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Extraction of phenolic compounds from red grape marc for use as food lipid antioxidants

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Abstract

Grape marc resulting from red winemaking was extracted with a mixture of ethyl acetate and water in order to obtain its phenolic compounds with a view to their use as food lipid antioxidants. Crushed and uncrushed marcs were extracted for 5, 10, 20 and 30 min in order to determine the minimum time required for ensuring maximal extraction of phenols. The results reveal a higher extraction of these compounds by the ethyl acetate acting on crushed marc, so the cost of this last operation can be largely compensated. The antioxidant lipid activity of the phenolic compounds of this extract was determined by using the Rancimat method on refined olive oil, and compared with the induction periods of BHT, BHA, propyl gallate and pure standards of those phenols identified in the above mentioned organic extract. In this test, the phenols of the extract show a lower antioxidant activity than the synthetic food antioxidants, although close to that of BHT, mostly as a result of its catechin content. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Winemaking by-products; Phenolic compounds; Food antioxidants

1. Introduction

Diminishing the environmental impact of industrial wastes has been a subject of increasing concern in recent years. Specifically, many food industries have an adverse environmental impact because of the presence in their wastes of residual phenols from the plant raw materials they use. In industrial liquid effluents, these compounds considerably increase biochemical and chemical oxygen demands, with detrimental effects on the flora and fauna of discharge zones. In solid residues for obtaining organic fertilizers, relatively high levels of phenolic compounds are a problem because of their inhibition of germination properties.

However, polyphenols have many favourable effects on human health, such as the inhibition for the oxidization of low-density lipoproteins, thereby decreasing the risk of heart diseases (Frankel, Kanner, German, Parks, & Kinsella, 1993; Frankel, Waterhouse, & Teissedre, 1995; Fuhrmann, Lavy, & Aviram, 1995; Teissedre, Waterhouse, Walzam, German, Frankel, Ebeler, & Clifford, 1996), antiinflammatory activity (Landolfi, Mower, & Steiner, 1984; Moroney, Alcanaz, Forder, Carey, & Hoult, 1988), and anticarcinogenic properties (Bailey & Williams, 1994; Huang & Ferraro, 1991; Huang & Ferraro, 1992; Teissedre et al., 1996). On the other hand, the activity of these compounds as food lipid antioxidants is well known; this has promoted studies of extracts from various plants containing them (Budowski, 1964; Gadow, Joubert, & Harsmann, 1997; Harel & Kanner, 1984; Pratt, 1965; Pratt & Birac, 1979; Pratt & Hudson, 1990; Shahidi & Wanasundara, 1992; Sheabard & Neeman, 1988; Zhao, Li, He, Cheng, & Wenjuran, 1989). Thus, phenolic compounds can be considered to be added-value by-products, justifying their isolation from the industrial waste.

The recovery of phenols from plant tissues has so far been accomplished with various solvents including ethanol, methanol, ethyl ether and ethyl acetate. This last is especially suitable for the extraction and their use in the food industry. Ethyl acetate is an efficient solvent for phenols and, in fact, is customarily used in available analytical methods for their determination (Merida, Moyano, Millán, & Medina, 1991; Ramey, Bertrand, Oough, Singleton, & Sanders, 1986; Salagoity-Auguste & Bertrand, 1984). Also, because of its low polarity, it preferentially extracts those phenols that are readily dissolved in the lipid fraction of the food. Moreover, the

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low boiling point facilitates its removal and reuse. Finally, any possible residue is scarcely toxic since at levels around mg/l this compound is a typical component of fermented drinks.

The purpose of this work was to obtain phenolic compounds from red grape marc with a view to their utilization as lipid antioxidants for foods, and in order to find a use for winemaking wastes.

2. Material and methods

2.1. Samples

Industrial marc (skins and seeds) from red grapes in a 1:1 ratio of the Cabernet Sauvignon and Tempranillo varieties grown in the Montilla-Moriles region (southern Spain) was used. The marc was obtained after 6 days of maceration for the red vinification at 20°C.

