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Optimization of continuous phenol extraction from *Vitis vinifera* byproducts

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Abstract

Grape byproducts are a cheap source of antiradical phenols, extractable as food preservatives or dietary supplements for disease prevention. However, efficiency of extraction strongly depends on operative conditions. In this work, the effects of three critical variables – flow-rate (between 2 and 3 ml/min), sample quantity (between 2.5 and 7.5 g) and particle size (between 0.5 and 5.5 mm) – on extraction efficiency were studied. Process conditions maximizing the antiradical activity of the phenol extract were found to be 2 ml/min flow rate, 2.5 g sample quantity and 0.5 mm particle size. In addition, extraction promoted the formation of phenolic compounds not naturally occurring in the grape residue. Chromatographic profiles of extracts confirmed that these novel compounds were formed as a consequence of phenol polymerisation.

Keywords: Grape (*Vitis vinifera*); Continuous extraction; Polyphenols; Antiradical capacity

1. Introduction

Extraction is one of the most widely used unit operations in the food industry. Its main use is for obtaining certain desired components initially retained in a food matrix (Barzana et al., 2002). Molecules obtained by extraction may be used as food additives or for exerting peculiar beneficial effects on human health (Osada, Hoshina, Nakamura, & Sugano, 2001).

In the past several years, a large number of scientific reports have described the properties of phenolic compounds from numerous natural products. In particular, natural phenols have been reported with excellent properties as food preservatives (Valenzuela, Nieto, Cassels, & Speisky, 1992) as well as having an important role in protection against a number of pathological disturbances, such as atherosclerosis, brain dysfunction and cancer (Gordon, 1996; Steinberg, 1992). For this reason, a great effort has been made to characterize phenols occurring in different plant tissues. Special interest has always been paid to grape polyphenols, not only for their health-beneficial properties but also their sensory properties in wine (Bonilla, Mayen, Merida, & Medina, 1999; González-Paramás, Esteban-Ruano, Santos-Buelga, Pascual-Teresa, & Rivas-Gonzalo, 2004). However, very few literature reports focus on the outcome of extraction conditions on either, (1) the overall process efficiency or (2) phenol composition of the extract.

Extraction efficiency is known to be a function of process conditions. Several factors affect the concentration

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of desired components in the extract: Temperature, liquid–solid ratio, flow rate and particle size. For instance, phenolic content of almond hulls extracts was found to be three times higher when a batch liquid–solid extraction was performed at 50 °C in comparison to that at 25 °C. Time contact and liquid–solid ratio were also reported to be significant variables (Pinelo, Rubilar, Sineiro, & Núñez, 2004a; Rubilar, Pinelo, Franco, Sineiro, & Núñez, 2003). The positive or negative role of each factor in the mass transfer of the process is not always obvious and is often conditioned by factors, such as packaging phenomena, saturation effects or thermal degradation (Fan, Morris, & Wakeman, 1948; Tulbentci, 1986).

Since phenols are highly reactive, extraction conditions could profoundly modify the phenolic profile of the extract. Two typical zones are generally observed in HPLC chromatograms of grape extracts and derivatives: A non-polymeric phenol zone and a polymeric phenol one (Kantz & Singleton, 1990; Peng et al., 2001). In the former, catechin, epicatechin and quercetin are the most usually identified monomeric phenols (Pérez-Magariño & Gonzlez-San Jos, 2004; Yilmaz & Toledo, 2004). The latter are known to have a strong tendency to undergo polymerization reactions that promote important structural changes, and, as a consequence, variations in their properties. In fact, previous findings reveal the important roles of temperature, time and solvent properties in the development of phenol polymerisation. Pinelo, Manzocco, Núñez, and Nicoli (2004b) have reported an initial increase in the antiradical capacity of catechin and quercetin solutions during the first period of storage at 22, 37 and 60 °C. When the reaction time was prolonged, the value of this parameter decreased, concomitantly with the formation of phenol polymers. For this reason, extraction conditions could favour the formation of phenol polymers, whose concentration in the extract would be much higher than that in the original food matrix. In the light of these considerations, the reactivity of extracted species under the conditions chosen for extraction should be strictly considered in order to carry out a good identification of the original compounds present in such matrix.

