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Removal of Polyphenols from Wine Sludge Using Cloud Point Extraction

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ABSTRACT
Cloud point extraction (CPE), a promising and simple technique for the separation of organic compounds using surfactants (Genapol X-080 [oligoethylene glycol monoalkyl ether] and PEG 8000 [polyethylene glycol with molecular weight of 8000]), was used to recover polyphenols from wine sludge (wine production waste). The effect of various parameters such as surfactant concentration, temperature, and pH on the percentage of phenol recovery and phase volume ratio during phenol separation from wine sludge was investigated, and the derived optimum parameters were used as the basis for the selection of CPE conditions. When a two-step CPE with a total of 4% v/v of Genapol X-080 (pH = 3.5, temperature = 55 °C, and time = 30 min) or 10% v/v of PEG 8000 (pH = 2.5, temperature = 55 °C, and time = 30 min) was applied the phenol recovery values achieved were 75.8 or 98.5%, respectively. Phenols recovered from wine sludge using the above surfactants maintained high antiradical activity as determined by the 1,1-diphenyl-2-picrylhydrazyl method.

IMPLICATIONS
CPE is a promising and simple technique for the separation of organic compounds using surfactants. This technique was used in the work presented here to recover polyphenols from wine sludge (wine production waste). Phenolic compounds are considered as high added-value byproducts (as food additives) and the use of low-cost industrial wastes (such as wine sludge) could greatly reduce production costs and environmental pollution.

INTRODUCTION
Grapes are one of the world's largest fruit crops. More than 80% of grapes are used to make wine.[1] The wine industry is an important sector in the economy of some countries, especially those from the Mediterranean area.[2] Because of the high organic load and large volumes with a pronounced seasonable variability, the environmental impact of wastes from the wine industry is noticeable.[3] Their extreme toxicity arises from their high biological oxygen demand, acidity, and high organic content (including acids, carbohydrates, phenols, and unsaturated compounds).[4]

Despite the above described pollutant character, grapes, wine, grape seeds, and skin extracts are reported to exert favorable effects on human health (protection against cardiovascular disease, antiinflammatory activity, and anticarcinogenic effects) because of their phenolic content.[5–11] Wine-making wastes such as marc (the residue after pressing for white wines or vinification for red wines) and stalks are rich in phenols. Phenolic compounds can be considered as high added-value byproducts, and the use of low-cost industrial waste could greatly reduce the production costs and increase the profit margin of the products.[12,13] Furthermore, the activity of these compounds as food antioxidants is well known. The addition of antioxidants is a method of increasing shelf life, especially of fats, oil, and fat-containing food products.

Because synthetic antioxidants (e.g., butylated hydroxyanisole [BHA] and butylated hydroxytoluene [BHT]) have restricted use in foods because of their toxicological effects on various species and suspected carcinogenic potential, the search of natural and safe antioxidants, especially of plant origin, has greatly increased in recent years.[14]

Until recently, the extraction of phenolic compounds from wastes was not only complicated and costly[15] but also not friendly to environment because it requires large quantities of toxic and flammable organic solvents. Other methods (e.g., liquid-solid phase extraction, solid-phase extraction, supercritical fluid extraction,[15–18] ultrasonically assisted solvent extraction,[19] and accelerated pressurized and microwave-assisted extraction techniques) are not satisfactory for analytical purposes or for industrial production of phenolic antioxidants for dietary applications because they lead to lower phenolic recovery and require expensive equipment[15] or high energy demand (electrical and/or thermal).

Surfactants appear as a good solution to the above mentioned problems[15] because inexpensive equipment is needed, the temperatures used and the energy consumption are relatively low, and there is no need for organic solvents. Additionally, when the surfactants used are of low or no hazard (edible), there is no need for separation of polyphenols from them; thus, the cost of procedure is greatly reduced. The polyphenols are also protected from the environment and any possible alternation (e.g., oxidation). As indicated by Katsoyannos et al.,[20] micellar systems using nontoxic surfactants (nonionic, without branched aliphatic chains or aromatic moieties) are appropriate for the isolation of natural antioxidants (phenols), which then can be used in dietary applications.[15] Micellar system properties and cloud point extraction (CPE) parameters were described by Carabias-Martinez et al.[21] Seronero[22] and Mahugo Santana et al.[23] used surfactants to extract and preconcentrate chloro- and nitrophenolic compounds from aqueous samples. CPE was combined with the microwave-assisted micellar extraction for the preconcentration of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and phenolic derivatives from natural, river, and seawater.[24]

