabey & Mazza, 2010; Sant' Anna, Brandelli, Marczak, & Tessaro, 2012; Yilmaz & Toledo, 2004), occasionally assisted by ultrasounds (González-Paramás, Esteban-Ruano, Santos-Buelga, de Pascual-Teresa, & Rivas-Gonzalo, 2004; Yilmaz & Toledo, 2004); enzymatic methods (Meyer, Donovan, Pearson, Waterhouse, & Frankel, 1998), and even flash chromatography to a fractional extraction (Torres, Varela, García, Carilla, & Matito, 2002). Other techniques employed in the extraction of polyphenols from semi-solid and solid samples of different plant origin have been critically reviewed very recently (Ignat, Volf, & Popa, 2010). Pressurized solvent extraction (PSE) has not been included among these techniques.

One common limitation of all the proposed methods is the use of temperatures well below the boiling point of the extraction solvents when applied to polyphenols. However, in the PSE technique, a heated and pressurized extraction solvent is driven into an extraction vessel containing the sample. The pressurized solvent at high temperature increases the solubility of the analyte and also the desorption kinetic rate of the analyte from the sample matrix, accelerating in this way the extraction process. Thus, PSE reduces the overall solvent consumption and sample preparation time.

Temperature is an important parameter that favuors PSE. Although the use of high temperatures can reduce selectivity and cause compound degradation as well (Giergielewicz-Mozajska, Dabrowski, & Namiesnik, 2001), the stability of phenolic compounds using superheated solvents was demonstrated by Palma, Piñeiro, and Barroso (2001, 2002) in grapes. However, grape marc is a matrix that not exactly matches the original grapes (humidity, chemical composition) since it is mainly determined not only by the grape variety of origin but mostly by the winemaking process.

Most of the polyphenols extraction methods have been developed and applied to red winemaking products (grapes, must, wine

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or byproducts) since their general higher polyphenol content. However, a comparative study of polyphenol screening of red and white grape pomace (Kammerer et al., 2004) concluded that besides the lack of anthocyanins in white grape marc, no important differences between red and white varieties were observed. The applications of PSE to grape pomace are up to now, to extract anthocyanins (Srinivas, King, Monrad, Howard, & Zhang, 2011) and procyanidins (Monrad, Howard, King, Srinivas, & Mauromoustakos, 2010).

The aim of this work is, therefore, to optimise a PSE method to extract polyphenolic compounds from the bagasse obtained as byproduct after the winemaking of white wine. To our knowledge this is the first application of PSE to white grape marc polyphenols extraction. The experimental domain will be composed by a set of preliminary experiments, and then Screening and Response Surface Methodology (RSM) will be used for the fine tuning of the most influential factors. The response variables evaluated for each set of experimental conditions are the concentration of gallic acid, catechin and epicatechin (sometimes referred to as total non-hydroxycinnamates (Makris, Psarra, Kallithraka, & Kefalas, 2003)), as well as, total polyphenols, total flavanols, total hydroxycinnamates and total flavonoids.

The selection of these response variables for the PSE optimisation is justified in two ways. On the one hand, the spectrophotometric indexes, based on well-established reactions, have proven to be excellent screening tools in the optimisation processes carried out by means of experimental design and also to get fast and cheap guess analysis, as well as very good parameters on a comparative basis (Makris et al., 2007). Regarding the individual polyphenols, grape seeds and skins are good sources of gallic acid (a benzoic acid) and catechin and epicatechin (monomeric flavanols) (Yilmaz & Toledo, 2004). These are the three major polyphenols in the obtained PSE extracts of white grape marc. In addition these polyphenols, among others, act particularly well as H-atom donors, showing the antioxidation ability frequently cited as the key property underlying prevention and/or reduction of several oxidative stress-related and age-related diseases (Quideau et al., 2011). Very recently Yoo, Saliba, Prenzler, and Ryan, (2012), an interesting study about wines functionally enhanced with catechin-rich extracts has been published; and even, there are studies that show that it is safe to use grape skin and seed extracts as components of the human diet (Bentivegna & Whitney, 2002). The optimised method has been applied to a set of 13 bagasse samples from Albariño grapes (*Vitis vinifera sp*) cultivated in Galicia (NW Spain) and used for the production of high quality white wines. Further, the antiradical activity of the grape marc extracts was evaluated and the possible correlations with the concentration of the major polyphenols were also investigated.

2. Materials and methods

2.1. Chemicals

Materials used as dispersant phases were: washed sea sand (200–300 μm, Scharlau, Barcelona, Spain) and C_{18} (9–12% carbon, Aldrich, St. Louis, USA). Extraction solvents used were methanol HPLC grade and n-Hexane (95%) (Panreac, Castellar del Vallès, Barcelona, Spain); acetone HPLC grade and formic acid (98–100%) (Merck, Darmstadt, Germany) and acetonitrile (LC–MS Chromasolv, Fluka, Steinheim, Germany). Ultrapure water was produced in the laboratory with a Milli-Q gradient system (Millipore, Bedford, MA, USA). The Folin&Ciocalteu phenol reagent was obtained from Sigma–Aldrich (Steinheim, Germany). Other chemicals that are needed to determine the spectrophotometric indexes were DMACA (*p*-dimethylamino-cinnamaldehyde, Sigma, St. Louis, USA), sodium hydroxide (NaOH, Merck), sodium nitrite ($NaNO_2$, PRO-BVS), sodium carbonate (Na_2CO_3, Panreac) and aluminum trichloride ($AlCl_3$, Merck). 2,2-Diphenil-1-picrylhydrazyl (DPPH, Sigma, St. Louis, USA) was used to determine the scavenging activity of the grape marc extracts.

Pure polyphenolic of gallic acid 99% (CAS 149-91-7), catechin 99% (CAS 154-23-4), epicatechin 97% (CAS 490-46-0) and chlorogenic acid 98% (CAS 327-97-9), were all supplied by Sigma–Aldrich (St. Louis, USA). Individual standard stock solutions of 2.000–8.000 μg mL^{-1} were prepared in methanol. Working solutions in water containing the target analytes (1–200 μg mL^{-1} gallic acid; 5–700 μg mL^{-1} catechin; 25–500 μg mL^{-1} epicatechin) were obtained by appropriate dilution. Solutions were stored at −20 °C protected from light.