In this work, a study of the extraction process of distilled white-grape byproducts by two different approaches is proposed. First, the influence of three critical variables (flow rate, sample amount and particle size) on the efficiency of the extraction process was evaluated. Process conditions maximizing polyphenolic content and antiradical activity were considered as optimal extraction conditions. Secondly, since the latter parameter depends on both the chemical structure and conformation of the species contained in extract, the evolution of extract phenol profile during the overall extraction time was studied by HPLC analysis.

2. Materials and methods

2.1. Sample preparation

The starting material, provided by Bodegas Miguel Torres, S. A. (Villafranca del Penedés, Spain) was the residue of the grape after distilling white Garnacha grapes (*Vitis vinifera*). It consists of skins, seeds and small amount of stems. Samples were ground in a knife mill. Powdered samples were sieved to select particles smaller than 3 and 0.5 mm and stored at room temperature until used.

2.2. Continuous extraction

Deionized water was used as a solvent for polyphenol extraction from grape residue. Samples were extracted in an immersion extractor of 4.5 cm i.d. and 10 cm height. The extractor was kept at 50 °C by a thermostated external water bath. Previous studies of the optimisation of the extraction conditions on several natural residues, such as almond hulls, pine sawdust or apple byproducts (Pinelo et al., 2004a; Rubilar et al., 2003) showed that 50 °C was the optimal temperature for achieve this extraction process. It is true that increasing temperatures favoured extraction by , enhancing solubility of solute and increasing the extraction coefficient, however, this cannot be increased indefinitely; the stability of phenolic compounds and the denaturation of membranes can occur (above 50 °C), with dramatic effects on antiradical capacity (Cacace & Mazza, 2003; Schwartzberg & Chao, 1982). A condenser was fitted to avoid solvent losses. Extraction was accomplished by continuously pumping fresh solvent through the cake bed. The solvent was pumped upward from the bottom. The scheme of the immersion extractor appears in Fig. 1, and the experimental set-up is shown in Fig. 2. The outlet extract was sampled at intervals of 30 min for further analyses.

2.3. Analytical determinations

2.3.1. Determination of total polyphenolic compounds

The total phenolics were assayed colorimetrically by the Folin–Ciocalteu method, as modified by Singleton and Rossi (1965). 2.5 ml of tenfold diluted Folin–Ciocalteu reagent, 2 ml of 7.5% sodium carbonate, and 0.5 ml of phenolic extract were mixed. The absorbance was measured at 765 nm after 15 min heating at 45 °C. A mixture of water and reagents was used as a blank. The content of phenolics was expressed as gallic acid equivalents and was always referred to material weight.

2.3.2. Determination of antiradical capacity

The antiradical activity was measured, following the methodology described by Brand-Williams, Cuvelier, and Berset (1995); the bleaching rate of a stable free rad-

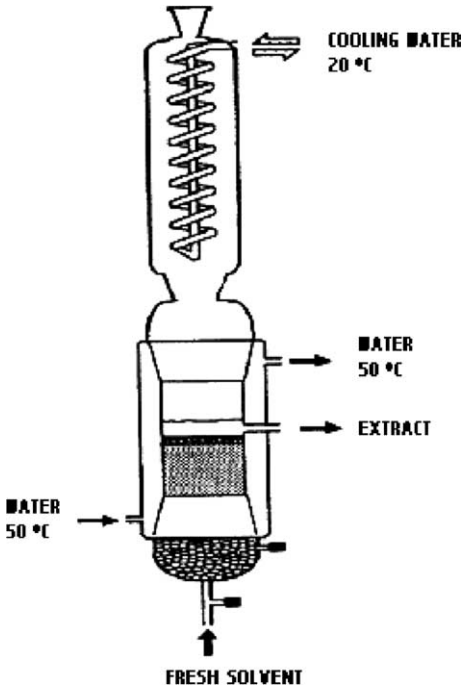

Fig. 1. Immersion stirred extractor.

ical, 2,2-diphenyl-1-picrylhydrazyl (DPPH·) is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH· absorbs at 515 nm, decreasing its absorption upon reduction by an antiradical or a radical specie.

