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A Simple Method To Separate Red Wine Nonpolymeric and Polymeric Phenols by Solid-Phase Extraction

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Simple polyphenols and tannins differ in the way that they contribute to the organoleptic profile of wine and their effects on human health. Very few straightforward techniques to separate red wine nonpolymeric phenols from the polymeric fraction are available in the literature. In general, they are complex, time-consuming, and generate large amounts of waste. In this procedure, the separation of these compounds was achieved using C18 cartridges, three solvents with different elution strenghts, and pH adjustments of the experimental matrices. Two full factorial 2^3 experimental designs were performed to find the optimal critical variables and their values, allowing for the maximization of tannin recovery and separation efficiency (SE). Nonpolymeric phenols such as phenolic acids, monomers, and oligomers of flavonol and flavan-3-ols and anthocyanins were removed from the column by means of an aqueous solvent followed by ethyl acetate. The polymeric fraction was then eluted with a combination of methanol/acetone/water. The best results were attained with 1 mL of wine sample, a 10% methanol/water solution (first eluant), ethyl acetate (second eluant), and 66% acetone/water as the polymeric phenols-eluting solution (third eluant), obtaining a SE of ca. 90%. Trials with this method on fruit juices also showed high separation efficiency. Hence, this solid-phase extraction method has been shown to be a simple and efficient alternative for the separation of nonpolymeric phenolic fractions and the polymeric ones, and this method could have important applications to sample purification prior to biological testing due to the nonspecific binding of polymeric phenolics to nearly all enzymes and receptor sites.

KEYWORDS: C18 cartridges; red wines; solid-phase extraction; tannins; purification

INTRODUCTION

Condensed tannins (proanthocyanidins) and pigmented tannins constitute the majority of wine phenolics. The predominating tannins in grapes and wines are composed of units of dihydroxylated catechins, i.e., (+)-catechin and (−)-epicatechin, linked by C4−C6 or C4−C8 bonds and are sometimes esterified by gallic acid on C3, especially (−)-epicatechin (*1*). In general, tannins have been associated with organoleptic characteristics of wine. Condensed tannins, in particular, are among the compounds responsible for its astringency. Monomeric flavonoids are primarily bitter, but as the molecular weight increases upon polymerization, astringency and bitterness can increase up to 25−30 times (*2*). Casalini et al. (*3*) demonstrated that the latter compounds also exert a protective effect on oxidative DNA damage, thus having a beneficial influence on carcinogenesis prevention. As regards the anthocyanins, which are the compounds responsible for the red color of wines (*4*), it has been suggested that they could modulate the astringency perception either directly or through certain reactions occurring during aging (*5*). Evidence supporting the incorporation of anthocyanins into tannins during winemaking has been reported since the early 1990s (*6*).

Very few techniques to separate simple polyphenols from the polymeric fraction of wine, which basically consists of tannins and anthocyanins associated with tannins, exist in the literature. One of the most widely used is based on a chromatographic method employing Sephadex LH-20, as described by Kantz and Singleton (*7, 8*). This technique provides excellent separation efficiency, but the use of chromatographic columns is complex, time-consuming, and generates large amounts of waste. Solid-phase extraction (SPE) techniques have been previously used to isolate wine phenolics and other components of wine (e.g., organic acids, sugars, etc.) (*9*−*11*), but little work has been done on optimizing the separation and recovery of both polymeric and nonpolymeric phenols. For this reason, the use of SPE in the separation of these fractions was investigated in this work.

MATERIALS AND METHODS

Samples. Six wines produced in the experimental cellar of the Department of Viticulture and Enology, University of California, Davis, and two fruit juices purchased in a local market were used in these experiments. The chosen wines included four red wines (Cabernet Sauvignon 1992, Cabernet Sauvignon 1999, Cabernet Sauvignon 2004,

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and Port 1982) and two white wines (Chardonnay 1992 and Chardonnay 2004). The selected juices were cranberry (Mountain Sun) and apple juice (Martinelli's). The experiments were conducted April−June 2005.