2.2. Grape marc samples

Bagasse samples from Albariño grapes (*V. vinifera sp*) used for the production of high quality white wines were kindly donated by different wineries of Galicia (NW Spain). Twelve samples belonged to four sub-areas of the Denomination of Origin Rias Baixas: Ribeira do Ulla (AL-01), Val do Salnés (AL-02, AL-07, AL-04, AL-05, AL-12-e, AL-06), O Rosal (AL-11), Condado de Tea (AL-10, AL-09, AL-03, AL-12-f); and one sample belonged to the Denomination of Origin Ribeira Sacra (AL-08). The O Rosal AL-11 sample was the bagasse used for the PSE optimisation procedure.

The grape bagasse was collected in each particular winery on the day of its production immediately after the pressing process, placed into plastic freezer bags, sealed, and stored at −20 °C. The whole grape marc was used in the experiments, that is, seeds and skins were not separated because the objective is to process the winemaking byproduct directly as it is produced. Freezing the sample prior to the extraction is advisable since ice crystals produce lesions in the cellular structure and consequently facilitate the exit of the cellular components and thus the process of extraction (Escribano-Bailon & Santos-Buelga, 2003). For the homogenisation of the samples, the grape marc was ground with a conventional electric coffee grinder (Moulinex).

To calculate the moisture content of the grape marc samples, 3 g of bagasse were dried in an oven at 105 °C. The sample was weighed before and after the dryness step. This operation was carried out in triplicate. All data were expressed on dry weight (dw).

2.3. Pressurized solvent extraction (PSE)

Extractions were performed on an ASE 150 (Dionex, Co., Sunyvale, CA, USA), equipped with 10-mL stainless steel cells and 60-mL collection vials. One cellulose filter (Dionex) was placed at each end of the PSE cell. The sample, previously ground in a mortar with the selected dispersant in a ratio 1:2, was introduced into the 10 mL extraction cell, where previously 1 g of clean sand (200–300 μm grain size, Sigma–Aldrich) was placed. Finally, the dead volume of the cell was also filled with sand. The cell was tightly closed and placed into the PSE system. Extractions were performed without preheating the cell. The extraction pressure was set to 1500 psi, the flush volume was 60%, and the purge time was set to 100 s. The number of extraction cycles and the need of a cleaning step were assessed by preliminary experiments. The time of each cycle, the sample size, the extraction temperature, the nature of the dispersant, and the nature and composition of the extraction solvent were all the variables optimised by experimental design. The applied experimental conditions in the PSE optimisation process are summarized in Table 1. The obtained extracts were made level to a final volume of 25 mL with MeOH and then passed through a 0,45 μm Polyvinylidene Fluoride (PVDF) filter (Simplepure, USA).

Table 1
Summary of the experimental conditions applied in the PSE optimisation process and optima values selected for the proposed extraction method, (in brackets, the factor code in the screening design).

	Preliminary experiments[b,c]	Design type[c]		Optima[c]
		Screening	Response surface	
Dispersant (A)[a]	C18	C18/Sand	Sand	Sand
Solvent (B)[a]	MeOH	ACN/MeOH	MeOH	MeOH
Temperature (°C) (C)	80	60–110	60–120	105
Solvent (%) (D)	80	30–90	10–90	63
Cycle time (min) (E)	5	5–15	5	5
Sample size (g) (F)	1	0.5–2	1	1
Cycle number	1–5	2	2	2

[a] Non-continuous variables.
[b] Clean-up solvent: n-hexane:acetone (1:1); clean cycles (1).
[c] Fixed factors: pressure (1500 psi); rinse volume (60%); purge time (100 s).

2.4. Polyphenols identification by LC–MS–MS

The extracts were analysed by LC–MS/MS for an accurate identification of the major polyphenols. The liquid chromatographic system used was a Finnigan Surveyor™ HPLC Thermo Fisher Scientific (Madrid, Spain), which contains a LC pump plus, a degasser, and PDA detector. TSP AS3000 was used as the autosampler. The experimental conditions include the use of a 3.9 mm × 150 mm, 4 µm, 60 Å, WatersNova-Pak C_{18} column. The injection volume was 20 µL. The two mobile phase solvents were (A) 1% formic acid/water and (B) 1% formic acid/methanol. The mobile phase gradient program started with 5% B, changed to 20% B at 20 min, and then changed to 100% B at 25 min. The entire HPLC run time was 25 min with a flow rate of 1.0 mL/min and 50 °C column temperature. Electrospray mass spectrometry was performed with a TSQ Quantum Discovery triple stage quadrupole mass spectrometer from Thermo Fisher Scientific (Madrid, Spain). Column effluent was monitored using the Selected Reaction Monitoring (SRM). Polyphenols were detected in the negative mode (ESI-NI) and thus, producing mainly the $[M-H]^-$ pseudomolecular ions. The ESI-MS/MS was operated with a scanning range of m/z 100–600. The capillary voltage was set to 3.0 kV and the capillary temperature was set to 270 °C. High purity nitrogen (99.9%) was used as sheath gas and auxiliary gas at 40 psi and 10 psi and 350 °C, respectively. Argon was the collision gas at 30 psi. The m/z values for the parent/product ions pairs (MRM mode) were 169/125 for gallic acid and 289/205, 289/245 for both catechin and epicatechin. Since only one transition was available for gallic acid, its identity was confirmed via this transition and the retention time. The corresponding tube lens offset was 90 V and the collision energies were 20 eV for m/z 125 and 245, and 16 eV for m/z 205.