A volume of 1.80 ml of 6.1×10^{-5} M DPPH· methanol solution was used. The reaction was started by the addition of 200 μl of sample. The bleaching of DPPH· was followed at 515 nm (Uvikon 860, Kontron Instruments, Milano, Italy) at 25 °C for 20 min. In all cases, the DPPH· bleaching rate was proportional to the sample concentration (added to the medium). The following equation was chosen in order to obtain the rate of DPPH· bleaching, k (Nicoli, Manzocco, & Calligaris, 2000)

$$\frac{1}{A^3} - \frac{1}{A_0{}^3} = 3kt$$

where A_0 is the initial optical density and A is the optical density at increasing time, t. The antiradical activity was expressed as k ($-\mathrm{OD}^{-3}\ \mathrm{min}^{-1}$ mg of dry matter^{-1}).

2.3.3. High-pressure liquid chromatography

The HPLC apparatus with a pump, PU-980, connected to a quaternary gradient unit LG-1580-04, a JASCO UV-1575 UV–Vis detector and a Rheodyne model 7725 loading sample injector with a 20 μl sample loop were used to determine the phenolic composition of the samples. The column (250 mm × 4.6 mm) was a C18 Hypersil ODS (5 μm particle size) (Supelco).

The two solvents used to make the gradient were: (A) 0.5% acetic acid Milli-Q water solution and (B) methanol. The solvent gradient in volumetric ratios of solvents A and B was as follows: 0–10 min, 95A/5B; 10–60 min, 50A/50B; 60–80 min, 30A/70B; 80–90 min, 95A/5B. Detection was carried out using 280 nm as the preferred wavelength.

Flow rate was set at 0.7 ml/min. Three determinations were made on each extrac obtained. Standards of gallic acid, catechin, epicatechin, quercetin and resveratrol were purchased from Sigma (St. Louis, MO).

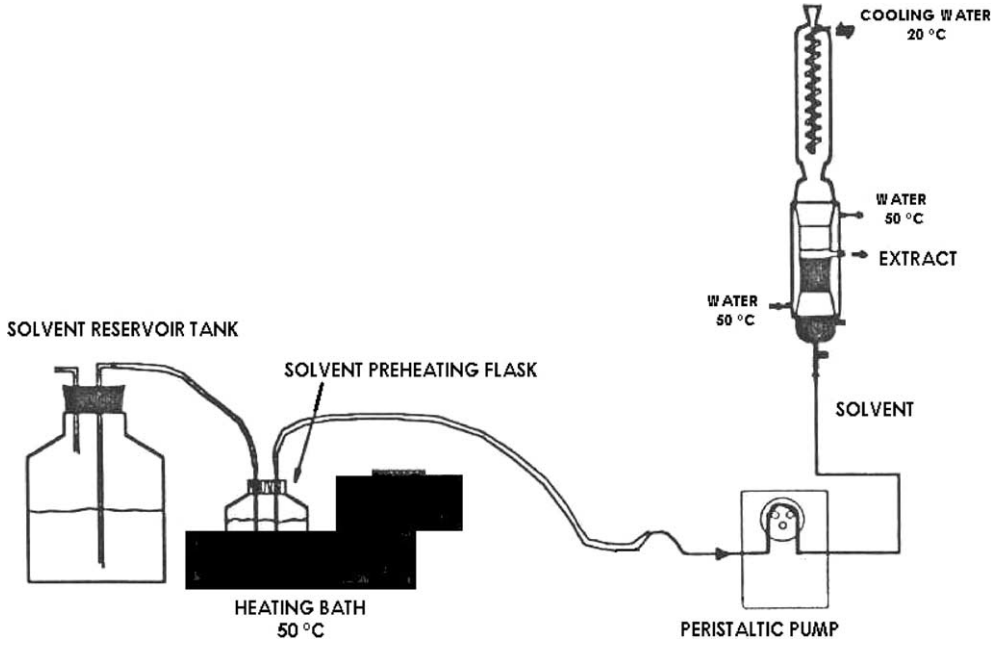

Fig. 2. Experimental extractor set-up.