SPE. On the basis of adsorption, wine polyphenols can be separated into three fractions: (i) phenolic acids, (ii) catechins, flavonols, and anthocyanins, and (iii) polymeric fractions. Initially, each wine sample was dealcoholized and adjusted to pH 7.0. After the neutral C18 Sep-Pak cartridge (Fisher Scientific, 1 g) was preconditioned by using 2 mL of methanol and then water, the wine and juice samples were passed through by means of a SPE vacuum device from Supelco, which allowed 12 samples to be handled simultaneously. As the wine was eluted from the column, the colors of the different eluting fractions, corresponding to the various polyphenol classes, were readily observed. The phenolic acids (appearing brown in color) were not adsorbed to the hydrophobic C18 stationary phase and were collected in the first eluant (fraction 1). The hydrophobic polyphenols were adsorbed onto the column and not eluted by water. Following this step, 2 mL of 0.01 M HCl was added to the cartridge to acidify the matrix, and a solvent of lower polarity (ethyl acetate) was used to elute catechins, "reddish" anthocyanins, and flavonols (second fraction). Finally, mixtures of acetone, water, and methanol at different proportions were used to remove the polymeric fraction. Depending on the final solvent mixture used, this fraction contained differing concentrations and proportions of monomeric catechins, other flavonoids (e.g., anthocyanins), along with the polymeric phenols.

Total Phenolics. Total phenolics were determined by a modified Folin−Ciocalteu method (*12*). Each sample (0.2 mL) was mixed with 1 mL of 10-fold diluted Folin−Ciocalteu reagent and 0.8 mL of 7.5 w/v sodium carbonate solution. After 2 h, the mixture was measured at 765 nm.

High-Performance Liquid Chromatography (HPLC). An HP 1090 apparatus with a Phenomenex Luna Silica 2 (particle size, 5 μm; 250 mm × 4.60 mm i.d.) column, protected by a guard column (10 mm × 4 mm) containing the same material (EM Science) was used to determine the nature of the phenolic compounds. A normal phase chromatography method, developed by Kennedy and Waterhouse (*13*), that uses a binary gradient with mobile phases containing methylene chloride−methanol−formic acid−water, (A) 0:97:2:1 and (B) 83:14:2:1, both containing 20 mM heptanesulfonic acid, was employed. The elution conditions were as follows: 0.75 mL/min, linear gradients from 0 to 34% A in 30 min, from 34 to 100% A in 5 min, and 100% A for 10 min. The column was reequilibrated with B for 10 min before subsequent injections. The injection volume was 10 μL for each sample. Samples were analyzed at 280 nm.

Experimental Design. A full factorial 2^3 experimental design (*14*) was used to evaluate the effect of three different variables in the separation of red wines phenolic fractions. Variables studied in the first experimental design (ED I) were as follows: (i) wine volume (W), 1, 2, and 3 mL; (ii) volume of each eluant (E), 4, 6, and 8 mL; and (iii) % acetone in the last eluant (A), 20, 60, and 100%. Variables studied in the second experimental design (ED II) were as follows: (i) % methanol in the first eluant (M), 10, 30, and 50%; (ii) % acetone in the last eluant (B), 12, 39, and 66%; and % methanol in the last eluant (C), 0, 40, and 80%.

All variables were codified so that their values ranged between +1 and −1, taking zero as the central point. Variables were then codified as follows for ED I: $W = (W - 2)/1$; $E = (E - 6)/2$; and $A = (A - 60)/40$. Codes for ED II were as follows: $M = (M - 30)/20$; $B = (B - 39)/27$; and $C = (C - 60)/40$.

Data were adjusted to a response surface R: $R = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{123}X_1X_2X_3$, where R represents the value of the phenol recovery (P) or tannin recovery (T). Recovery was defined as the ratio between the phenol/tannin quantity eluted at the conditions of each experiment and the maximum quantity capable of being eluted at the optimal conditions of the experimental design. X_i is the value of the variable in the coded form, a_0 is the value of the objective function in the central point conditions, a_1, a_2, and a_3 represent the principal effect associated with each variable, and a_{12}, a_{13}, a_{23}, and a_{123} represent the crossed effects among variables.

Separation Efficiency. Separation efficiency of tannins (TSE) and nonpolymeric phenols (NPSE) from the other species were assessed chromatographically as follows: TSE = [(polymeric phenols peak area)/(polymeric phenols peak area + monomeric and oligomeric phenols area)] and NPSE = [(monomeric and oligomeric phenols area)/(polymeric phenols peak area + monomeric and oligomeric phenols area)].

Polymeric phenols were never detected in the ethyl acetate fraction of any experiment. Therefore, only the polymeric phenols peak areas corresponding to the first and last fraction were used to calculate TSE and NPSE, respectively.