2.5. Polyphenols Analysis by HPLC–DAD

A 5 mL aliquot of PSE grape marc extracts were concentrated to a final volume of 0.5 mL under a N_2 stream (VLM EC1 Sample Concentrator), keeping the extract at a temperature of 40 °C. Finally, the concentrated extract was filtered through 0.22 µm PVDF filter (Simplepure, USA) and analysed by high performance liquid chromatography using a Varian Prostar HPLC equipped with a diode array detector (DAD). The experimental conditions were similar to those described in Section 2.4. Polyphenols were detected at 280 nm and identified by comparison of their retention times and UV spectra to those of pure standards (Fig. 1).

2.6. Determination of total polyphenols (TP)

The amount of total polyphenols in grape bagasse extracts was determined according to the Folin–Ciocalteu (FC) colorimetric method (Singleton & Rossi, 1965). TP were quantified from a calibration curve prepared with gallic acid standard solutions in concentrations ranging from 3 to 20 mg L^{-1} (R^2 = 0.9982) and expressed as mg of gallic acid equivalents in the liquid extract (mg L^{-1} GAE). TP sample concentrations were expressed as mg gallic acid per g of dry weight of bagasse (mg gallic/g dw).

2.7. Determination of total flavanols (TF)

The measurement of total flavanols were adapted from Psarra et al. for white wines (Psarra, Makris, Kallithraka, & Kefalas, 2002). TF concentration was estimated from a calibration curve of catechin, obtained by plotting known concentrations of the standard in methanol (1–16 mg L^{-1}) against A_{640} (R^2 = 0,9991). Results in the extract were expressed at catechin equivalents (mg L^{-1} CTE). Final concentrations were expressed as mg catechin per g of dry weight bagasse (mg catechin/g dw).

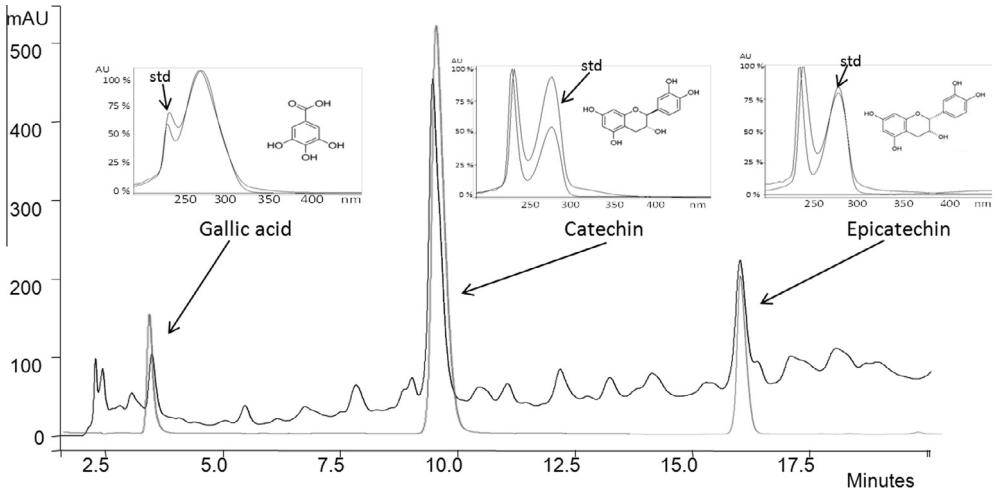

Fig. 1. Overlaid chromatograms (λ = 280 nm) and spectra comparison of a mixture of the polyphenols standards (grey line) and the PSE extract of an *Albariño* grape marc sample (AL-07) (black line) obtained by HPLC–DAD.

2.8. Determination of total hydroxycinnamates (THC)

The analysis of hydroxycinnamates in the PSE extracts was also adapted from Psarra et al. (2002). Results were expressed as chlorogenic acid equivalents (mg L^{-1} CGAE), using the linear regression equation obtained by plotting known concentrations of CGA (5–30 mg L^{-1} CGAE) against A_{320} (R^2 = 0,9968). Final concentrations were expressed as mg chlorogenic acid per g of dry weight bagasse (mg GCA/g dw).

2.9. Determination of total flavonoids content (TFC)

Total flavonoids were measured according to the colorimetric assay used by Kim, Chun, Kim, Moon, and Lee (2003). TF concentration was estimated from a calibration curve of catechin, obtained plotting known concentrations of catechin in water (5–200 mg L^{-1}) against A_{520} (R^2 = 0,995). Results in the extract were expressed as catechin equivalents (mg L^{-1} CTE). Final concentrations were expressed as mg catechin per g of dry weight bagasse (mg catechin/g dw).

2.10. DPPH radical scavenging activity

2,2-Diphenyil-1-picrylhydrazyl (DPPH) scavenging activity was determined using a modified method of Brand-Williams, Cuvelier, and Berset (1995) against Trolox®. DPPH 0.1 mM was dissolved in 100% methanol. The grape marc extracts, 0.1 mL, were added to 3.9 mL of the methanolic DPPH solution. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The decrease in absorbance of the resulting solution was monitored at 515 nm at 30 min. The antiradical activity (AA) was determined using the following equation ($y = 0.5223x + 0.0276$; R^2 = 0,999) obtained from linear regression after plotting the A_{515} of known solutions of Trolox against concentration (0.08–1 mM). The DPPH radical scavenging activity of the PSE extracts was expressed as mM Trolox g^{-1} of grape marc (dry weight). The radical stock solution was prepared daily.

2.11. Statistical analysis

The experimental design applied in the optimisation of the PSE method, and the subsequent data analysis were performed using Statgraphics XV Centurion software package (Manugistics Inc., Rockville, MD). To firstly define the experimental domain for the PSE we used a screening design. Six factors and 7 response variables (namely, the concentration of gallic acid, catechin and epicatechin and the total polyphenols content (TP), the total flavanols content (TF), the total hydroxycinnamates content (THC), and the total flavonoids content (TFC)) were selected. response surface methodology (RSM) was then used for the fine tuning of the most influential factors and the same dependent variables. The results obtained were evaluated by analysis of variance (ANOVA), which measures whether a factor contributes significantly to the variance of the response. A comparison of means based on LSD (least significant difference) Fisher test was used to determine significant differences between the levels within each factor.