For the extraction of the phenolic compounds, an amount of 250 g of grape marc (approximately 50 g dry matter) was treated with 1 l of a mixture of 500 ml ethyl acetate and the water volume required to complete the water content in the marc up to 500 ml. In this way, the ratio between the solvents was always 1:1 (v/v) and that of each to marc dry matter 10:1 (v/w).

The extraction efficiency was examined at four different times: 5, 10, 20 and 30 min, in two different extraction batches consisting of crushed and uncrushed grape marcs, respectively. So, for each time and grape marc treatment, two phases were obtained, ethyl acetate and aqueous phases. All experiments were carried out at 20°C in triplicate.

2.2. Analytical methods

The total phenols content was determined by the absorbance of the organic and aqueous extracts at 280 nm in a Beckman DU600 spectrophotometer with a 10 mm pathlength. The latter extract was adjusted to pH <1 with 1 M HCl prior to measurement in order to have the anthocyanins in the same form. All absorbance values were corrected for the dilutions made.

2.3. Extraction of phenolic compounds

In order to separate the anthocyanins of the other phenols in aqueous extracts, avoiding interferences of the former in the HPLC determinations, the method of Ramey et al. (1986) was used. These extracts were subjected to extraction with ethyl acetate after adjusting the pH to 2 and 7; then the phenols were redissolved in methanol, after evaporation of the solvent, for subsequent chromatographic analysis. Organic extracts were also redissolved in methanol after evaporation of the ethyl acetate.

Anthocyanins were purified in the aqueous extracts by using a SEP-PAK C18 cartridge (Waters Associates) that was preconditioned at pH 7 according to Jaworski and Lee (1987). Samples were passed, and subsequently eluted with 16% CH_3CN at pH 2 (Oszmianski, Ramos, & Bourzeix, 1988). The fraction thus obtained was evaporated to dryness and then dissolved in methanol for its injection in HPLC.

2.4. Identification

The identification of the phenolic compounds was achieved by comparing with the retention times of the standards, UV spectra obtained by HPLC Rapid Scanning Detector (Spectra-Physics mod. Focus) and calculation of UV absorbance ratios after coinjection of samples and standards (Mayen, Merida, & Medina, 1994; Mayen, Merida, & Medina, 1995). Commercial standards were purchased from Sigma-Aldrich Chem. Co. (Madrid, Spain) and Sarsynthese Co. (Genay, France). The standards purity was 95–99%. Each compound was quantified by comparison with a calibration curve obtained with the corresponding standard, except that the procyanidins were quantified as catechin and anthocyanins were quantified as malvidin-3-glucoside.

2.5. HPLC analyses

A 50 μl aliquot of each fraction is injected into a Spectra-Physics SP880 high performance liquid chromatograph, furnished with a C_{18} column (220×4.6 mm, 5 μm particle size). The chromatographic conditions used were as follows:

2.5.1. Phenolic acids

Flow-rate, 2 ml/min; variable UV-V detector with a working wavelength of 280 nm; mobile phases, 2% aqueous acetic acid and acetonitrile. The elution program involved a gradient from 0.1 to 5% CH_3CN in 5 min, isocratic elution for 10 min, up to 15% CH_3CN in 5 min, isocratic elution for 10 min, up to 100% CH_3CN in 10 min, isocratic elution for 10 min.

2.5.2. Flavan-3-ol derivatives

Flow-rate, 2 ml/min; variable UV-V detector with a working wavelength of 280 nm; mobile phases, 2% aqueous acetic acid and acetonitrile. The elution program involved gradient elution from 0.1 to 15% CH_3CN in 15 min, isocratic elution for 5 min, up to 20% CH_3CN in 5 min, up to 30% CH_3CN in 5 min, up to 100% CH_3CN in 10 min, isocratic elution for 10 min.