Statistical Analysis. The results reported in this work were the averages of at least three measurements. The coefficients of variations, expressed as the percentage ratio between standard deviations (SD) and the mean values, were found to be <10 units in all cases. Significant variables ($p < 0.05$) were calculated by multivariate linear regression using SPSS statistical program version 12.0 (SPSS Inc.).

RESULTS AND DISCUSSION

In the first experimental design, the effects of three critical variables, namely, wine volume, volume of each eluent, and acetone percentage in the third eluant, were evaluated for phenol and tannin recoveries from wine. The recovery of phenols in this case was achieved by passing water, ethyl acetate, and a methanol/acetone blend through the preconditioned neutral C18 Sep-Pak cartridge subsequently. Acidic polyphenols and other ionizable species such as organic acids become ionized at pH 7.0. They do not adsorb to the lipophilic packing material and thus are easily eluted by the aqueous fraction. After the column has been reconditioned to acidic conditions, flavonols, catechins, and anthocyanins are eluted by ethyl acetate. Finally, an aqueous methanol/acetone blend was employed to recover the polymeric fraction.

As can be observed in **Table 1**, the values of phenolic concentration in the third solvent were higher than those detected for previous eluants. This presumably indicates that, in the wine selected for the experimental design (Cabernet Sauvignon 99), a major proportion of polymeric phenols in comparison with the nonpolymeric ones occurs. The relative proportions of these phenolic fractions can vary largely as a function of the wine considered. Phenolic acids are normally lower than 40%, while anthocyanins commonly represent about 10% of the total phenols (*15*). The highest values of both phenol and tannin recoveries were attained under the conditions of experiment 3, where the lowest volume of wine (1 mL) and 20% acetone were employed. Multivariable linear regression analysis of tannin and phenol recovery data resulted in the following response functions:

$$T = 75.205 - 7.995\ W - 18.705\ A \qquad \text{(confidence level} \geq 95\%)$$

$$F_{mod} = 9.722$$

where $p < 0.022$ and $R^2 = 0.944$.

$$P = 69.854 - 11.751\ W - 13.289\ A \qquad \text{(confidence level} \geq 95\%)$$

$$F_{mod} = 13.656$$

$p < 0.012$ and $R^2 = 0.960$.

These models, whose response surfaces are plotted in **Figures 1** and **2**, bear out that the recoveries of both phenols and tannins are favored by using both decreasing values of wine volume and acetone percentage in the last eluant. In fact, the highest value of tannin recovery was accomplished when 1 mL of wine and 20% acetone were employed. The increasing recoveries derived from using lower quantities of wine in SPE separation

Table 1. Total Phenolics Concentration (mg/L Gallic Acid Equivalents) and Recovery Values (%) in Experimental Design I for Cabernet Sauvignon 99 (Highest Values in Bold)

exp. no.	volume (mL)		% acetone in last eluant	phenol concentration of eluant			recovery	
	wine	eluants		1st[a]	2nd[b]	3rd[c]	phenol	tannin
1	1	4	20	89 ± 7[d]	103 ± 6	246 ± 7	85 ± 6	93 ± 5
2	3	4	20	124 ± 4	222 ± 16	611 ± 24	66 ± 3	82 ± 5
3	**1**	**8**	**20**	**67 ± 1**	**60 ± 5**	**122 ± 8**	**97 ± 8**	**99 ± 2**
4	3	8	20	109 ± 1	131 ± 7	309 ± 1	72 ± 4	84 ± 4
5	1	4	100	86 ± 6	113 ± 5	151 ± 13	64 ± 5	62 ± 3
6	3	4	100	120 ± 1	210 ± 3	380 ± 9	40 ± 3	52 ± 5
7	1	8	100	61 ± 4	59 ± 1	76 ± 2	69 ± 5	62 ± 5
8	3	8	100	91 ± 7	121 ± 6	151 ± 4	44 ± 3	41 ± 3
9	2	6	60	89	155	278	74	84
10	2	6	60	94	183	251	75	76
11	2	6	60	94	123	271	69	82
12	2	6	60	94	183	251	75	76

[a] Water. [b] Ethyl acetate. [c] Acetone/methanol blend. [d] SD.

