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On the extraction and antioxidant activity of phenolic compounds from winery wastes

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Abstract

Winery waste (from red winemaking, variety Agiorgitiko) was extracted under various conditions using different solvents. The minimum time required for ensuring maximum extraction of phenols was 180 min at a solvent to sample ratio 9:1 v/w and at pH 1.5. The antioxidant activity of solvent extracts was investigated by DPPH radical scavenging method, by determination of peroxide value on virgin olive oil and by the Rancimat method on sunflower oil. Ethanol extract exhibited the highest antioxidant activity compared to the other solvent extracts, to synthetic food antioxidants BHT, ascorbyl palmitate and to the natural food antioxidant, vitamin E. No correlation was found between antioxidant activity and total phenol content. HPLC analysis of the extracts showed that gallic acid, catechin and epicatechin were the major phenolic compounds in winery waste. Hydroxytyrosol, tyrosol, cyanidin glycosides and various phenolic acids such as caffeic, syringic, vanillic, *p*-coumaric and *o*-coumaric acids were also identified.

Keywords: Grape waste; Extraction time; Solvent; pH; Antioxidants; Phenolics

1. Introduction

The grape is one of the major fruit crops worldwide and its harvest is about 60 millions tonnes per year (Schieber, Stintzing, & Carle, 2001). About 80% of the harvest is utilized for winemaking and the grape waste consists the 20% of the weight of processed grapes (Mazza & Miniati, 1993). Phenolic compounds of grapes are responsible for some of the major organoleptic properties of wines, in particular colour and astringency, as well as flavour and body (Minussi et al., 2003; Perez-Magarino & Gonzalez-San Jose, 2006). The phenolic composition of wines depends on the variety of grapes and on the vinification conditions (Cheynier, Hidalgo Arellano, Souquet, & Moutounet, 1997; Gonzalez-Neves et al., 2004; Perez-Magarino & Gonzalez-San Jose, 2006). However, winemaking leads to the generation of large quantities of wastes (around 5–9 million tonnes per year, worlwide), which considerably increase the chemical oxygen demand (COD) and the biochemical oxygen demand (BOD_5) due to a high pollution load (high content of organic substances such as sugars, tannins, polyphenols, polyalcohols, pectins and lipids) with detrimental effects on the flora and fauna of discharged zones (Bonilla, Mayen, Merida, & Medina, 1999; Louli, Ragoussis, & Magoulas, 2004; Schieber et al., 2001). Therefore, treatment and disposal of winery waste is a serious environmental problem and winery waste must find another use other than as animal feed or as fertilizers.

A drastic change in consumer demand has occured recently; naturally processed, additive-free and safe products are requested (Bianco & Uccella, 2000). Consumers prefer safe, more palatable and traditional products, which are accepted as natural without other additives (Bianco & Uccella, 2000). So, the substitution of currently used synthetic food antioxidants (many of them are suspected for carcinogenesis) by natural ones interests the food technologist. Grape wastes can be used for the extraction of poly-

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phenols with a view to their use in foods (Lapornik, Prosek, & Wondra, 2005; Yilmaz & Toledo, 2005). Phenolic compounds, which are plant secondary metabolites and the main antioxidant compounds in grapes and grape products, can be divided into two groups: (i) phenolic acids and related compounds and (ii) flavonoids. There is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidants (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). Furthermore, there is a number of different mechanisms by which phenolics may act as antioxidants: via free radical scavenging, hydrogen donation, singlet oxygen quenching, metal-ion chelation or as substrates for attack by superoxide (Hamilton, Kalu, Prisk, Padley, & Pierce, 1997; Robak & Gryglewski, 1988). Phenolic antioxidants function primarily as terminators of the free radical reactions, depending on their activity with respect to the ability to interfere with the chain propagation reactions by rapid donation of a hydrogen atom to lipid radicals. Alternative mechanisms only become important at very low oxygen pressures, very low rates of chain initiation or very high concentrations of antioxidant (Robards et al., 1999). The major phenolic compounds in grape wastes are anthocyanins, catechins, glycosides of flavonols and phenolic acids. So, grape wastes can be used for extraction of polyphenols for use as food lipid antioxidants in order to prevent the formation of off-flavour and toxic compounds resulting from lipid oxidation. Polyphenols not only show antioxidant activity but other properties such as anticancer, antiallergic, antimutagenic and antiageing activity (Frankel, Waterhouse, & Teissedre, 1995; Jayaprakasha, Singh, & Sakariah, 2001; Jayaprakasha, Selvi, & Sakariah, 2003; Teissedre, Frankel, Waterhouse, Peleg, & German, 1996; Waterhouse, 1994).