Nonionic surfactants, in particular those without branched aliphatic chains or aromatic moieties, are considered as edible by the U.S. Food and Drug Administration (FDA).[25] One of these surfactants, Genapol X-080 (oligoethylene glycol monoalkyl ether), has been previously applied to extract vitamins A and E from human serum and whole blood[26] and nitro- and chlorophenols from seawater.[23] Hey et al.[27] used PEG-8000 (polyethylene glycol with molecular weight of 8000) in combination with Triton X-114 to remove polyphenols during the purification of banana polyphenol oxidase. Recently, CPE with various surfactants (Triton X-114 and Genapol X-080) was successfully applied for the recovery of phenols and tocopherols from olive mill wastewater.[15,20] The surfactants used in the work presented here, Genapol X-080 and PEG 8000 are nonionic surfactants with a minimum critical concentration required to form micelles (CMC) of 0.05–0.35 and 0.01–0.40 mM, respectively. Their cloud point temperatures (CPTs) are 25–42 and 32–55 °C, respectively.[15]

The aim of the work presented here was to apply CPE using Genapol X-080 and PEG 8000 as surfactants for the separation of phenolic compounds directly from the liquid fraction of wine sludge wastes. To the authors' knowledge, this technique (CPE) has not been previously applied for the extraction of added-value byproducts from wine industry wastes.

MATERIALS AND METHODS

Materials

Wine sludge, derived from red grape variety "Agiorgitiko" (Nemea, Greece), was supplied from the pilot plant of the Oenology Department of the Technological Educational Institute of Athens. It was centrifuged (20 min at 4000 rpm or 4486 × *g*) using a Hermle Labortechnick Z 200A, filtered to remove solids, and refrigerated (at 4 °C) until use.

PEG 8000, Genapol X-080, Folin-Ciocalteu reagent, methanol, ethyl acetate, *n*-propanol, and sodium carbonate (Na_2CO_3) were purchased from Merck. DPPH (1,1-diphenyl-2-picrylhydrazyl) was purchased from Sigma-Aldrich. Gallic acid was purchased from Panreac. The enzymatic test for the determination of alcohol was obtained from Boehringer Mannheim/R-Biopharm.

CPE Procedure

The procedure used was a modification of the method of Gortzi et al.[15] Before CPE, 5% sodium chloride (NaCl) was added to the sample to facilitate the phase separation process because it increases the density of the bulk aqueous phase and reduces the CPT. The mixture of sample (10 mL), salt, and surfactant (Genapol X-080 or PEG 8000 at concentrations 2, 5, 10, and 20% by volume) contained in tapered glass tubes was vigorously agitated for 1 min, followed by equilibration at 55 °C and a pH value of 2.5 (PEG 8000) or 3.5 (Genapol X-080) for 30 min in a S/N 70 water bath (Konidaris S.A.). The sample was then centrifuged (5 min at 3500 rpm or 3705 × *g*) and the phases were separated by decanting (first extraction step). The surfactant-rich phase was highly viscous. The volumes of the water and surfactant phases were recorded after centrifugation and used for the calculation of polyphenol recovery. After decanting, the nonextracted phenols contained in the water phase were extracted using the same procedure (second extraction step). Every CPE experiment was repeated three times under the same conditions; thus, all recovery values represent mean values of three extraction experiments.

The phenol recovery by the surfactant from the sample was calculated as[15]:

$$\text{Recovery (\%)} = \frac{\text{CsVs}}{\text{CoVo}} \times 100 = \frac{\text{CoVo} - \text{CwVw}}{\text{CoVo}} \times 100 \qquad (1)$$

where, C_o represents the phenol concentration in the initial sample of volume V_o, C_w represents the phenol

concentration in the water phase of volume V_w, and C_s represents the phenol concentration in the surfactant phase of volume V_s.