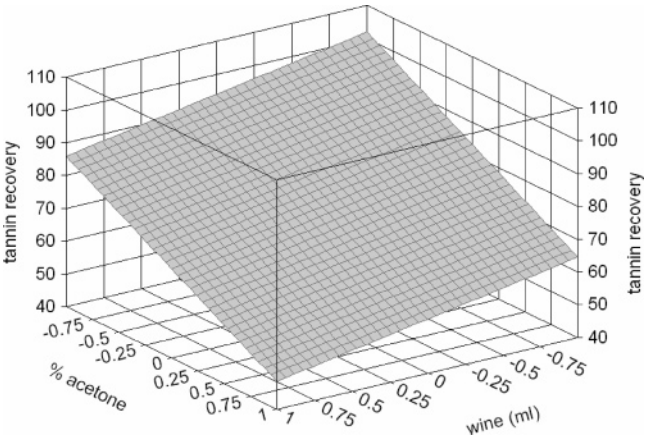

Figure 1. Response surface for tannin recovery as a function of the wine volume and percentage of acetone in the third eluant (methanol/acetone).

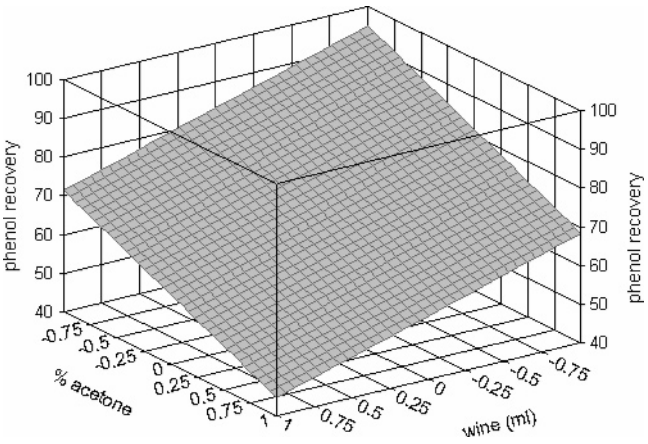

Figure 2. Response surface for phenol recovery as a function of the wine volume and percentage of acetone in the third eluant (methanol/acetone).

were already reported in previous works. For instance, Alamo et al. (*16*) found higher recoveries of free molecular phenols in polymeric cartridges when 2 mL instead of 5 mL of wine were employed. This can be explained on the basis of the limited capacity of the cartridges stationary phase to retain all phenolic compounds when these are in excess. As a consequence, the loss of the compounds of interest, probably due to low initial retention, yields a decrease in recovery.

No effect of the eluant volume was found, suggesting that 4 mL was enough to remove each phenolic fraction. As regards

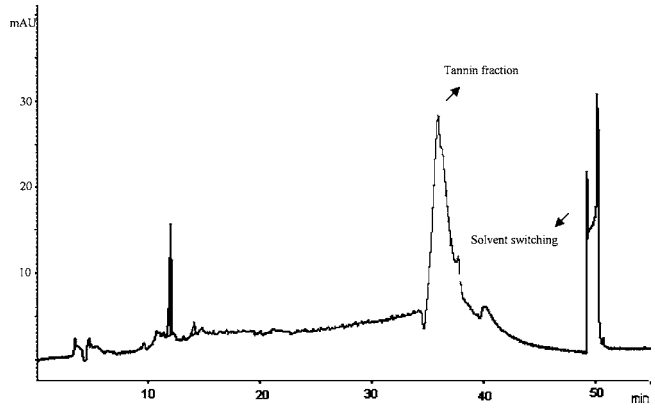

Figure 3. Chromatogram corresponding to the third fraction (methanol/acetone) of experiment 3 (wine volume, 1 mL; eluants volume, 4 mL; and % acetone, 20%).

the solvent employed to remove the polymeric fraction of phenolics from the column, both methanol and acetone have been traditionally used for the extraction and/or desorption of phenols from natural sources and polymer matrixes (*17*, *9*). In our case, the increased percentage of methanol in the last eluant correlated positively with the values of phenol and tannin recovery. It could be therefore inferred that methanol has a higher capacity to desorb tannins from C18 cartridges.