2.4. Mathematical tools

2.4.1. Experimental design

A full factorial 2^3 experimental design was developed to evaluate the effects of flow rate (F), sample amount (C) and particle size (G) (Box, Hunter, & Hunter, 1999). Flow rate values varied between 2 and 3 ml/min, sample quantity varied between 2.5 and 7.5 g and particle size between 0.5 and 5.5 mm (this latter was assessed as the particle size medium value of the whole individual residue). Variables were codified in the way that their value ranged between +1 and 1, taking, as the central point, the zero value

$$F = (\mathbf{F} - 2.5)/0.5,$$

$$C = (\mathbf{C} - 5)/2.5,$$

$$G = (\mathbf{G} - 3)/2.5.$$

(Table 1) shows the factorial design matrix, with variables in both coded and non-coded form, for better comprehension.

Data were adjusted to a response surface R

$$R = a_0 + a_1F + a_2C + a_3G + a_{12}FC + a_{13}FG + a_{23}CG + a_{123}FCG,$$

where a_0 is the value of the objective function under the central point conditions, a_1, a_2, a_3 represent the principal effect associated with each variable and a_{12}, a_{13}, a_{23}, a_{123} represent the crossed effects among variables.

2.4.2. Statistical analysis

The results reported in this work are the averages of at least three measurements, and the coefficients of variations, expressed as the percentage ratios between standard deviations (SD) and the mean values, were found to be <10 in all cases. Significant variables ($P < 0.05$) were calculated by linear regression using SPSS statistical programme version 10.0 (SPSS Inc., Chicago, Illinois).

3. Results and discussion

A typical evolution of polyphenol concentration as a function of extraction time is shown as an example in Fig. 3. Data presented are relevant to experiment 5 of the factorial experimental design (Table 1) but similar results were obtained in all cases. In order to evaluate the outcome of each experiment, an index (OPC) comprising a quantitative evaluation of total polyphenols extracted for each experimental condition was proposed. It was assessed by numerical integration of the area under the polyphenol concentration curve as a function of extraction time (Table 2). The highest OPC value was obtained when extraction was carried out under the conditions of experiment 5 (lower flow rate, lower sample amount and lower particle size). Likewise, Fig. 4 shows the evolution of antiradical capacity of grape extracts with respect to the extraction time. Also, in this case, results correspond to conditions of experiment 5. It is possible to distinguish two phases in the extraction process; in the first one, the antiradical capacity of extracts decreases concomitantly with the increase in extraction time (this seems obvious, taking into account the phenolic depletion of solid matrix). When the extraction time was prolonged (beyond 4 h in this case) a stabilisation of this parameter was reached. These phases are typically present in continuous extraction processes. Ferreira, Nikolov, Doraiswamy, Meireles, and Petenate (1999) reported a similar trend in extraction of essential oil from black pepper (*Pipper nigrum* L.).

Table 3 shows the area under the antiradical activity curve as a function of extraction time, which was also calculated by numerical integration. By analogy with the OPC index, this was named as the OAC index. Also, in this case, best results were obtained under the same operational conditions as experiment 5, which was also associated with maximum OPC value. Applying statistical analysis with the OAC values, the following significant model was obtained

Table 1
Extraction conditions of the experimental design

Experiment	Flow rate (ml/min)	Sample amount 2.5 (g)	Particle size (mm)	Flow rate (F)	Sample amount(C)	Particle size (G)
1	3	2.5	0.5	1	−1	−1
2	3	7.5	0.5	1	1	−1
3	3	2.5	5.5	1	−1	1
4	3	7.5	5.5	1	1	1
5	2	2.5	0.5	−1	−1	−1
6	2	7.5	0.5	−1	1	−1
7	2	2.5	5.5	−1	−1	1
8	2	7.5	5.5	−1	1	1
9	2.5	5	3	0	0	
10	2.5	5	3	0	0	
11	2.5	5	3	0	0	0
12	2.5	5	3	0	0	0

Not coded/coded variables.

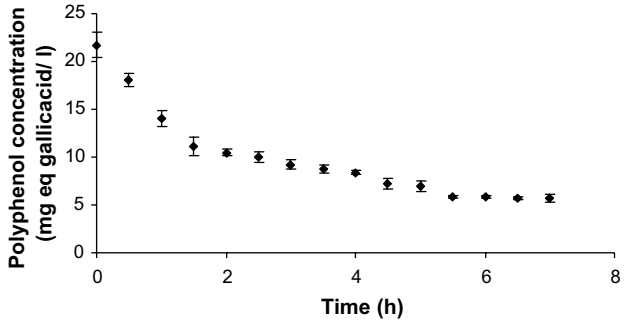

Fig. 3. Polyphenol concentration as a function of extraction time of 2.5 g grape residue with 0.5 mm particle size at 2 ml/min water flow rate (experiment 5, Table 1).