3. Results and discussion

3.1. Optimisation of PSE parameters

Parameters that can potentially influence the efficiency of the PSE process, are several specific PSE variables such as temperature and pressure of the extraction, flush volume, extraction and purge time, as well as the number of cycles; in addition to solvent nature and sample size as in conventional solid–liquid extractions. Pressure is used to increase the contact between the extracting solvent and the sample, but it generally has a negligible effect on the extraction yield (Carabias-Martínez, Rodríguez-Gonzalo, Revilla-Ruiz, & Hernández-Méndez, 2005). Thus, all experiments were conducted at 1500 psi, which is the standard operating pressure in PSE extractions (Dionex, 2004). Flush volume and purge time were also set at their standard values, 60% and 100 s, respectively (Dionex, 2004). The influence of the remaining variables was studied as described below.

Baseline improvements in the HPLC chromatograms were described when introducing a cleanup step in PSE using low polarity solvents (Papagiannopoulos & Mellenthin, 2002). In this way, the possibility to obtain cleaner extracts was also tested. The cleanup process used a mixture of n-hexane:acetone (1:1) for 1 to 3 cycles of 5 min, previous to the extraction of the polyphenols with methanol in water at 80%. The chromatograms obtained with and without cleanup were compared and since no additional improvement was observed, the cleanup step was finally discarded.

The number of extraction cycles applied was tested using 1, 2 or 5 cycles. The use of several static cycles that introduce fresh solvent during the extraction process assists in keeping a favourable extraction equilibrium (Fernandez-Alvarez et al., 2009); especially for samples with very high analyte concentration or for those in which the matrix hampers the solvent diffusion (Dionex, 2004). In our case, extraction efficiency was slightly improved using 2 cycles, while the use of 5 static cycles led to an increased background, and was then discarded. This was consistent with the results obtained by Luthria (2006), who optimised the extraction of polyphenols in parsley, extracting already 80% of the total content in the first cycle of extraction using 70% methanol in water as extraction solvent, and over 95% in the second cycle. Therefore, we decided to perform 2 extraction cycles to guarantee a higher efficiency.

Following these preliminary experiments, the optimisation of other variables was accomplished using experimental design tools: spectrophotometric indexes (TP, TF, THC, and TFC) and chromatographic responses of gallic acid, catechin, and epicatechin were the dependent variables evaluated for each set of experimental conditions.

3.1.1. Screening

The selected design was a Placket–Burman of 6 factors at 2 levels 2^6 * 3/16, which implied 14 experiments. The studied factors and their corresponding levels are summarized in Table 1. In contrast to other screening designs, this one allows running continuous and non-continuous factors together. Two center points were added to increase the freedom degrees to evaluate the experimental error.

An analysis of variance (ANOVA) was used to assess whether main factors or their interactions showed a statistically significant contribution to the variance of the response, and the obtained results are given in Table 2. These results can be simply interpreted analysing the signification data along with the graphical outcomes of the experimental design shown in Fig. 2a and b. In the Pareto charts, the standardized effects are plotted in decreasing order of absolute magnitude and the line drawn on the chart indicates whether an effect is statistically significant at the specified significance level (in this case, 95%). Thus, Fig. 2a shows that the type of dispersant (A), the type of solvent (B) and the time of each cycle (E) have no significant effects. For this reason, the shorter cycle time (5 min) was selected, making experiments as fast as possible and increasing sample throughput. As sand and methanol are both comparatively economic to C18 and acetonitrile, respectively; and considering also the increasing difficulty in obtaining commercial acetonitrile, both were selected for the following optimisation

Table 2
F and *p* values obtained in the analysis of variance for the Placket–Burman design of 6 factors at 2 levels.

	Factors											
	Dispersant (A)		Solvent (B)		Temperature (C)		Solvent ratio (D)		Cycle time (E)		Sample size (F)	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Gallic acid	0.03	0.87	0.39	0.56	8.85	0.03[a]	1.89	0.23	3.1	0.14	4.89	0.08
Catechin	0.13	0.74	0.47	0.52	8.96	0.03[a]	0.03	0.87	2.27	0.19	5.1	0.07
Epicatechin	0.81	0.41	3	0.14	12.91	0.01[a]	1.2	0.32	2.53	0.17	5.01	0.07
TP	0.11	0.75	0.28	0.62	5.39	0.07	7.03	0.04[a]	0.26	0.63	0.01	0.94
TF	1.29	0.31	0	0.99	8.24	0.03[a]	3.3	0.13	0.29	0.61	0.61	0.47
TFC	0.1	0.77	0.49	0.51	1.88	0.23	2.78	0.16	0.75	0.42	0.02	0.88
THC	0.18	0.69	2.33	0.19	10.65	0.02[a]	10.31	0.02[a]	2.66	0.16	0.02	0.88

TP, total polyphenols; TF, total flavanols; THC, total hydroxycinnamates; TFC, total flavonoids.
[a] Significant effects for a confidence level of 95%.

tests. In addition, methanol is part of the mobile phase in HPLC analysis and is therefore more compatible. The amount of sample (F) did not prove significance for any of the variables studied, and as its effect was somewhat opposite between spectrophotometric and chromatographic variables, we opted for a compromise with the intermediate value of the parameter (1 g) considered an adequate and manageable amount of grape marc for testing.

The most influential and significant variables were the temperature (C), and the proportion of organic solvent in the liquid elution phase (D) (Table 2, Fig 2a); and thus both require fine tuning in the search for their optimal values, which was conducted by a response surface type design.

3.1.2. Response Surface Methodology (RSM)

Once the experimental domain was confined, the proportion of organic solvent in the elution mixture (factor A) and the temperature (factor B) were fine-tuned using the Response Surface Methodology (RSM). The objective was to simultaneously optimise the levels of these factors to attain the best PSE performance. A face-centered central composite design 2^2 was chosen, so that the axial distance is equal to 1 and the values of percentage and temperature originated in the matrix of experiments can be easily selected. Two central points were also added in order to increase the freedom degrees to evaluate the experimental error.