The 20% methanol/acetone fraction (third eluant) chromatogram obtained under the conditions of experiment 3 is shown in **Figure 3**. Cacao and epicatechin standards were used as references for the presence of monomers, oligomers, and polymers. Monomers and oligomers (containing up to three monomers) were found between 10 and 20 min of retention time, while the tannins retention time was at 35 min (*13*). In this fraction, the quantity of nonpolymeric species detected was significant (~8%) and their presence indicates that the previous solvents (water and ethyl acetate) were unable to remove all nonpolymeric phenols. Consequently, to avoid the low molecular weight species in the third eluant and enhance separation efficiency, a new experiment addressing the composition of eluant 1 as well as eluant 3 was run. Here, pure water as the first eluant was replaced with a methanol/water mixture in order to completely remove monomeric and oligomeric phenols. Furthermore, we undertook a search of the most favorable proportions of a ternary blend of methanol/water/acetone as the last eluant in order to enhance the total phenol recovery.

Table 2 shows the phenolic concentration of the different eluants as well as the phenol and tannin percentage recoveries

Table 2. Total Phenolics Concentration (mg/L Gallic Acid Equivalents) and Recovery Values (%) in Experimental Design II for Cabernet Sauvignon 99 (Highest Values in Bold)

exp. no.	% methanol in first eluant	% acetone in last eluant	% methanol in last eluant	phenol concentration of eluant			recovery	
				1st[a]	2nd[b]	3rd[c]	phenol	tannin
13	50	2.4	80	404 ± 5[d]	18 ± 1	25 ± 0	85 ± 4	10 ± 1
14	10	2.4	80	96 ± 9	85 ± 8	217 ± 9	75 ± 2	88 ± 1
15	50	13.2	80	384 ± 5	20 ± 2	28 ± 2	82 ± 5	11 ± 1
16	10	13.2	80	99 ± 3	87 ± 8	229 ± 8	79 ± 3	93 ± 4
17	50	12	0	392 ± 1	22 ± 2	10 ± 1	81 ± 5	4 ± 1
18	10	12	0	89 ± 8	90 ± 1	75 ± 6	48 ± 3	30 ± 1
19	50	66	0	350 ± 13	21 ± 3	33 ± 1	77 ± 6	13 ± 1
20	**10**	**66**	**0**	**90 ± 9**	**91 ± 9**	**232 ± 2**	**87 ± 6**	**94 ± 6**
21	30	23.4	40	242	78	127	85	51
22	30	23.4	40	218	67	144	81	58
23	30	23.4	40	232	70	139	83	56
24	30	23.4	40	232	75	134	85	55

[a] Methanol/water blend. [b] Ethyl acetate. [c] Methanol/acetone/water blend. [d] SD.

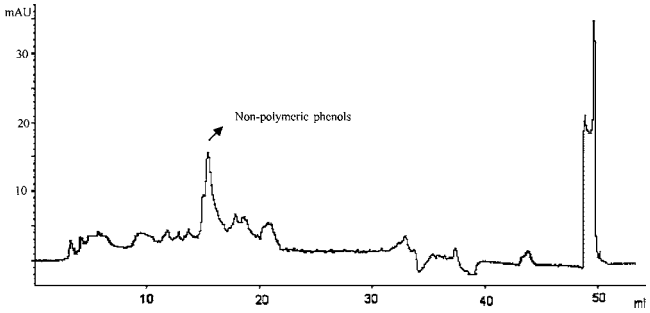

Figure 4. Chromatogram corresponding to the first fraction (10% methanol/water) of experiment 20 (% methanol, 10%; % acetone in last eluant, 66%; and % acetone/water in last eluant, 100%).

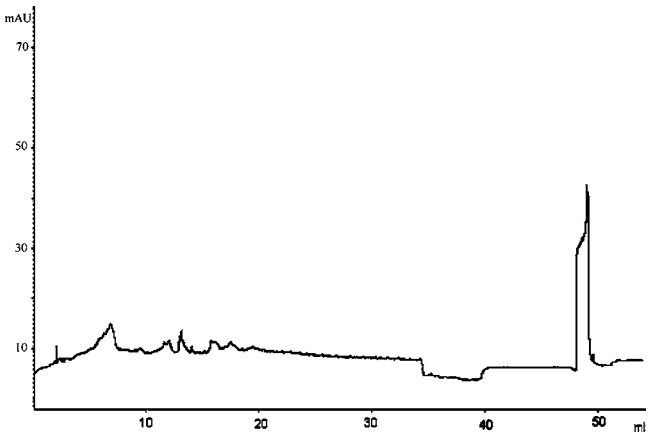

Figure 5. Chromatogram corresponding to the second fraction (ethyl acetate) of experiment 20 (% methanol, 10%; % acetone in last eluant, 66%; and % acetone/water in last eluant, 100%).