Determination of Total Polyphenols

Polyphenols were photometrically determined by the Folin-Ciocalteu procedure according to Katsogiannos et al.[20] A calibration curve was prepared using the absorbance of 10 standard solutions (with concentrations of 1.0–10 mg/L of gallic acid) prepared in 25-mL flasks. During the preparation of these solutions, 0.5 mL of Folin-Ciocalteu reagent was added in each flask. After 3 min, 1 mL of Na_2CO_3 (35% w/w) was added. The flasks were then filled with a mixture of 5% methanol and 95% water and left to stand for 1 hr in the dark. Finally, the absorbance of each solution was measured at 725 nm using a Hitachi U-2000 spectrophotometer. A solution including all of the reagents without the addition of gallic acid was used as a blank. The polyphenols of the samples or phases were extracted with ethyl acetate (three successive extraction steps with 4-fold solvent volume) and *n*-propanol (two successive extraction steps with 2-fold solvent volume). The solvents were then evaporated and the polyphenols were dissolved in methanol:water (5:95). Finally, their concentration was photometrically determined using the calibration curve ($y = 0.1077x + 0.0076$ and $R^2 = 0.9994$).

Determination of Water, Total Solids, and Alcohol Content of Wine Sludge Sample

The wine sludge sample was removed from the refrigerator and left to stand for 2 hr to reach ambient temperature. Water content and total solids were determined gravimetrically after drying at 103 °C for 24 hr (according to the procedures of standard methods[28]). Alcohol content was determined according to the method of Saalfeld and Freund.[29]

Determination of the Effect of pH, Temperature, and Surfactant Concentration on CPE Efficiency

The effect of percent surfactant concentration (%SC) (2–20%) on the efficiency of CPE was determined by percent recovery of phenols from wine sludge (V_s/V_w) and the concentration of phenols in the separated surfactant phase (Ps). Also, the effect of pH value on the CPE efficiency was investigated in the pH range of 2.5–5.5 (monitored by a Hanna P210 instrument) using 5% PEG 8000 and 2% Genapol X-080 during the CPE procedure. The temperature effect was investigated in the range 25–65 °C and monitored by a GTH 175/Pt digital thermometer (Greisinger Electronic GmbH) again using 5% PEG 8000 and 2% Genapol X-080 during the CPE procedure.

Determination of the Antiradical Activity

The antiradical activity of the phenols trapped in the surfactant phase as well as those remaining in the sample after treatment with surfactant were estimated according to the DPPH method of Tsaknis and Lalas[30] and compared with the antioxidant activity of the initial sample (wine sludge).

Statistical Analysis

Results are displayed as means of three determinations of three simultaneous assays in all methods.

Table 1. Effect of %SC on V_w, V_s, V_s/V_w, percent phenol recovery from wine sludge, and Ps (mg/L) using PEG 8000 and Genapol X-080 as surfactants.

Surfactant Percent	V_w (mL)	V_s (mL)	V_s/V_w [a]	Percent Phenol Recovery	Ps
PEG 8000					
2	7.8	2.5	0.32	63.6	5.7
5	7.7	2.7	0.35	84.0	6.3
10	7.6	2.9	0.38	88.5	3.6
20	5.2	5.5	1.06	90.1	0.4
Genapol X-080					
2	8.9	1.1	0.12	64.0	5.5
5	7.1	2.9	0.41	77.5	5.3
10	6.5	3.5	0.54	89.7	3.5
20	4.5	5.5	1.22	94.9	2.7

Notes: [a]Phase volume ratio after centrifugal separation.

RESULTS AND DISCUSSION

The possibility for use of CPE with Genapol X-080 or PEG 8000 for the separation of polyphenolic compounds from wine sludge was determined. When a nonionic surfactant water solution is heated above a temperature known as the CPT it becomes turbid and the solution is separated in two phases: a viscous surfactant phase and a water phase. When the surfactant molecule concentration increases above the CMC, colloidal-sized clusters (micelles) are spontaneously formed in various shapes (depending on the specific surfactant and solution conditions).[15] Many theories were proposed for the explanation of the separation and include increase in micellar size when temperature is increased,[31,32] change in micellar interactions,[32] and the dehydration process.[33] However, the mechanism by which separation occurs is not yet clear.[21] Additionally, the micellization progress, including the percentage of encapsulation and the factors influencing it, are still the subject of some debate.[15] The CMC parameters, aggregation number, and structural aspects depend on the microstructure of the nonionic surfactant.[15,24] It has also been shown that the clouding and phase-separation procedure is reversible and micelles are merged with the water phase, re-creating a homogeneous system when the initial solution conditions are established.[34]