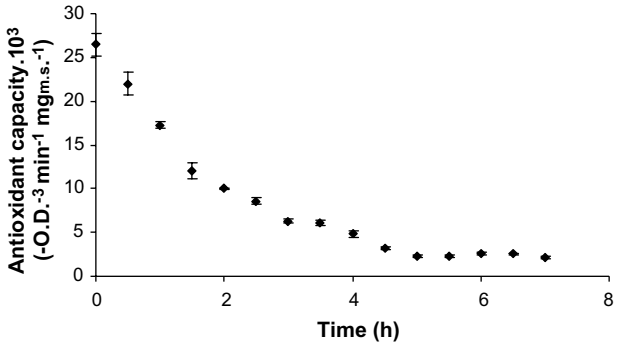

Fig. 4. Antiradical capacity as a function of extraction time of 2.5 g grape residue with 0.5 mm particle size at 2 ml/min water flow rate (experiment 5, Table 1).

$$\mathrm{OAC} \times 10^3 = 3.31 - 4.53F - 3.70C - 5.91G,$$
$$F_{\mathrm{mod}} = 41.28, \quad P < 0.000, \quad R^2 = 0.902$$

In the light of these results, only a proportional effect of each single variable on the overall antiradical capacity was detected. The surface response plots of the overall antiradical capacity, as a function of particle size and sample amount, are shown in Figs. 5 and 6 for extractions carried out with 2 and 3 ml/min flow rates, respectively. As can be observed, the OAC evolution are similar in both cases, being higher at lower flow rate.

The influence of each parameter can easily be explained. In particular, low values of flow rate (2 ml/min) allowed more concentrated extracts to be obtained. According to mass transfer principles, since the driving force of the extraction process is considered to be the solute concentration gradient between the solid and the solvent involved, the effect of low flow during continuous extraction is analogous to that of low solvent-to-solid ratio during batch extraction. In fact, the higher the flow rate used (or solvent-to-solid ratio), the higher the concentration gradient. In this regard, previous findings showed that the percentage of total phenolic compounds (with respect to material weight on dry basis) obtained in aqueous pine sawdust batch extraction was higher when the liquid–solid ratio was reduced from 10:1 to 5:1, keeping constant the other extraction conditions (Pinelo et al., 2004a). Considering Table 2, the highest phenol concentration (and the highest antiradical capacity) was obtained when low sample amounts were extracted (2.5 g). The Folin–Ciocalteu assay showed that increase of the sample amount did not only prevent the expected increase in phenol concentration, but also promoted a decrease in this parameter. This difference can be justified by the occurrence of packaging phenomena during extraction. High solid amounts could beget preferential flow channels and offside zones, promoting a decrease in surface contact between solid and liquid. As a consequence, a decrease in mass transfer was observed. Finally, the highest OPC value was associated with low particle size (0.5 mm). Actually, decrease in particle size promotes an increase of surface contact between solid and extractant. The same effect was observed in phenolic extraction from different juice press residues and grape pomace extracts (Meyer, Jepsen, & Sorensen, 1998; Meyer, 2002).

Moreover, in the OAC response function, no crossed effects between variables were observed, implying no influence among them. This indicates that extracts with

Table 2
Area under the polyphenol concentration curve as a function of extraction time (OPC) for each experiment

Experiment	Flow rate (ml/min)	Sample amount (g)	Particle size (mm)	OPC (mg eq gallic acid l^{-1} h) $\times 10^3$
1	3	2.5	0.5	31.4 ± 1.56
2	3	7.5	0.5	25.4 ± 1.41
3	3	2.5	5.5	28.8 ± 0.63
4	3	7.5	5.5	22.5 ± 1.96
5	**2**	**2.5**	**0.5**	**52.7 ± 3.32**
6	2	7.5	0.5	30.0 ± 0.32
7	2	2.5	5.5	32.0 ± 0.23
8	2	7.5	5.5	24.9 ± 1.32
9	2.5	5	3	33.7 ± 2.30
10	2.5	5	3	32.7 ± 0.25
11	2.5	5	3	35.0 ± 1.21
12	2.5	5	3	35.0 ± 1.33

Maximum OPC values and relevant extraction variables in bold.