Temperature is an important parameter that generally favours PSE extractions, as it is used to break the analyte-matrix bonds. However, the use of high temperatures can reduce selectivity (Giergielewicz-Mozajska et al., 2001) and cause some compound degradation as well. Palma et al. (2001) concluded that using PSE at high temperatures, namely 100 °C, phenolic compounds suffer as much as 10% degradation even for the most oxidizable ones; but it can be worse with even higher temperatures (e.g. 150 °C). Thus, this continuous factor was evaluated at three levels: 60, 90 and 120 °C.

The choice of an appropriate elution solvent composition is another essential aspect for an efficient extraction. The solvent must solubilize the target analytes while leaving the sample matrix as intact as possible (Dionex, 2004). Methanol and aqueous methanol are commonly used solvents for extracting polyphenols, and the process efficiency is influenced by the concentration of the organic solvent (Escribano-Bailon & Santos-Buelga, 2003). This factor was then tested at three levels: 10%, 50% and 90% MeOH in water.

The final number of experiments was ten, and the selected experimental design allows evaluating the second order interactions between factors. The analysis of the obtained data lead to the ANOVA results shown in Table 3. As it can be seen, temperature was significant for three of the four spectrophotometric indexes and catechin, whereas the percentage of methanol in the extraction solvent was significant just for the gallic acid extraction. The interaction of factor A (solvent) with itself was also significant (Table 3). This interaction results from the quadratic behaviour of this factor. Such parabolic behaviour can be graphically seen in Fig. 2b, showing that the maximum of the factor does occur at some intermediate value.

The strategy to select the optimal values for the considered parameters was a multiple response optimisation that led to the following values: a temperature of 105 °C and a 63% of methanol in the extraction solvent. Regarding the spectrophotometric variables, the temperature could be higher, but for the individual polyphenols, a decrease in extraction efficiency is observed when a certain temperature is exceeded (quadratic factor), behaviour already described (Palma et al., 2001). On the other hand, the differences in extraction yields regarding the solvent composition may be explained by the action of polyphenol oxidase, which activity is sensitive to methanol. A low methanol proportion in the extraction solvent does not completely inactive polyphenol oxidase in fruits, thus reducing some polyphenols extraction yields (e.g. catechins) (Escribano-Bailon & Santos-Buelga, 2003). Table 1 also summarizes the RSM optimised experimental conditions used for the extraction of the grape marc samples.

3.2. Method performance

The quality parameters of the proposed PSE method were estimated in order to verify that the optimised method is suitable for the quantitative determination of polyphenols in grape marc samples. The HPLC–DAD instrumental linearity was evaluated injecting nine concentration levels in triplicate of each individual standard at its corresponding concentration range (gallic acid: 5–200 $mg\,L^{-1}$; catechin: 10–700 $mg\,L^{-1}$; epicatechin: 25–500 $mg\,L^{-1}$); and the response function was found to be linear with R^2 values of 0.9978, 0.9988 and 0.9961; respectively. Recovery was calculated by comparing replicate determinations of spiked and unspiked samples; and the results, expressed as percentage ± standard deviation: gallic acid: 91.3 ± 11; catechin: 100.8 ± 11; epicatechin: 104.6 ± 9; were corrected with the measured original concentrations of the target analytes in the real sample. Method precision ($n = 3$) was studied within-a-day (repeatability) and among-days (reproducibility) and expressed in terms of RSDs, correspondingly: gallic acid (5% and 17%); catechin (15% and 18%); and epicatechin (17% and 5%). The instrumental detection (LOD) and quantification (LOQ) limits were calculated as the concentration giving a signal-to-noise ratio of 3 ($S/N = 3$) and 10 ($S/N = 10$), respectively; and the obtained values were at the low mg/L level (LOD: 0.8, 2.7 and 2.5; and LOQ: 2.6, 8.9, 8.3) for gallic, catechin and epicatechin, respectively. Finally, the LOD and LOQ of the overall method were also determined, giving values of 0.09 and 0.29 $mg\,g^{-1}$ dw for gallic acid; 0.33 and 1.10 $mg\,g^{-1}$ dw for catechin; and 0.36 and 1.19 $mg\,g^{-1}$ dw for epicatechin. Similarly, linearity and precision were estimated for the spectrophotometric indexes used in this study. The response function was found to