obtained under the conditions of the second experimental design. The highest tannin recovery was reached under the conditions of experiment 20, where 10% methanol/water and 66% acetone/water mixtures were employed as first and third eluants, respectively. The chromatograms corresponding to the three fractions obtained under the conditions of this experiment are plotted in **Figures 4**−**6**. As expected, most of the phenols detected in the **Figure 4** were nonpolymeric (NPSE ∼90%), although a little amount of polymeric phenols was also eluted by the 10% methanol/water solution. The polymeric fraction was not observed in the second eluant (ethyl acetate). In the third fraction chromatogram, only polymeric phenols were detected, showing that the use of water/methanol as a first eluant

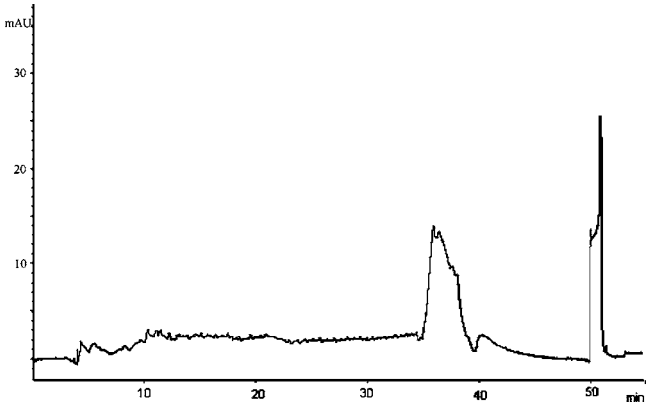

Figure 6. Chromatogram corresponding to the third fraction (methanol/acetone/water) of the experiment 20 (% methanol, 10%; % acetone in last eluant, 66%; and % acetone/water in last eluant, 100%).

was able to remove all nonpolymeric species. The model equations obtained after linear regression corresponding to tannin recovery are as follows:

$$T = 47.259 - 34.1\ \mathrm{M} + 10.657\ \mathrm{A} + 9.112\ \mathrm{A\ C}$$

$$F_{\mathrm{mod}} = 20.72$$

where $p < 0.005$ and $R^2 = 0.973$.

The response surface of tannin recovery, when 10% methanol/water was employed as a first eluant, is plotted in **Figure 7**. In a sense, methanol and acetone appeared as having similar capacities to elute tannins, but in fact, they are different. When the percentage of acetone was low in the last eluant, a higher quantity of methanol was required to enhance the tannin recovery. In contrast, when acetone percentage was high, methanol did not favor the tannin recovery, showing the need for water to effectively solubilize polyphenols.

As regards phenol recovery, statistical analysis gave the following model equation and relevant plot (**Figure 8**):

$$P = 78.433 - 5.381\ \mathrm{M} - 5.609\ \mathrm{M\ A}$$

$$F_{\mathrm{mod}} = 3.098$$

where $p < 0.086$ and $R^2 = 0.844$.

The high values of phenol concentration detected in the first fractions of experiments 1, 3, 5, 7, and 9−12 suggest the undesired elution of polymeric species when solvents with a

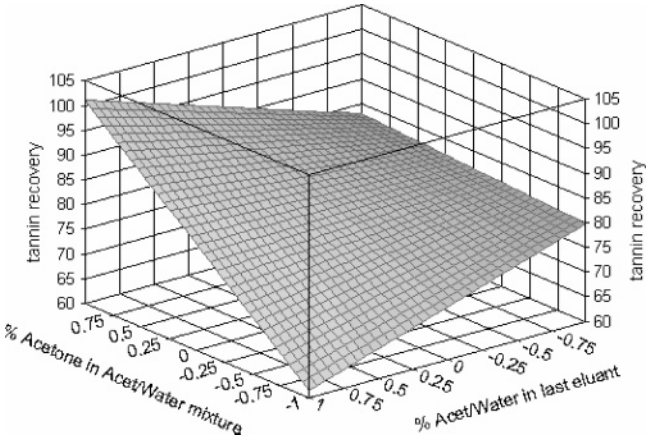

Figure 7. Response surface for tannin recovery as a function of the methanol/acetone/water relative proportions in the last eluant.