Table 3
Area under the antiradical capacity curve as a function of extraction time (OAC) for each experiment

Experiment	Flow rate (ml/min)	Sample amount (g)	Particle size (mm)	OAC ($-OD^{-3}\ min^{-1}\ mg^{-1}\ h) \times 10^{-3}$
1	3	2.5	0.5	29.8 ± 1.23
2	3	7.5	0.5	21.8 ± 0.65
3	3	2.5	5.5	29.3 ± 0.44
4	3	7.5	5.5	20.3 ± 1.11
5	**2**	**2.5**	**0.5**	**55.6 ± 2.66**
6	2	7.5	0.5	35.9 ± 2.14
7	2	2.5	5.5	38.9 ± 1.03
8	2	7.5	5.5	27.0 ± 0.88
9	2.5	5	3	29.5 ± 1.25
10	2.5	5	3	30.5 ± 1.39
11	2.5	5	3	36.2 ± 1.30
12	2.5	5	3	31.0 ± 0.77

Maximum OAC values and relevant extraction variables in bold.

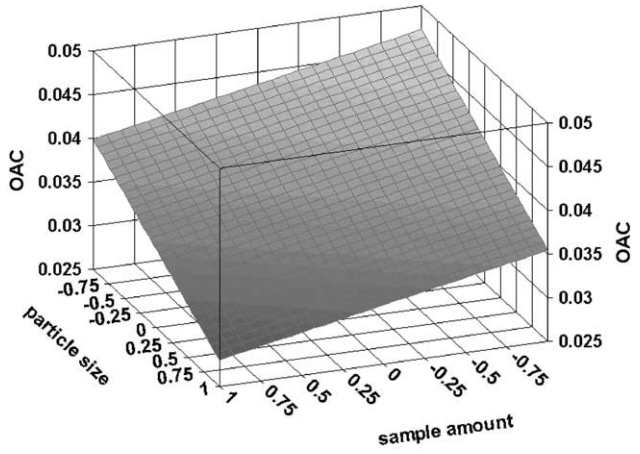

Fig. 5. Response surface plot of antiradical capacity as a function of sample amount and particle size for extraction with 2 ml/min water flow rate.

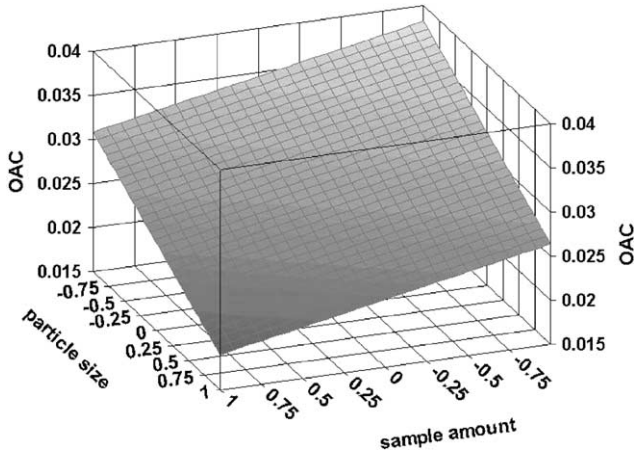

Fig. 6. Response surface plot of antiradical capacity as a function of sample amount and particle size for extraction with 3 ml/min water flow rate.

enhanced antiradical capacity could be obtained by promoting changes of the values of each single variable, irrespective of whether the other one is varied.