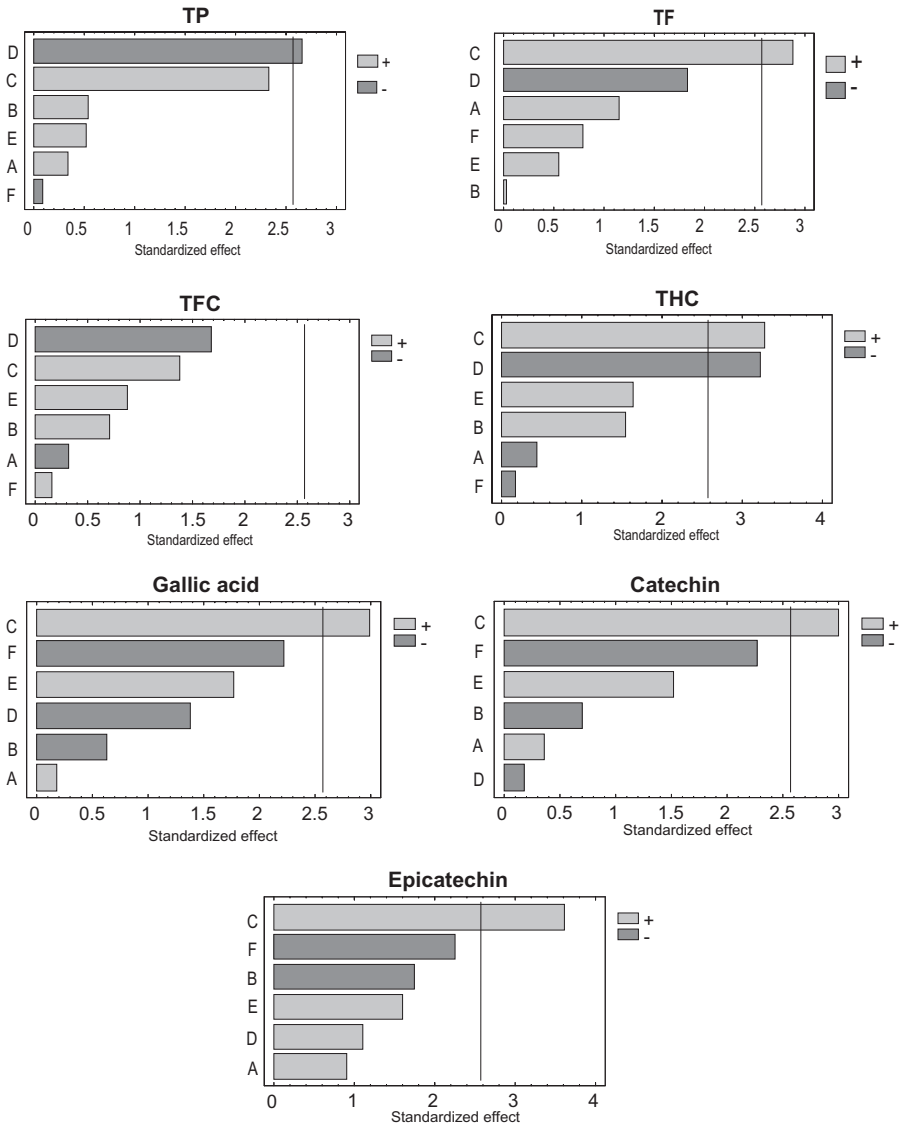

Fig. 2a. Pareto charts corresponding to the screening design. Factor codes: dispersant (A); solvent (B); temperature (C); solvent ratio (D); cycle time (E); and sample size (F).

be linear with determination coefficients (R^2) higher than 0.995. RSDs for the within-a-day precision ranged from 5% to 8%, while the RSDs for the among-days precision ranged from 4% to 7%.

3.3. Application of the optimised method to white grape marcs

The proposed method was employed for the analysis of 13 grape marc samples from the variety Albariño collected from 12 wineries located in Galicia (NW Spain). All samples belonged to the DO Rias Baixas with only one exception (AL08) belonging to DO Ribeira Sacra. Table 4 presents the results obtained.

The average values of the samples for the spectrophotometric indexes (Table 4) were 40.33 mg GAE/g dw for TP, 14.21 mg CTE/g dw for TF, 28.47 mg CTE/g dw for TFC, and 3.82 mg CGAE/g dw for THC, respectively. In all Albariño samples, flavonoids (TFC) represent about 70% of TP content, flavanols (TF) are within the group

Table 3
F and *p* values obtained in the analysis of variance for the response surface design of 2 factors at 3 levels.

	Factors				Interactions					
	A: % methanol		B: temperature		AA		AB		BB	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Gallic acid	10.86	0.03[a]	2.40	0.20	19.01	0.0121[a]	0.80	0.4203	1.31	0.3163
Catechin	0.03	0.87	13.74	0.021[a]	12.23	0.0250[a]	0.48	0.5279	2.46	0.1918
Epicatechin	1.39	0.30	2.16	0.216	2.08	0.2227	0.25	0.6406	1.09	0.3563
TP	0	0.96	30.76	0.005[a]	27.88	0.0062[a]	4.95	0.0901	1.22	0.3309
TF	0.01	0.93	50.90	0.002[a]	15.78	0.0165[a]	7.46	0.0524	3.91	0.1190
TFC	2.18	0.21	56.86	0.002[a]	4.42	0.1034	0.66	0.4612	4.35	0.1055
THC	0.19	0.69	3.62	0.13	15.07	0.0178[a]	0.40	0.5597	0.03	0.8793

TP, total polyphenols; TF, total flavanols; THC, total hydroxycinnamates; TFC, total flavonoids.
[a] Significant effects for a confidence level of 95%.