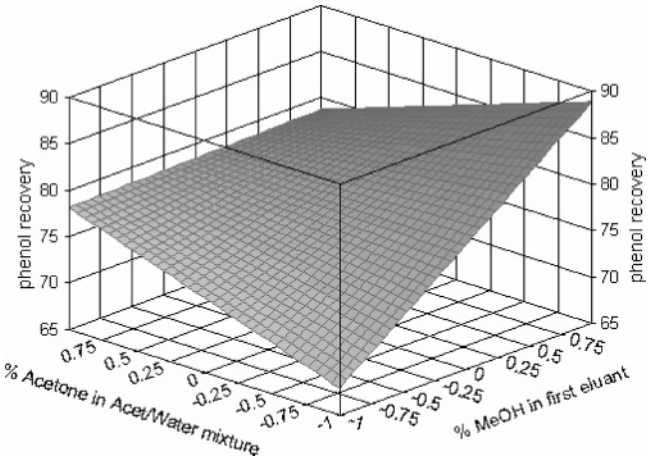

Figure 8. Response surface for phenol recovery as a function of the methanol percentage and percentage of acetone in the last eluant.

Table 3. Total Phenolic Concentration (mg/L Gallic Acid Equivalents) of Wine and Juice Samples and Phenol Recoveries (%) under the Conditions of Experiment 20

sample	total phenols	recovery	NPSE[a]	TSE[b]
Cavernet Sauvignon 99	1959 ± 67[c]	86 ± 6	92 ± 4	97 ± 3
Port 82	993 ± 35	93 ± 4	88 ± 2	90 ± 1
Cabernet sauvignon 92	2043 ± 128	85 ± 2	90 ± 2	93 ± 3
Cabernet sauvignon 04	1744 ± 123	83 ± 5	96 ± 2	87 ± 2
Chardonnay 92	599 ± 21	79 ± 2		
Chardonnay 04	492 ± 3	98 ± 4		
cranberry juice	3025 ± 199	86 ± 8	80 ± 4	84 ± 4
apple juice	1085 ± 44	60 ± 1	89 ± 5	92 ± 5

[a] Separation efficiency of nonpolymeric phenols assessed as indicated in the Materials and Methods. See **Figure 4**. [b] Separation efficiency of tannins assessed as indicated in the Materials and Methods. See **Figure 6**. [c] SD.

methanol percentage higher than 10% were employed. The chromatograms of these fractions (not shown) reveal high levels of polymeric phenols in these eluted fractions.

Having reached an optimum series of solvents, the efficiency of experiment 20 separation conditions was tested in a short list of wines and juices, and the results are shown in **Table 3**. Nonpolymeric and tannin separation efficiency were assessed by HPLC analysis. High separation rates were attained in the red wines assayed (Port 1982, Cabernet Sauvignon 1992, and Cabernet Sauvignon 2004), reaching efficiencies ~90% in all cases. The differences in separation efficiency between Cabernet Sauvignon of different ages could be explained on the basis of the changes that they undergo with time, mainly due to oxidation. Monomeric and oligomeric phenols tend to undergo polymerization reactions with time (*18*), thus decreasing their quantity in more aged wines. No tannin fraction was observed in chromatograms corresponding to the white wines, Chardonnay 1992 and Chardonnay 2004.

The method of tannin separation based on the conditions of experiment 20 also provided successful results in the fruit juice samples selected. Cranberries and apples are examples of fruits with high tannin contents. In fact, cranberry extract was reported to contain 5 μg of total tannins/g of dry weight, while 16.4 μg of total tannins/g of fresh weight was found in apple extract (*19*, *20*). High values of tannin separation efficiency (84 and 91.76%, respectively) were accomplished by using this method, although low recoveries of apple phenolics were obtained. This suggests that the method, with some modification, may be suitable to the separation of phenolics in diverse fruit extracts or drinks.

The application of simple reversed phase SPE separation technology can be used to quickly separate phenolics based on molecular size, using appropriate solvents. This should be useful in purifying phenol-containing samples for many purposes but, in particular, samples for studying biological or health-related properties. Polymeric phenols are not absorbed by mammals but are able to bind to and affect nearly any enzyme or receptor, producing irrelevant results, so removing "tannins" from samples before such assays is most important.

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