2.5.3. Flavonols

Flow-rate, 1.5 ml/min; variable UV-V detector with a working wavelength of 365 nm; mobile phases, 2% aqueous acetic acid and acetonitrile. The elution program

involved gradient elution from 0.1 to 15% CH_3CN in 15 min, isocratic elution for 5 min, up to 20% CH_3CN in 5 min, up to 30% CH_3CN in 5 min, up to 40% CH_3CN in 10 min, up to 100% CH_3CN in 10 min, isocratic elution for 10 min.

2.5.4. Anthocyanins

Flow-rate, 1 ml/min; variable UV-V detector with a wavelength working of 520 nm; mobile phases, 10% aqueous formic acid and acetonitrile. The elution program involved gradient elution from 5 to 9% CH_3CN in 5 min, up to 11% CH_3CN in 10 min, up to 15% CH_3CN in 25 min, up to 20% CH_3CN in 10 min, up to 30% CH_3CN in 15 min and, finally, up to 40% CH_3CN in 5 min.

2.6. Determination of antioxidant activity

A Rancimat 679 instrument from Metrohm AG (Herisau, Switzerland) was used to determine the antioxidant lipid activity of the phenols contained in the ethyl acetate extract of crushed red grape marc. The air flow-rate and temperature used were 15 l/h and 100°C, respectively. Oxidations were conducted on refined olive oil in the presence and absence (Test) of a concentration of 100 mg of phenolic compounds (from the extract) per kilogram of oil. This concentration was also used to determine the induction periods of BHT, BHA and propyl gallate, as well as of the pure standards of the following compounds present in the extract: gallic acid, protocatechuic acid, vanillic acid, catechin, epicatechin, quercetin-3-galactoside, quercetin-3-glucoside, kampherol-3-glucoside, isorhamnetin-3-glucoside, quercetin and kampherol.

2.7. Statistical procedures

Student's *t*-tests were performed on the replicated samples by using Statgraphics Statistical Computer Package (Statistical Graphics Corp.).

3. Results and discussion

Figs. 1 and 2 show the total phenols content, measured as absorbance at 280 nm, for the red grape marc extracts in water and ethyl acetate at the four extraction times assayed. As a result of the increased contact surface, crushing the grape marc favoured the extractions with both solvents at all times, particularly increasing the absorbances for the ethyl acetate phase. Taking into account that the phenol contents extracted by this solvent were dramatically higher as a result of crushing the marc, the cost of this operation can be assumed.

It should be noted that, because of polarity differences between solvents, the two resulting phases at all extraction times need not be of the same qualitative or quantitative composition. As is well known, the anthocyanins are not soluble in ethyl acetate, allowing the separation of colorants (in the aqueous phase) from other phenols (organic phase). These latter are more soluble in edible fat on account of their low polarities and are without problems of colour alteration of the foods.

In order to minimize the extraction period while ensuring a maximal concentration of phenols, Student's *t*-tests were performed between consecutive times for each extract. Ethyl acetate extracts of uncrushed marc exhibited significant differences ($p < 0.05$) from 0 to 5 min but none beyond the latter point. An extraction time of 5 min was thus adopted as optimum. Similarly, the better times for the other experiments were found to be 10 min for the organic extracts of crushed marc, and 20 and 5 min for the aqueous extracts of crushed and uncrushed marc respectively (see Figs. 1 and 2). At these optimum times, both phases exhibited significant differences ($p < 0.001$) in absorbance between their respective treatments with crushed and uncrushed grape marc. Taking into account that, in practice, the extractions

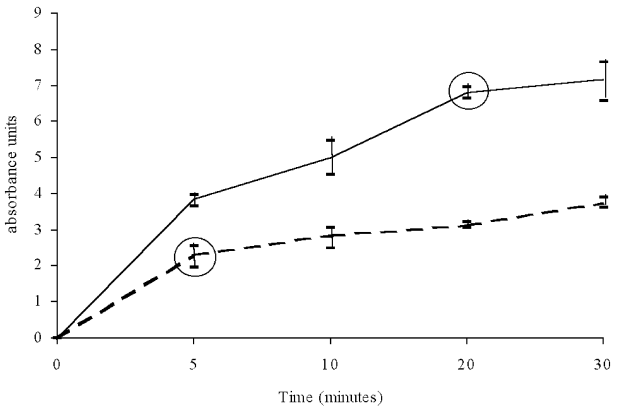

Fig. 1. Absorbance at 280 nm of the aqueous extracts from red grape marc. Circles indicate optimum times of extraction (— crushed grape marc; - - - uncrushed grape marc).