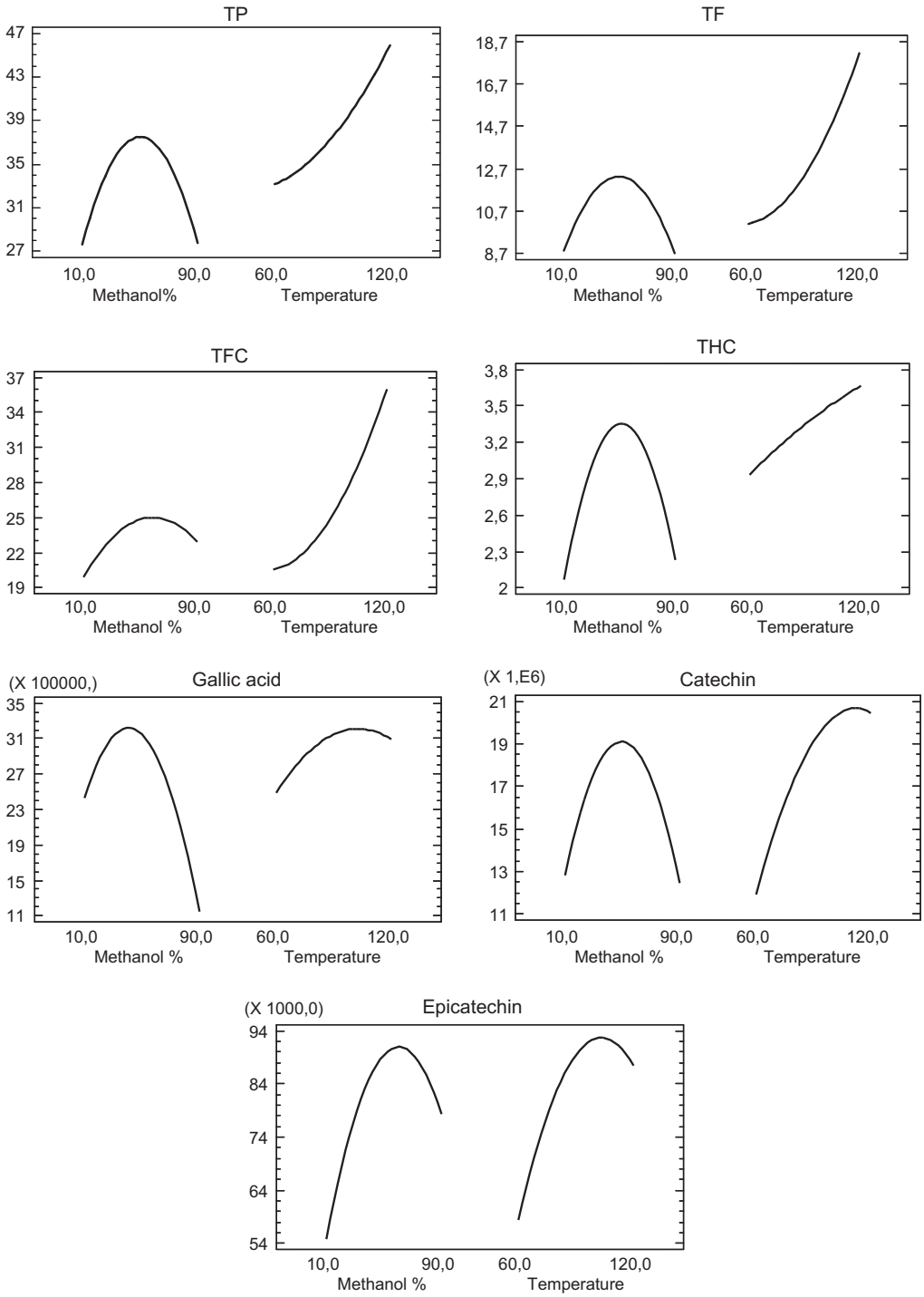

Fig. 2b. Main effects plots corresponding to the RSM design. Factor codes: methanol% (A); temperature (B).

of flavonoids (TFC) and represent approximately 50% of this group, and finally, hydroxycinnamates represent on average 10% of the TP. It is important to highlight the TP content of the white Albariño grape marc samples, with an average value of 40 and a maximum of 53.5 mg GAE/g dw. These data are similar to those obtained by Makris et al. in white grape marc pomace from Greece (Makris et al., 2007), and equal or even higher than those obtained for red grape bagasse by the same authors (TP content of 54.02 mg GAE/g dw of Agiorgitiko variety) and also by Negro et al. (TP content of 41.9 mg GAE/g dw of Negro amaro variety) (Negro, Tommasi, & Miceli, 2003). Indeed, Makris et al. (2007) reported equivalent data (TP, TF and TFC) for white grape pomace from *V. Vinifera* variety Roditis: 48.26 mg GAE/g dw, 12.58 mg CTE/g dw, and 35.22 mg CTE/g dw, respectively. In all these referenced cases, the polyphenols extraction procedure involves several steps as maceration, stirring, filtering and centrifugation.

The flavanols content of various winery byproducts, derived from red and white varieties was studied by González-Paramás, Esteban-Ruano, Santos-Buelga, de Pascual-Teresa, and Rivas-Gonzalo (2003) using classical extraction methodology based on ultrasounds assisted maceration, finding polyphenols contents very different among grape pomace extracts, from about 2.9 mg/g dw

Table 4
Analysis of real grape marc samples (*v. Albariño*).

	Gallic acid mg/g dw[a]	Catechin mg/g dw	Epicatechin mg/g dw	TP mg GAE/gdw	TF mg CTE/gdw	TFC mg CTE/gdw	THC mg CGAE/gdw	AA mM Trolox/gdw
AL01	0.09 ± 0.01	3.60 ± 0.68	1.52 ± 0.32	34.8 ± 0.3	15.3 ± 0.3	36.6 ± 5.8	4.4 ± 0.1	2.67 ± 0.07
AL02	0.17 ± 0.03	4.13 ± 0.39	2.98 ± 0.16	53.5 ± 1.8	20.4 ± 0.7	43.5 ± 4.5	4.7 ± 1.0	3.21 ± 0.08
AL03	0.09 ± 0.01	1.75 ± 0.09	1.13 ± 0.23	35.2 ± 1.6	18.6 ± 3.7	28.7 ± 2.9	3.4 ± 0.6	2.89 ± 0.59
AL04	0.12 ± 0.02	2.72 ± 0.35	2.00 ± 0.34	43.8 ± 1.5	14.9 ± 0.9	26.6 ± 1.8	3.8 ± 0.3	2.43 ± 0.08
AL05	0.11 ± 0.00	2.51 ± 0.39	1.69 ± 0.06	41.6 ± 4.4	12.4 ± 0.7	23.1 ± 4.1	3.5 ± 0.0	2.82 ± 0.13
AL06	0.11 ± 0.01	3.10 ± 0.37	1.53 ± 0.18	46.9 ± 0.5	15.6 ± 0.4	30.8 ± 1.5	4.1 ± 0.2	3.06 ± 0.18
AL07	0.06 ± 0.00	1.91 ± 0.28	1.19 ± 0.12	37.2 ± 0.5	10.4 ± 1.7	21.2 ± 0.2	4.0 ± 0.1	2.44 ± 0.06
AL08	0.12 ± 0.02	2.96 ± 0.35	1.84 ± 0.11	45.9 ± 5.7	15.6 ± 3.1	27.7 ± 5.4	4.4 ± 0.5	2.72 ± 0.18
AL09	0.07 ± 0.01	1.73 ± 0.28	1.30 ± 0.07	29.1 ± 3.4	10.5 ± 1.8	19.8 ± 0.0	3.7 ± 0.1	2.68 ± 0.01
AL10	0.10 ± 0.02	3.32 ± 0.54	1.86 ± 0.29	36.9 ± 0.9	11.6 ± 1.8	24.1 ± 0.5	3.3 ± 0.5	3.05 ± 0.14
AL11	0.12 ± 0.02	2.93 ± 0.52	2.02 ± 0.11	41.6 ± 2.8	15.1 ± 1.6	28.6 ± 0.1	3.1 ± 0.2	2.83 ± 0.17
AL12-e	0.12 ± 0.00	2.93 ± 0.47	1.71 ± 0.17	36.9 ± 5.6	12.5 ± 2.7	30.0 ± 6.5	3.7 ± 0.1	4.14 ± 0.75
AL12-f	0.07 ± 0.01	2.56 ± 0.37	1.37 ± 0.18	40.9 ± 1.3	11.8 ± 0.8	29.4 ± 2.7	3.5 ± 0.4	3.13 ± 0.11
Average	0.10 ± 0.03	2.79 ± 0.79	1.72 ± 0.5	40.3 ± 6.3	14.2 ± 3.0	28.5 ± 6.3	3.8 ± 0.5	2.93 ± 0.44