Species responsible for the antiradical capacity of extracts were studied in the following chromatographic analysis. Fig. 7 shows the evolution of the phenolic profile of extracts obtained under the optimal conditions of experiment 5. In Fig. 7(a), the chromatogram obtained after 30 min of extraction time is plotted. Non-polymeric compounds, such as gallic acid, catechin, epicatechin and quercetin, were identified before the 60 min of retention time. Catechin was the prevalent phenolic monomer, being 3 times more abundant than the second one found, i.e., gallic acid. This result is in agreement with previous findings of monomeric phenols in grape extracts. Despite their relative proportions depending on grape variety, the presence of catechin, epicatechin, gallic acid and quercetin is usual in these studies (Burns et al., 2000; Shui & Peng-Leong, 2002). It is noteworthy that, after 30 min of extraction time (Fig. 7(a)), no peaks associated with the presence of polymeric species were detected. The phenolic profile of the extract obtained after 90 min of extraction time is plotted in Fig. 7(b). Although in minor quantities, monomeric phenols identified were the same as those observed in Fig. 7(a) (see scale differences). The order of decreasing quantity was catechin > gallic acid > epicatechin > quercetin. In this case, polymeric species were detected about 65 min as a wide peak. In light of the features of the equipment used to carry out extraction (see Section 2), it is important to note that each solvent fraction within the extractor has a different residence time, due to the recirculation and preferential pathways. This indicates that the extracts always contained a mixture of freshly extracted phenols and others remained in the extractor from the outset. So, two explanations could be given for this peculiar 'polymeric' evolution: (1) Lower-molecular weight phenols were possibly more easily extractable during the first stages of the process, whilst

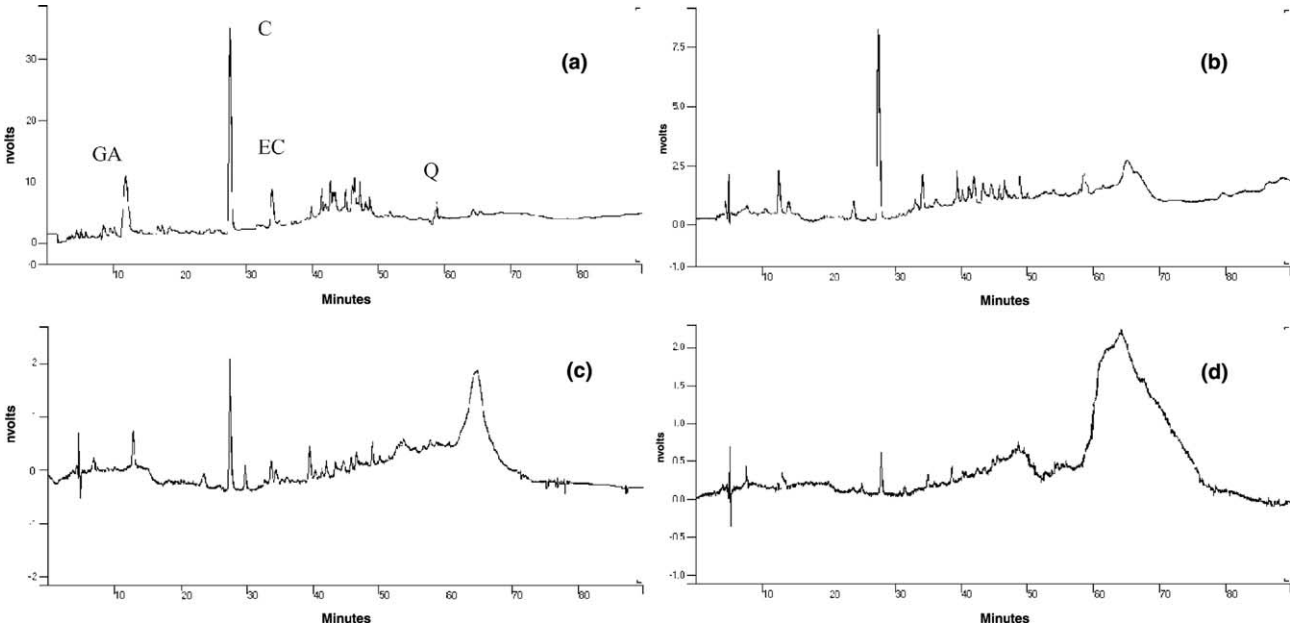

Fig. 7. Chromatograms of extracts of 2.5 g grape residue with 0.5 mm particle size at 2 ml/min water flow rate (experiment 5, Table 1): (a) 30 min extraction time, (b) 90 min extraction time, (c) 3 h extraction time, (d) 6 h extraction time. GA, gallic acid; C, catechin; EP, epicatechin; Q, quercetin.

polymers would be retained in the food matrix until later extraction times. (2) The presence of polymers in the extract could be attributed to polymerization reactions occurring in the extractor bulk. In fact, as previously noted, certain flavonoids posses a strong tendency to form polymers. Catechin and quercetin model solutions were actually reported to easily polymerise during storage (Pinelo et al., 2004b), increasing, as a consequence, the overall antiradical activity of the reacting solution. In fact, Nicoli et al. (2000) have reported an increase (above 50%) of the initial antiradical activity of an aqueous catechin solution during storage at 25 °C. This acute difference in antiradical activity was explained by the formation of polymeric species.