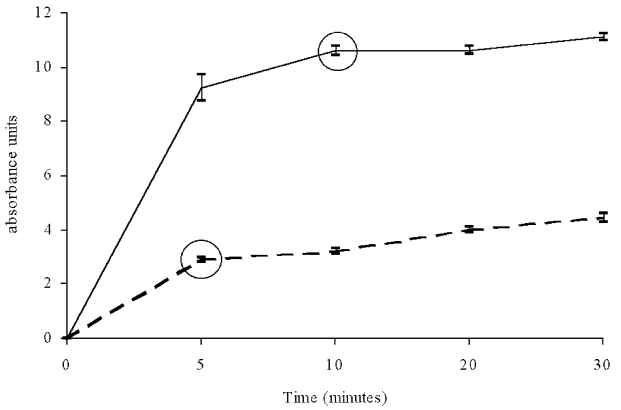

Fig. 2. Absorbance at 280 nm of the ethyl acetate extracts from red grape marc. Circles indicate optimum times of extraction (— crushed grape marc; - - - uncrushed grape marc).

with a mixture of two solvents only can be carried out at the same time, an extraction time of 5 min was chosen for the uncrushed grape marc treatment and 20 min for the crushed treatment (where the aqueous phase showed a better efficiency measured in terms of absorbances).

Table 1 lists the phenolic compound contents, quantified by HPLC, in the four extracts studied at the two extraction times mentioned above. As expected, the extracts obtained from crushed grape marc showed higher contents in phenolic compounds than those from uncrushed grape marc for each solvent. On the other hand the phenolic acid fractions and the quercetin-3-galactoside were only extracted by ethyl acetate, while flavan-3-ol dimers (procyanidins) and anthocyanins were only in the aqueous phase. The remaining compounds, flavonols and flavan-3-ol monomers (catechin and epicatechin) partitioned between both phases.

Fig. 3 shows the percentage of each compound extracted by the organic and aqueous phases in relation to the overall amount extracted by both for the crushed grape marc treatment. It also shows the significant differences between the means for each compound in the two phases, calculated by Student's *t*-tests. Flavan-3-ol monomers were largely recovered in the ethyl acetate phase, exceeding 88% of the overall extracted by both solvents, and with significant differences at $p < 0.001$ in relation to the aqueous phase. Catechin was the compound present at the highest concentration in the organic phase (Table 1). All the flavonols studied were also extracted preferentially in this phase (also at $p < 0.001$), where they occurred in proportions exceeding 78%.

The aqueous phase was found to contain rather small amounts of phenols (Table 1), so their antioxidant lipid activity must reasonably be very low. In addition, it should be borne in mind that these phenolic compounds would be scarcely soluble in edible fat on account of their higher polarities. On the other hand, the aqueous phase contained anthocyanins, which are red-coloured compounds; this is a severe constraint to their use as food additives since they can undesirably alter the colour of some types of food. Besides, the cost required to remove anthocyanins from the aqueous extract is unjustified because of the above-mentioned low content in phenols.

Taking into account that crushing the marc facilitated extraction of the phenols and that their total concentrations in the ethyl acetate phase were much higher than those in the aqueous phase (112 vs 10.7 mg/l), the organic phase obtained from crushed grape marc was used to perform the fat rancidity test by the Rancimat method. Although this technique has been questioned by some authors (Frankel, 1993), it is preferred in this work because it is a common procedure in the food industry and governmental analytical laboratories.