The aims of this work were to investigate the effect of extraction time, extracting solvent type and concentration and extraction pH on the phenol content of winery waste extracts and to evaluate the antioxidant activity of winery waste extracts in order to develop an effective procedure for the recovery of phenolic compounds from winery wastes with a view to their utilization as lipid antioxidants for foods.

2. Materials and methods

2.1. Samples

Winery waste (grape skin and seeds) from red winemaking, variety Agiorgitiko, was provided by a winery, located in Nemea (Peloponese), in October of 2004. The samples were promptly analysed for pH, moisture, total solids and ash using the APHA, AWWA, WPCF (1985) methods. Fat was determined by a modification of HACH, ASTM and IRSA procedures (APHA–AWWA–WPCF, 1989). Ten grams of winery waste sample was acidified with H_2SO_4 to pH < 2. After the addition of 1–2 g NaCl, the sample was extracted four times with 20 ml petroleum ether for 15 min each time. Then, centrifugation took place for 2 min at 3000 rpm. Petroleum ether of combined extracts was evaporated in a water bath (50–60 °C) and finally, drying in an oven at 55 °C for 1 h took place. The extracted fat was determined gravimetrically. Reducing sugars were determined according to the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956), using glucose and arabinose as standards. Then, the samples were dried at 60 ± 0.5 °C in an air-circulating tray dryer (Apex SSE17M, London, England), ground to fine powder in a Brabender grinder and stored at −20 °C for further experimentation.

2.2. Reagents and standards

Methanol, ethanol, acetone, isopropanol and ethyl acetate used were analytical reagent grade and purchased from Merck (Darmstadt, Germany). Acetonitrile, acetic acid and water were HPLC grade and purchased from Merck (Darmstadt, Germany). Folin–Ciocalteau phenol reagent and free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH) were purchased from Sigma Chemical Co. (Sigma–Aldrich Company Ltd., Great Britain). Phenolic standards were of purity of 98–99% and purchased from Sigma Chemical Co. (Sigma–Aldrich Company Ltd., Great Britain).

2.3. Extraction of phenolic compounds

The extraction of phenolic antioxidants was performed using methanol, ethanol, mixture of ethanol to water 1:1, acetone, isopropanol and ethyl acetate as solvents and under various conditions of pH, solvent to sample ratio and extraction period, at ambient temperature. Grape waste was acidified with HCl (pH from 1.5 to 3.6) and extracted for 1.5 h with *n*-hexane at a ratio of 10:1 (v/w) by three-step extraction, in an orbital shaker (Orbital Shaker SO1, Stuart Scientific, UK), at ambient temperature for fat removal. The extract was filtrated using GF/F filter paper Buchner funnel and the filtrate, which contained the lipids, was removed. The residue was reextracted continuously or by steps with different extracting solvents (methanol, ethanol, mixture of ethanol:water 1:1, isopropanol and ethyl acetate), at different proportions of solvent volume to sample mass (from 3:1 to 12:1 v/w), for different extraction times (from 30 min to 24 h) in the orbital shaker, at ambient temperature. The new extract was filtrated using GF/F filter paper Buchner funnel and the filtrate was obtained. The combined filtrates were evaporated to dryness in a rotary evaporator (Ika-Werke RV06-ML, Germany) and the residue redissolved in methanol and kept at −20 °C until subsequent analyses.

2.4. Phenol content determination

The total phenol content of winery waste and winery waste extracts was determined colorimetrically at 725 nm,

using the Folin–Ciocalteau reagent according to a modification of the Gutfinger (1981) method. Methanolic solution of grape waste extract (0.1–0.3 ml), 20 ml deionized water and 0.625 ml of Folin–Ciocalteau reagent were added to a 25 ml volumetric flask. After 3 min, 2.5 ml of saturated solution of Na_2CO_3 (35%) were added. The content was mixed and diluted to volume with deionized water. After 1 h, the absorbance of the sample was measured at 725 nm against a blank by using a double-beam ultraviolet–visible spectrophotometer Hitachi U-3210 (Hitachi, Ltd., Tokyo, Japan). Gallic acid served as the standard for preparing the calibration curve ranging from 60 to 140 μg/25 ml assay solution.