Before CPE experiments, the water, total solid, alcohol, and total phenol content of wine sludge used in this work were determined. The samples contained (%) 90.4 ± 0.5 water, 2.1 ± 0.8 total solids, 3.4 ± 0.9 alcohol, and 4.1 ± 0.2 total phenols. Next, the effect of %SC was investigated at 2, 5, 10, and 20%. The equilibration time was 30 min because it was shown that longer equilibration does not have any significant effect on the extraction.[23] The ratio of the volumes of the surfactant-rich phase to the aqueous phase (V_s/V_w) was determined by means of centrifugation after CPE completion because they were necessary for the phenol mass balance and thus for the calculation of the phenol recovery (percent of phenols recovered from the sample by the surfactant).[20] Table 1 indicates the results of the effect of surfactants (PEG 8000 and Genapol X-080) concentration on the V_w, V_s, V_s/V_w, percent phenol recovery from wine sludge, and Ps. A significant impact of surfactants on Ps, V_s/V_w, and

Table 2. Effect of pH value on CPE efficiency using 5% PEG 8000 and 2% Genapol X-080.

pH	Percent Phenol Recovery	V_s/V_w	Ps
PEG 8000			
2.5	75.7	0.45	7.8
3.5	75.8	0.42	7.7
4.0	74.4	0.36	7.5
4.5	65.0	0.46	7.3
5.5	67.0	0.54	6.7
Genapol X-080			
2.5	60.2	0.35	6.3
3.5	63.6	0.31	6.6
4.0	60.1	0.52	6.4
4.5	57.3	0.59	6.0
5.5	54.9	0.63	5.8

Table 3. Effect of temperature on CPE efficiency using 5% PEG 8000 and 2% Genapol X-080.

Temperature (°C)	Percent Phenol Recovery	V_s/V_w	Ps
PEG 8000			
25	0	–	–
45	71.1	0.20	10.3
55	82.4	0.21	11.3
65	65.0	0.23	6.2
Genapol X-080			
25	0	–	–
45	61.3	0.21	7.2
55	66.2	0.12	8.5
65	55.2	0.27	6.4

percent phenol recovery was observed that depended on the surfactant concentration. Mahugo Santana et al.[23] reported that the recoveries increase as the concentration of surfactant increases, although the changes are not the same for all compounds. It should be mentioned that a low ratio indicates a more economical procedure (less surfactant used) and an easier extraction (if necessary) of the polyphenols entrapped in the surfactant. As also indicated in previous work,[15] when surfactant concentration increases, recovery values also increase. However, the use of surfactant concentrations higher than 10% of the initial wine sludge volume leads to unacceptably high V_s/V_w values (V_s becomes >30% of the initial wine sludge volume) and therefore the cost of the operation increases (surfactant cost and operation cost). The %SC value of 5% PEG 8000 seems to be optimum for CPE because the corresponding values of V_s/V_w and Ps are maximal and the percent phenol recovery value is high enough. In the case of Genapol X-080 the results seem to follow the same trend, although a %SC of 2% appears to be optimum for CPE. The use of higher %SC (20% for both surfactants) increases the percent phenol recovery by 7.3% (PEG 8000) or 48.3% (Genapol X-080) but reduces the Ps by 93 and 50.9%, respectively.

The effect of pH on CPE efficiency was also determined. The percent phenol recovery and Ps were investigated in a pH range of 2.5–5.5 using 5% PEG 5000 and 2% Genapol X-080 during the CPE procedure. A significant impact on Ps, V_s/V_w, and the percent phenol recovery from wine sludge was observed. As was indicated by Mahugo Santana et al.,[23] pH modification increases the percentage of extraction for most solutes and is the most noticeable change for more polar compounds. pH values in the range 2.5–3.5 seem to be favorable for CPE because the values of phenol recovery and Ps in this pH range are maximum. Specifically, the optimal pH values for PEG 8000 and Genapol X-080 were 2.5 and 3.5, respectively (Table 2).