Fig. 4 shows the induction periods of refined olive oil for the phenols contained in the above-mentioned extract. It also shows the induction periods for BHT, BHA, propyl gallate and pure standards of the phenols present in the extract. For the phenolic acids assayed,

Table 1
Contents in phenolic compounds (mg/l) in the ethyl acetate and aqueous phases extracted from the crushed and uncrushed grape marc

	Uncrushed marc		Crushed marc	
	Ethyl acetate	Water	Ethyl Acetate	Water
Gallic acid	0.853 ± 0.185	< 0.001	1.23 ± 0.121	< 0.001
Protocatechuic acid	0.224 ± 0.067	< 0.001	0.472 ± 0.027	< 0.001
Vanillic acid	4.87 ± 0.691	< 0.001	6.25 ± 1.59	< 0.001
Catechin	37.5 ± 4.57	0.439 ± 0.087	50.2 ± 2.50	0.660 ± 0.139
Epicatechin	4.12 ± 0.307	0.512 ± 0.103	7.95 ± 0.944	1.01 ± 0.109
Procyanidin B1	< 0.001	0.177 ± 0.010	< 0.001	0.168 ± 0.010
Procyanidin B2	< 0.001	0.350 ± 0.089	< 0.001	0.359 ± 0.037
Procyanidin B3	< 0.001	0.343 ± 0.047	< 0.001	0.536 ± 0.159
Procyanidin B4	< 0.001	0.494 ± 0.084	< 0.001	0.710 ± 0.179
Quercetin-3-galactoside	7.71 ± 1.41	< 0.001	10.1 ± 1.22	< 0.001
Quercetin-3-glucoside	8.97 ± 1.44	3.21 ± 0.291	13.7 ± 2.92	3.85 ± 0.413
Kampherol-3-glucoside	5.37 ± 0.906	0.824 ± 0.023	8.18 ± 0.333	1.12 ± 0.170
Isorhamnetin-3-glucoside	3.63 ± 0.565	1.58 ± 0.177	6.82 ± 0.944	1.35 ± 0.177
Quercetin	0.370 ± 0.058	0.044 ± 0.004	1.49 ± 0.117	0.062 ± 0.002
Kampherol	2.39 ± 0.578	0.048 ± 0.003	5.49 ± 0.439	0.059 ± 0.006
Delphinidin-3-glucoside	< 0.001	0.014 ± 0.002	< 0.001	0.047 ± 0.014
Cyanidin-3-glucoside	< 0.001	0.017 ± 0.000	< 0.001	0.018 ± 0.001
Petunidin-3-glucoside	< 0.001	0.012 ± 0.000	< 0.001	0.009 ± 0.000
Peonidin-3-glucoside	< 0.001	0.035 ± 0.006	< 0.001	0.055 ± 0.001
Malvidin-3-glucoside	< 0.001	0.272 ± 0.009	< 0.001	0.544 ± 0.007
Peonidin-3-(6-acetyl)-glucoside	< 0.001	0.033 ± 0.002	< 0.001	0.073 ± 0.001
Malvidin-3-(6-acetyl)-glucoside	< 0.001	0.009 ± 0.000	< 0.001	0.029 ± 0.003