2.5. HPLC analysis

Identification of the phenolic compounds of extracts was performed by HPLC analyses according to a modification of the McDonald et al. method (2001), using a Waters system (Waters Chromatography Division, Massachusetts, MA 01757, USA) equipped with a variable UV/VIS detector set at 280 nm. Separations were achieved on a Shimadzu Pathfinder® AS silica 100, 5.0 μm RP column (150 × 4.6 mm, id 5 μm). The flow rate was 1.0 μl/min. The mobile phase used was 0.01% acetic acid in water (A) versus methanol:acetonitrile:acetic acid (95:5:1 v/v/v) (B) for a total running time of 80 min and the gradient changed as follows: solvent B started at 5% for 2 min, then increased to 25% in 8 min, to 40% in 10 min, to 50% in 10 min, to 100% in 10 min, held for 22 min and returned to initial conditions over 18 min. The data were processed by a Waters Baseline 815 program and the qualification was performed by external standard calibration.

2.6. Antioxidant activity

2.6.1. DPPH radical method

The antioxidant activity of phenol extracts was evaluated by using the stable 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH) according to a modification of the method of Bandoniene, Murkovic, Pfannhauser, Venskutonis, and Gruzdiene (2002). Methanolic solution of phenol extracts (0.1 ml) and 3.9 ml methanolic solution of DPPH (0.0025 g/100 ml CH_3OH) were added in a cuvette and the absorbance at 515 nm (till stabilization) was measured against methanol by using a double-beam ultraviolet–visible spectrophotometer Hitachi U-3210 (Hitachi, Ltd., Tokyo, Japan). Simultaneously, the absorbance at 515 nm of the blank sample (0.1 ml methanol + 3.9 ml methanolic solution of DPPH) against methanol was measured. The radical scavenging activities of the tested samples, expressed as percentage inhibition of DPPH, were calculated according to the following formula proposed by Yen and Duh (1994):

$$\% \text{ Inhibition} = 100 \times (A - A_0)/A_0$$

where A_0 is the absorbance at 515 nm of the blank sample at time $t = 0$ min and A is the final absorbance of the test sample at 515 nm.

2.6.2. Determination of peroxide value

The ethanol, methanol and acetone extracts were added at different concentrations (50, 100 and 150 ppm) to commercial virgin olive oil. Then, all the samples were put in an oven at 85 °C where thermal oxidation took place. Every 24 h, for a period of four days, the samples were analyzed for peroxide value in order to monitor the oxidation process. The peroxide value was determined according to the EEC method (EEC Regulation No. 2568/91, L-248/05-09-1991). In a stoppered conical flask, 2.000 g of sample were weighed and 10 ml chloroform, 15 ml acetic acid and 1 ml potassium iodide 10% were added. The flask was shaken for 1 min and left in the dark for 5 min. Then, 75 ml of deionized water were added and titration took place with solution of sodium thiosulfate 0.01 N and 1% starch solution as index. Simultaneously, a blank run was carried out. The peroxide value expressed as mmoles of active oxygen per kg of sample was calculated by the following formula:

$$PV \text{ (mmoles/kg)} = [(V - V_0) \times T \times 1000]/m$$

where V is the volume (ml) of sodium thiosulfate solution for the sample, V_0 is the volume (ml) of sodium thiosulfate solution for the blank, T is the normality of sodium thiosulfate solution and, m is the sample weight (g).

2.6.3. Rancimat method

Ethanol extracts of winery waste were freeze-dried (Virtis 5 L, USA) and the freeze-dried extracts were added into commercial sunflower oil without any added antioxidant at concentrations ranging from 40 to 240 ppm. The antioxidant potential of these extracts was investigated and compared to the antioxidant potential of samples of commercial sunflower oil containing synthetic (BHT, ascorbyl palmitate) and natural (vitamin E) antioxidants. The measurements were performed in a Rancimat 679 Instrument (Metrohm, Switzerland) with air flow-rate and temperature set at 20 l/h and 100 °C, respectively.

3. Results and discussion

3.1. Winery waste analysis

Chemical characterization of winery waste was a prior necessity in order to evaluate its potential, to determine the extraction yield and to be controlled qualitatively. In Table 1, data on chemical analysis of winery waste and especially, the total phenol content of winery waste extracts are shown.

HPLC analysis of extracts of winery waste showed a complex mixture of phenolics that was difficult to resolve. Several phenolic compounds, which are representative of the diverse structural types, were identified. Retention data

Table 1
Characterization and total phenol content of winery waste

Parameters	Value ± SD
pH	3.6 ± 0.2
Moisture (% w/w)	73.6 ± 2.6
Total solids (% w/w)	26.4 ± 1.8
Ash (% w/w)	4.6 ± 0.5
Fat (% w/w)	6.3 ± 0.8
Reducing sugars (% w/w)	1.5 ± 0.3
Total phenols (% w/w)[a]	
Ethanol:water 1:1 extract[b]	2.89 ± 0.27
Methanol extract[b]	2.77 ± 0.23
Ethanol extract[b]	1.93 ± 0.21
Acetone extract[b]	1.57 ± 0.23
Ethyl acetate extract[b]	0.17 ± 0.06
Isopropanol extract[b]	0.12 ± 0.02

[a] Total phenols dry weight, expressed as gallic acid equivalents.
[b] 3 h extraction.