The hypothesis that phenol polymers are formed in the extractor is supported by additional experiments of batch extraction. In particular, extraction of a sample having the properties indicated by experiment 5 (2.5 g of sample amount and 0.5 mm of particle size) was carried out in batch for increasing times. Fig. 8 shows the chromatogram corresponding to the extract obtained after a batch extraction for 3 h. In this case, non-polymeric phenols were scarcely observed, suggesting that most phenolic monomers, despite being present in the food matrix (as shown in Fig. 7) have polymerized. Comparing Fig. 8 with Fig. 7(c) (corresponding to the same extraction conditions but continuous), a higher presence of monomeric phenols was observed when extraction was carried out continuously. In fact, although higher reaction times promoted the formation of polymeric compounds, small amounts of monomeric phenols were also detected as a consequence of their

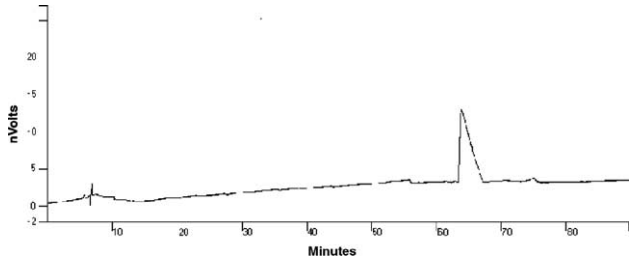

Fig. 8. Chromatogram of 3-h batch extracts of 2.5 g grape residue with 0.5 mm particle size.

fresh extraction from the plant matrix. From the Figures, it seems obvious that polymer formation was favoured with increasing time of extraction. Figs. 7(c) and (d) (corresponding to 3 and 6 h of extraction time, respectively), show a new reduction of the concentration of the phenol monomers, whilst a new increase of polymeric species was observed. It seems obvious that higher reaction times promote the formation of these polymeric compounds. Small amounts of monomeric phenols were also detected, arising by direct extraction. Previous findings showed that oligomers of flavonoids containing upto four monomers possessed a higher antiradical capacity than the original monomers. By contrast, a further enhancement of the degree of polymerisation promotes a decrease in the antiradical capacity of the molecule (Nicoli et al., 2000; Pinelo et al., 2004b). As previously indicated, non-polymeric phenols (monomers and oligomers) are detected before 60 min of retention time, whilst polymeric ones appear as a wide peak after this retention time. Since the latter have a lower

antiradical capacity, it would be interesting to avoid such long extraction times (above 4 h in this case), thus obtaining the phenols with higher antiradical capacity.

4. Conclusions

The residues or wastes from wine-making contain considerable quantities of antiradical phenols. This is of great importance for the winery industry, since the extracts of these byproducts are finding increasing applications as active substances for cosmetic and pharmaceutical compositions.

Industrially, the economical feasibility of the extraction process involves the search for optimum with extraction conditions, in order to maximize process efficiency. In this study, higher phenol contents and antiradical capacities of extracts were obtained when lowering flow rate, sample quantity and particle size.

Although the effects of varying certain variables on the antiradical capacity of extracts might seem obvious, this work reveals that other factors, such as packaging phenomena, saturation effects and operational factors, could have a non-expectable influence on the overall efficiency of the process. Besides, each particular system could involve other different variables that might be more suitable for that specific case.

Extraction conditions not only affect mass transfer phenomena occurring in the bulk of the extractor, but could also favour the formation of species (as a result of different chemical reactions) with a direct effect on the properties of extracts. In the particular case of phenols, the formation of polymers during advanced stages of extraction was observed. For this reason, the choice of extraction conditions should always be preceded by prior studies on reactivity of species subjected to characterization.

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