The temperature effect was investigated in the range 25–65 °C using 5% PEG 5000 and 2% of Genapol X-080 during the CPE procedure. Table 3 indicates the effect of temperature on percent phenol recovery from wine sludge, Ps, and V_s/V_w. A temperature of 55 °C is favorable for CPE because the values of phenols recovery and Ps are maximum. The lowest temperature used (25 °C) proved inadequate for the recovery of polyphenols, whereas the highest temperature used (65 °C) helped in the extraction, although in a much lower percentage than the other two temperatures (45 and 55 °C). The temperature of 55 °C showed the higher positive influence (for both surfactants) and for that reason was selected for the CPE procedure. Other authors (Mahugo Santana et al.[23]) used a much higher temperature (85 °C); however, the use of a temperature lower than 60 °C is important to avoid phenol degradation.

The data for V_s/V_w; time required for micelle formation; and pH, %SC, and temperature optimum values were the basis for the selection of conditions for CPE application. To maximize the yield of phenol separation from wine sludge, a double-step CPE procedure was undertaken at the optimal conditions (pH = 2.5, temperature = 55 °C, time = 30 min for PEG 8000 and pH = 3.5, temperature = 55 °C, time = 30 min for Genapol X-080) with 5% PEG 8000 or 2% Genapol X-080 by each CPE step. The results are summarized in Table 4. With a total consumption of 10% PEG 8000 the recovery of phenols was 98.5% in a double-step CPE, whereas 4% Genapol X-080 achieved 75.8%. Moreover, exceptionally low phase volume ratios were observed. PEG 8000 was proven a better surfactant than Genapol X-080 for the removal of polyphenols from wine sludge because it could extract

Table 4. Results of double-step CPE on wine sludge with 5% PEG 8000 or 2% Genapol X-080 by each step on percent phenol recovery and Ps (mg/L).

CPE Step	Percent Phenol Recovery	V_s/V_w	Ps
PEG 8000			
First step (5%)	88.6	0.2	6.6
Second step (5%)	98.5	0.3	5.7
Genapol X-080			
First step (2%)	65.8	0.1	5.6
Second step (2%)	75.8	0.1	5.1

Table 5. Effect of CPE treatment with PEG 8000 and Genapol X-080 on the antiradical activity (expressed as inhibition percent) of the phenols separated from wine sludge.

	Inhibition Percent		
Surfactant	Initial Sample	Water Phase	Surfactant Phase
PEG 8000 (5%)	56.2	20.3	65.0
Genapol X-080 (2%)	41.9	33.2	66.9

almost 22% more than the other surfactant (a third extraction step may be needed in the case of Genapol X-080). However, the quantity of PEG 8000 used was more than double that of the Genapol X-080. The choice of the surfactant will largely depend on its cost in market.

Finally, a qualitative test of the CPE treatment effect with PEG 8000 and Genapol X-080 on the antiradical activity of the separated phenols was performed. Wine sludge samples were treated with 5% PEG 8000 and 2% Genapol X-080 in a single-step CPE. After phase separation, the antioxidant activity values of the initial sample (wine sludge), the water phase, and the surfactant phase were determined by the DPPH method. As expected, water phases showed a lower reaction rate than the original untreated samples and surfactant phases (Table 5). Wine sludge phenols recovered by the surfactant showed (in the surfactant-phenols mixture) high antioxidant activity. However, the exact impact of surfactant on the antiradical activity of the recovered phenols cannot be evaluated based only on these indicative percent inhibition values and would demand a detailed investigation with various advanced methods for the estimation of antioxidant activity. In any case, the values in Table 5 (by means of the simple DPPH procedure) determine that phenols recovered by the surfactants used maintain high antiradical activity.

The CPE procedure offers an interesting alternative to the liquid-liquid or liquid-solid solvent extraction of phenols because of its simplicity; low time, labor, and equipment requirements; and the use of nontoxic extractants.[20] Further optimization of CPE conditions or use of other surfactants could increase the values higher than those achieved in this study and should be investigated.

CONCLUSIONS

The CPE procedure offers an interesting alternative to other methods of extraction of high added-value byproducts from wine sludge. Using the optimal conditions (surfactant concentration, temperature, pH, number of CPE steps) for each surfactant ($2 \times 2\%$ v/v of Genapol X-080 at pH = 3.5, temperature = 55 °C, time = 30 min or $2 \times 5\%$ v/v of PEG 8000 at pH = 2.5, temperature = 55 °C, time = 30 min) high phenol recovery values were achieved (75.8% or 98.5%, respectively). Phenols recovered maintained high antiradical activity (DPPH method). Further optimization of CPE conditions or use of other surfactants could increase the values higher than those achieved in this study and should be exploited.

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