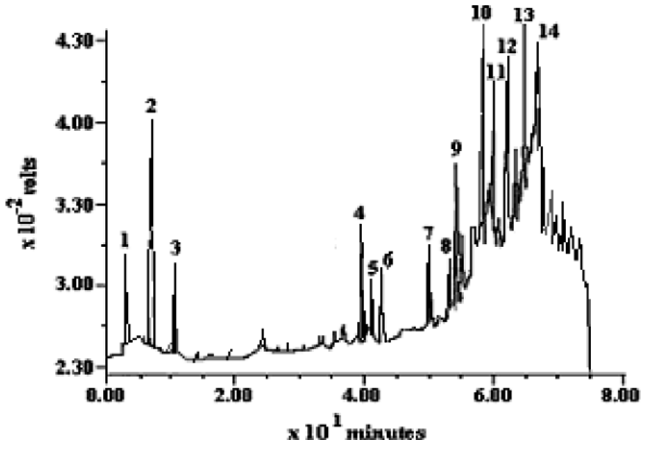

Fig. 1. Phenolics of ethanol winery waste extract by high performance liquid chromatography. Peak identities: 1, hydroxytyrosol; 2, gallic acid; 3, tyrosol; 4, protocatechinic acid; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, *o*-coumaric acid; 9, *p*-coumaric acid; 10, catechin; 11, epicatechin; 12-14, cyanidin glycosides.

are given in Table 2. Profiles of phenolic compounds, recovered from winery waste, were dominated by gallic acid, catechin and epicatechin peaks. In addition hydroxytyrosol, tyrosol, cyanidin glycosides and various phenolic acids such as caffeic, procatechinic, syringic, vanillic, *o*-coumaric, *p*-coumaric were also identified (Fig. 1). Moreover, the different extraction systems modified the phenolic composition quantitatively and not qualitatively.

3.2. Effect of extraction variables

Drying of winery waste before extraction was performed at a temperature of 60 °C, as increasing the temperature above 60 °C significantly reduced the yield of extracted phenols. The total phenol content was reduced by 10.3% and 15.7% at 80 °C and at 100 °C, respectively. Larrauri, Ruperez, and Saura-Calixto (1997) found a significant reduction in extractable polyphenols when red grape pomace peels were dried with air at 100 °C or higher. It was reported that drying at 100 °C caused a reduction of 18.6% and at 140 °C of 32.6% in the total extracted polyphenols.

Winery waste was ground before extraction in order to reduce particle size and increase the yield of extracted phenols and their antioxidant activity. Bonilla et al. (1999) reported that reduction in particle size of grape marc favoured solvent extraction of polyphenols and both mechanical crushing and enzyme demolition. Particle size reduction increased the antioxidant activity as a result of both increased extractability and enhanced enzymatic degradation of polysaccharides (Moure et al., 2001).

Extraction with a mixture of ethanol to water 1:1 lead to maximum phenol content, while isopropanol gave the lowest phenol content (Fig. 2 and Table 1). Methanol gave extracts with lower phenol content than those of a mixture of ethanol to water 1:1 (Fig. 2 and Table 1). Furthermore, relatively high phenol content was obtained using ethanol and acetone while ethyl acetate gave similar yields to isopropanol (Fig. 2 and Table 1). Comparing to extraction with a mixture of ethanol to water 1:1, yields of 95.9% and 66.8% were attained by the extraction of phenolic compounds with methanol and ethanol, respectively. Extraction with acetone gave a yield of 54.3%. However, very low phenolic yields were achieved by using ethyl acetate (5.9%) and isopropanol (4.2%) as extracting solvents. Eth-

Table 2
Retention times of phenolic compounds

Phenolic compounds	Retention time (min)
Hydroxytyrosol	3.5
Gallic acid	7.0
Tyrosol	10.5
Protocatechinic acid	40.0
Vanillic acid	41.5
Caffeic acid	43.0
Syringic acid	50.0
o-Coumaric acid	53.0
p-Coumaric acid	54.5
Catechin	60.8
Epicatechin	61.5
Cyanidin glycosides	63.5–70.0