TP, total polyphenols; TF, total flavanols; THC, total hydroxycinnamates; TFC, total flavonoids.
[a] Dry weight.

in white bagasse from Albillo and Viura varieties to a maximum of 19.9 mg/g dw in the Merlot red extract. The Albariño baggase PSE extracts showed TF content from 10.5 to 20.4 mg/g dw, which demonstrates their potential as a source of antioxidant flavanols and demonstrates the utility of the PSE proposed methodology.

As regards the non-hydroxycinnamate phenolics, gallic acid was the least abundant, with concentrations in the range of 0.07 to 0.19 mg/g dw, and an average content of 0.10 mg/g. Catechin content varied from 1.73 to 4.13 mg/g (mean 2.79 mg/g), and epicatechin content varied from 1.13 to 3.22 mg/g (mean 1.72 mg/g). Yilmaz and Toledo (2004) analysed the levels of these three polyphenols in seeds and skins, separatedly, from grapes of *V. vinifera* varieties Merlot (red) and Chardonnay (white). Our average data from grape marc of *V. vinifera* variety Albariño (white) shows contents greater than in Merlot for catechin (1.43 mg/g dw) and epicatechin (1.28 mg/g dw) and not so different for gallic acid (0.13 mg/g dw); while Chardonnay would present higher concentrations for the three species: 0.2, 4.18, and 4.65 mg/g dw, respectively; these reference data come from the sum of seeds and skins. Thus, all Albariño samples have higher catechin content and similar gallic content than the red Merlot, and some extracts (AL-02) reaches equivalent values to Chardonnay for gallic acid and catechin but not so for epicatechin. This means that the PSE methodology applied to raw Albariño grape marc samples is able to give extracts that are potentially as good sources of non-hydroxycinnamates, without the previous laborious separation of seeds and skin.

The sample AL08, belonging to DO Ribeira Sacra did not produce data that differ markedly from the DO Rias Baixas samples. ANOVA results were considered statistically significant when $p < 0.05$ and are expressed in terms of F and p as follows: TP (2.61, 0.12), TF (0.59, 0.45), TFC (0.09, 0.77) and THC (3.65, 0.066).This uniformity of the grape marc from Albariño variety in relation to their polyphenolic composition (despite being from different geographical zones) is an excellent new from the point of view of the possible reuse of such byproduct, as it makes much easier to adapt and scale an extraction process if the raw material that feeds it is homogeneous.

3.3.1. In Vitro antioxidant capacity

The antioxidant activity (AA) of the PSE grape marc extracts was also included in Table 4. AA values varied between 2.43 and 4.14 mM TRE/g dry weight. The obtained average value for the AA of Albariño extracts was 2.93 ± 0.19, slightly greater than that obtained for white grape pomace from Roditis variety, 2.22 ± 0.17 (Makris et al., 2007); and indeed, greater than reported data on the AA of Albariño wines (0.77–2.01 mM TRE) (Rodríguez-Bernaldo de Quirós, Lage-Yusty, & López-Hernández, 2009). It is well known that white wines contain significantly lower amounts of total polyphenols compared to red wines (Makris et al., 2003); however for the bagasse, the situation is the opposite as it can be deduced comparing these data with the available AA data of PSE red grape pomace extracts (mainly composed of anthocyanins), which vary from about 1.0 to less than 2.5 mM TRE/g dry weight (Monrad et al., 2010). These findings are consistent with both white and red winemaking process: the former is made from the free running juice and the latter is made maintaining the grape juice with grape seeds, stems and/or pulp at the first stage of wine fermentation. Thus, it is expected that less polyphenols are transferred to the juice during white winemaking, producing a bagasse keeping high antioxidant activity and opening the way for the reutilization of this wineries byproduct.

4. Conclusions

A PSE based methodology has been optimised to extract major polyphenols from white grape marc and to evaluate its content on the principal polyphenols families. The optimisation procedure was addressed by: screening the main influential factors, and by further tuning by RSM. The proposed PSE method performed well in terms of recovery (91–105%), linearity ($R^2 > 0.9978$), and inter and intra-day precision (RSD < 8% for indexes and <18% for individuals). LODs and LOQs were below 2.7 and 8.9 mg L^{-1}, respectively (instrumental), and below 0.36 and 1.19 mg g^{-1} dw, respectively (whole analytical method). The developed PSE method is more advantageous than those based on traditional techniques such as maceration/agitation, since similar or better results are faster obtained (less than 20 min per extraction), lowering solvent consumption. Gallic acid, catechin and epicatechin, positively identified by LC–MS and quantified by HPLC–DAD, were the main polyphenolic compounds in Albariño extracts. Developed methodology has been applied to different grape marcs obtained from Albariño, a white *V. vinifera* variety employed in NW Spain to elaborate high quality wines. Such extracts have several potential ways of exploitation, as a source of polyphenols notably non-hydroxycinnamates, with demonstrated antioxidant activity, even as raw marc extract; since they have similar or even greater radical scavenging activity than that of reported red grape marc extracts.

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