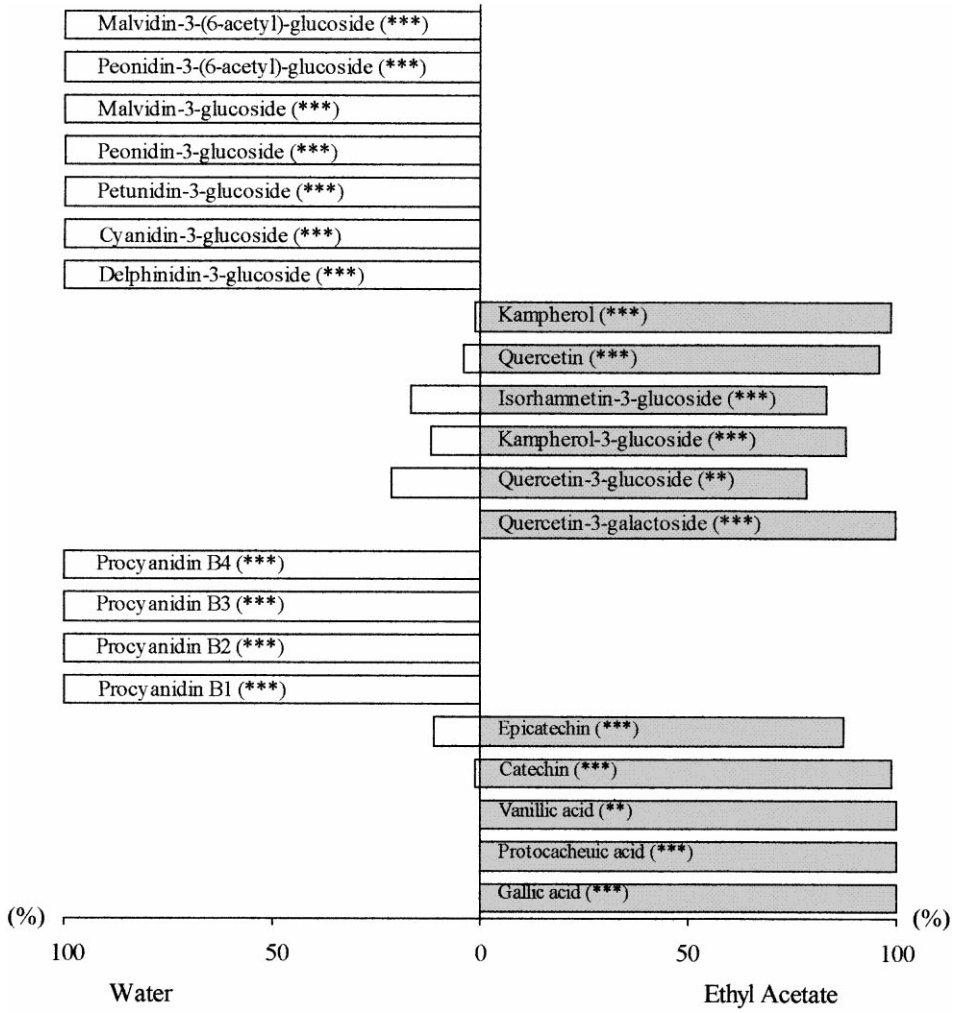

Fig. 3. Percent distribution of phenolic compounds in the ethyl acetate and aqueous phases for the crushed grape marc treatment (significant differences for each compound in the two phases are shown *** $p < 0.001$; ** $p < 0.01$).

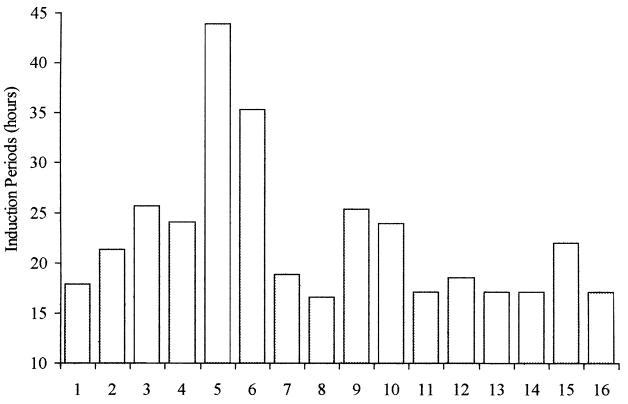

Fig. 4. Induction periods measured on refined olive oil for the Test [1], the phenols of the ethyl acetate phase obtained from crushed marc [2], the synthetic antioxidants BHA [3], BHT [4] and propyl gallate [5], and the pure standards of gallic acid [6], protocatechuic acid [7], vanillic acid [8], catechin [9], epicatechin [10], quercetin-3-galactoside [11], quercetin-3-glucoside, [12], kampherol-3-glucoside [13], isorhamnetin-3-glucoside [14], quercetin [15], kampherol [16].