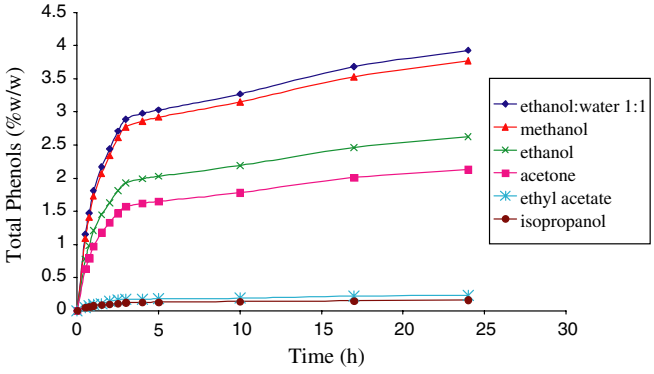

Fig. 2. Effect of solvent and extraction period on the quantity of extracted phenols from winery waste.

anol was selected as the most appropriate solvent for the extraction of phenolic compounds from winery waste for production of extracts with high phenol content and high antioxidant activity (Fig. 2 and Tables 1, 3 and 4). Jayaprakasha et al. (2001) reported that acetone or methanol alone did not give the optimum level of antioxidants in the extracts from *vinifera* grape seeds. Yilmaz and Toledo (2005) found that aqueous mixtures of either methanol, ethanol or acetone were better than a mono-component solvent for the extraction of phenolics from Muscadine seeds, a red *rotundifolia* species of *Vitis* and Lapornik et al. (2005) ascribed higher values of total polyphenols in ethanol and methanol extracts (70%) versus water extracts. Therefore, the above-mentioned results are in agreement with reported findings. Bonilla et al. (1999) reported selective extraction of flavan-3ol monomers, catechin and flavonols from grape marc, preferentially in the organic phase, whereas procyanidins were extracted in the aqueous phase. Increase of the extraction time resulted to an increase of the amount of extracted phenols (Fig. 2). From Fig. 2, it is evident that for all solvents an extraction time of 3 h was sufficient to obtain maximum phenol content. Further increase of the extraction period rendered the extraction procedure time consuming and uneconomical, without significant increases in the amount of extracted phenols and led to a decrease in antioxidant activity of the extracted phenols due to their long exposure to environmental factors (temperature, light and oxygen), which have a negative effect on the antioxidant activity. Larrauri, Sanchez-Moreno, and Saura-Calixto (1998) reported that the reduction in the free radical-scavenging activity, caused by exposure at high temperature, was more marked for red grape pomace peel than white grape pomace peel and these latter more than BHA.

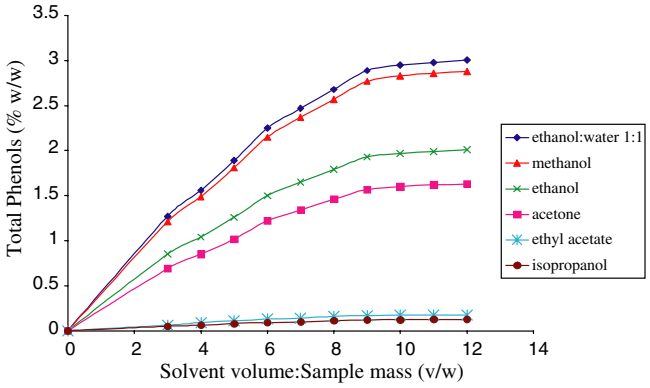

Fig. 3. Effect of solvent/sample proportion (v/w) on the quantity of extracted phenols from winery waste.

As it can be seen from Fig. 3, a solvent/sample proportion of 9:1 (v/w), was the most suitable for the maximum

Table 3
Antioxidant activity of extracts[a] of winery waste

Winery waste extract	Antioxidant activity, as % inhibition
Methanol extract	91.4 ± 1.05[b]
Ethanol extract	93.3 ± 1.57[b]
Ethanol/water extract	62.5 ± 0.76[b]
Acetone extract	90.5 ± 1.89[b]
Isopropanol extract	61.7 ± 2.34[b]
Ethyl acetate extract	50.7 ± 2.76[b]

[a] Extraction period: 3 h.
[b] Mean values ± SD.

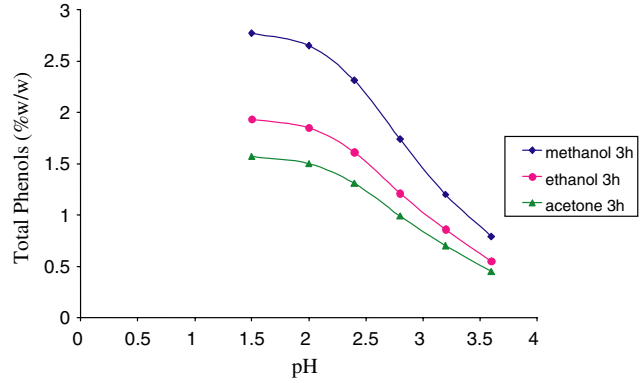

Fig. 4. Effect of pH on the quantity of extracted phenols from winery waste.

Table 4
Peroxide value of olive oil enriched or not with natural phenolic antioxidants ($T = 85$ °C)

Samples	Peroxide value (mmoles/kg) Time (h)			
	24	48	72	96
Olive oil	16.59 ± 0.23[a]	17.62 ± 0.41[a]	21.51 ± 0.52[a]	27.00 ± 0.38[a]
Olive oil + ethanol extract (50 ppm)	9.03 ± 0.14[a]	12.07 ± 0.27[a]	16.93 ± 0.44[a]	17.73 ± 0.11[a]
Olive oil + ethanol extract (100 ppm)	8.09 ± 0.25[a]	10.95 ± 0.09[a]	15.81 ± 0.13[a]	16.48 ± 0.08[a]
Olive oil + ethanol extract (150 ppm)	7.53 ± 0.16[a]	8.99 ± 0.33[a]	14.77 ± 0.05[a]	15.23 ± 0.12[a]
Olive oil + methanol extract (50 ppm)	10.07 ± 0.55[a]	13.17 ± 0.41[a]	18.01 ± 0.19[a]	18.85 ± 0.46[a]
Olive oil + methanol extract (100 ppm)	9.17 ± 0.07[a]	11.88 ± 0.06[a]	16.98 ± 0.14[a]	17.63 ± 0.34[a]
Olive oil + methanol extract (150 ppm)	8.41 ± 0.08[a]	10.96 ± 0.17[a]	15.81 ± 0.29[a]	16.52 ± 0.15[a]
Olive oil + acetone extract (50 ppm)	12.62 ± 0.40[a]	15.54 ± 0.26[a]	17.87 ± 0.13[a]	19.43 ± 0.56[a]
Olive oil + acetone extract (100 ppm)	11.78 ± 0.14[a]	14.27 ± 0.35[a]	16.70 ± 0.54[a]	18.13 ± 0.46[a]
Olive oil + acetone extract (150 ppm)	10.55 ± 0.21[a]	12.97 ± 0.18[a]	16.01 ± 0.09[a]	17.61 ± 0.04[a]

[a] Mean values ± SD.

extraction of phenolic compounds regardless of the extraction solvent.

Maximum phenol content had been achieved at pH 1.5. Further reduction of pH led to evaporation difficulties, without significant increase in the extraction yield and led to a decrease in antiradical activity of the extracted phenols. Baublis, Decker, and Clydesdale (2000) reported increased antioxidant activity of aqueous fractions from wheat bran after treatment at acidic conditions, probably due to altered phenol composition. The effect of pH on the amount of the extracted phenols for solvents showing higher extraction yields, is shown in Fig. 4.

Another extraction parameter, was the continuous and step extraction. The three-step extraction gave extracts with higher phenol content (2.59% w/w) than the two-step (2.37% w/w) and continuous extraction (1.93% w/w). In Fig. 5 an optimized extraction procedure, in pilot scale, for the recovery of phenolic compounds from winery waste is proposed.

3.3. Antioxidant properties of phenol extracts

Ethanol extracts of winery waste exhibited the highest antiradical activity, followed by the methanol extracts and the acetone extracts, whereas the ethyl acetate extracts showed the lowest antioxidant activity (Table 3). The different antioxidant activities of phenolic extracts can be attributed to different extracting solvent as the antioxidant activity depends on the type and polarity of the extracting solvent, the isolation procedures, the purity of active compounds, as well as the test system (Meyer, Heinonen, &