gallic acid exhibited the highest antioxidant activity, followed by protocatechuic and vanillic acids, this last being very scarcely active. This sequence is consistent with the results of Marinova and Yanishlieva (1992). In the flavonoid fraction, catechin was the most effective antioxidant, followed by epicatechin and quercetin and, in a lower degree, glycosyl flavonols and kampherol. Nevertheless, the above comments need a further explanation. All the phenols were tested at the same concentration by weight (100 mg/kg oil), which is the legal and commercial limit in European law for BHT; however, the chemical activities are dependent on their molar concentrations. Thus, the molar concentrations of catechin and quercetin tested were 0.345 and 0.331 mol/kg, respectively, the induction period being longer for the former, consistent with previous results of Gadow et al. (1997), who found the same activity sequence for equivalent weights of the two compounds in lard. By contrast, Hudson and Lewis (1983) arrived at the reverse sequence in terms of induction period for the two flavonoids in equivalent molar concentrations, also in lard.

As can be seen, the phenols of the extract exhibited a longer induction time than the oil sample containing no additives (Test). This antioxidant activity can mainly be ascribed to the catechin content in the extract since the gallic acid standard exhibited a much longer induction period but this compound was present at a much lower concentration. On the other hand, the induction period for the phenols of the extract was shorter than those for BHT, BHA and propyl gallate, so the mixture of natural phenols is less efficient than these synthetic additives.

Nevertheless, from the above comments, the use of natural phenols from red grape marc as food additives, can be advantageous. It is well known that maximum lawful levels for synthetic food additives are established from various toxicological parameters that need not be applicable to naturally-occurring compounds. Thus, in drinks such as white wine, the overall content of compounds such as those determined in the extract is between 200 and 300 mg/l, and in red wine it is over 1 g/l. In both cases it is considered to have beneficial effects on human health (Kanner, Frankel, Granit, German, & Kinsella, 1994; Frankel et al., 1995) and is in no way toxic. Likewise, many fruits and juices of widespread consumption also have similar or higher polyphenol concentrations and have never been banned for this reason. So the dried phenols from the extract could be used at higher levels than the synthetic phenols, thereby increasing their antioxidant effectiveness. On the other hand, the amounts of fat antioxidants added to foods do not always reach the maximum allowed levels; as a result, the antioxidant power of the phenols of the extract may in practice be similar to that of BHT used at a slightly lower concentration.

Economically, and toxically, the proposed method for extracting phenols from red grape marc with a view to their use as antioxidants for edible fat, is advantageous. Production costs are quite low since the boiling point of ethyl acetate facilitates its evaporation for recycling and ensures the absence of residual solvent in the product. In any case, solvent residues amounting to a few milligrams per litre should pose no problem since ethyl acetate is a typical component of fermented drinks, where it is frequently found at concentrations up to 100 mg/l.

Ethyl acetate extracts of crushed red grape marc contain only modest amounts of phenols, with an overall concentration a little over 1 g/kg dry matter. This level is much lower than that reported by Joubert (1996) for aspalathin in rooibos tea (15 g/kg dry matter), although close to the levels cited by Macheix, Fleuriet, and Billot (1990) for some fruits and plant tissues. It is well known that, in red winemaking, the must remains in contact with the marc for several days in order to extract the anthocyanins that give the wine its typical colour. As a result, the marc is partially depleted in phenolic compounds. However, taking into account that red grape is used in millions of tons by the countries producing red wines, the phenolic compounds extracted could be up to tens of thousands of kilograms, which could be used as natural antioxidants for hundreds of thousands of tons of foods, which needs further research. In this way an additional use of winemaking waste can be achieved, and might be compatible with other usage such as animal feed and organic fertilizers.

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