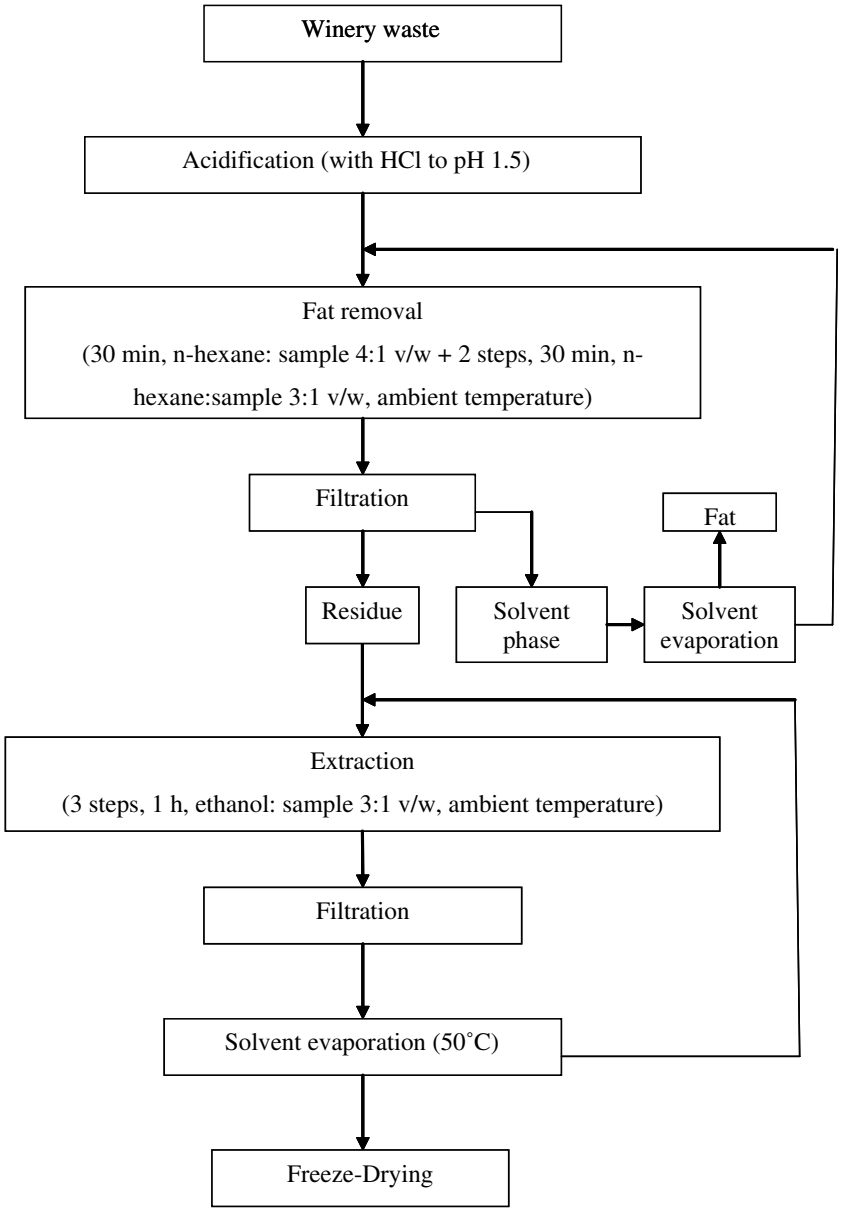

Fig. 5. Flow diagram of recovery of phenols from winery waste.

Frankel, 1998). Although the aqueous ethanol extract showed the highest phenol content, it did not exhibit the highest antioxidant activity. Koleva, Van Beek, Linssen, De Groot, and Evstatieva (2002) found that aqueous extracts of plants of the genus *Sideritis* exhibited lower antioxidant activity than methanol, ethyl acetate and butanol extracts (Koleva et al., 2002). No correlation was found between phenol content and antioxidant activity of extracts (Tables 1 and 3, Student's *t*-test, $P > 0.05$). Different results were reported on this aspect; whereas some authors found correlation between the polyphenol content and the antioxidant activity, others found no such relationship. Furthermore, no correlation between antioxidant activity and phenolic composition was found in citrus residues (Bocco, Cuvelier, Richard, & Berset, 1998), fruit berry, fruit wines (Heinonen, Lehtonen, & Hopia, 1998) or in plant extracts (Kahkonen et al., 1999). Yen and Duh (1995) found that the total phenol content differed significantly among peanut cultivars, although the specific antilipoperoxidant activity was similar.

The antioxidant activity of winery waste was significantly affected by the temperature of drying. Drying at 80 °C reduced the antioxidant activity of winery waste by 21% and at 100 °C by 33%, with respect to drying at 60 °C. According to Larrauri et al. (1997), the antioxidant activity of samples dried with air at 100 °C was reduced by 28% and, at 140 °C by half, with respect to drying at 60 °C.

The peroxide value of samples of olive oil enriched with natural phenolic antioxidant extracts is reported in Table 4. Increase of heating time resulted to an increase of peroxide value in all samples (Table 4). Higher inhibition of oxidation was achieved in samples containing natural phenolic antioxidants at a concentration of 150 ppm, even after four days (Table 4). The ethanol extract appeared to be a stronger antioxidant than the methanol and acetone extracts, being in agreement with the results obtained by the DPPH method. The antioxidant activity depends on the extract concentration. As a general trend, increased antioxidant activity was found with increasing extract concentration, but the concentration leading to maximum antioxidant activity is closely dependent on the extracts and, for the same extract, is dependent on the antioxidant activity test (Yen & Wu, 1999).

The induction periods of sunflower oil subjected to accelerated oxidation conditions without or with added antioxidant are reported in Table 5. BHT, ascorbyl palmitate and vitamin E were proven poor protectors against oil oxidation with induction times almost similar. Ethanol extracts of grape waste increased the induction time of sunflower oil from 7.45 h to 15.3 h. So, ethanol extracts exerted good protection against oxidation and their relatively strong protective effect in oily systems could be attributed to amphiphilic properties of phenolic constituents. It is generally assumed that the hydrogen donor ability and inhibition of oxidation are enhanced by increasing the number of hydroxyl groups in the phenol. So, the high antioxidant activity of ethanol extract can be also attributed to its major components, gallic acid and catechin (Fig. 1). Gallic acid, having three hydroxyl groups, exhibits exceptional antioxidant activity and catechin with the B-ring catechol and free 3-hydroxyl groups leads to high radical-scavenging capacity and strong radical absorption (Benavente-Garcia, Castillo, Lorente, Ortuno, & Del Rio, 2000). Moreover, the presence of an *o*-diphenol, as in caffeic acid and hydroxytyrosol, enhances the ability of the phenolic to act as an antioxidant, since *o*-diphenols may act as potent metal chelators (McDonald, Prenzler, Antolovich, & Robards, 2001). Yamaguchi, Yoshimura, Nakazawa, and Ariga (1999) compared grape seed extract with natural antioxidants, such as tocopherol and ascorbic acid and observed different effectiveness, depending on the assay. The combined use of natural phenol extracts with ascorbyl palmitate increased significantly the induction time of sunflower oil, a fact that shows that phenols and ascorbyl palmitate act synergistically and highly improve lipid stability. Synergistic actions between synthetic only, natural and synthetic and natural antioxidants have been observed. This effect is defined as the combined action which results in an increased antioxidant potential more than that expected from a mere additive effect (Moure et al., 2001). Meyer, Jepsen, and Sorensen (1998) found interactive effects between flavonoids and phenolic acids. However, the simultaneous presence of some compounds may present lower antioxidant activity than expected. Synergistic antioxidant effects were observed for mixtures of crude extracts of grape seed extracts and ascorbic acid

Table 5
Induction period at 100 °C of sunflower oil without or with the addition of synthetic and natural antioxidant

Sample	Induction period (h)	Protection factor[c]
Sunflower oil	7.45 ± 0.07[d]	1.00
Sunflower oil + ascorbyl palmitate[a]	9.97 ± 0.38[d]	1.34
Sunflower oil + BHT[a]	10.23 ± 0.13[d]	1.37
Sunflower oil + vitamin E[a]	9.20 ± 0.41[d]	1.23
Sunflower oil + ethanolic extract of winery waste[b]	15.27 ± 0.37[d]	2.05
Sunflower oil + ethanolic extract of winery waste[b] + ascorbyl palmitate[a]	37.93 ± 0.39[d]	5.09

[a] 200 ppm.
[b] 150 ppm.
[c] Protection factor: induction period of sample/induction period of sunflower oil.
[d] Mean values ± SD.

(Moure et al., 2001). In addition, the phenol extracts acted as antioxidants in a narrow range of concentrations of 50–200 ppm; outside the above range acted as prooxidants. As a general rule, the antioxidants extracted from plants can show prooxidant activity at low concentration and antioxidant activity over certain critical values (Wanasundara & Shahidi, 1998; Yen, Chen, & Peng, 1997).

Studies to incorporate the crude extracts from residual sources as antioxidant ingredients are scarce (Moure et al., 2001). Although the extracts of residual origin often exert high antioxidant activity, their quite intense flavour and colour or problems associated with their solubility and interaction with other food components (e.g. proteins) can limit their applications. Therefore, properties and taste must be suitable for incorporation into food products.

In conclusion, the high efficiency of natural phenolic extracts as potent antioxidants was confirmed, a fact which encourages the prospect of their commercialization as natural powerful antioxidants in foods in order to increase the shelf life of food by preventing lipid peroxidation and protecting from oxidative damage.

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