Low Molecular Weight Polyphenols in Cork of *Quercus suber*

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Low molecular weight polyphenols were studied by HPLC in samples of cork from different trees of Spanish *Quercus suber* and at different industrial processing stages. Gallic, protocatechuic, vanillic, caffeic, ferulic, and ellagic acids; protocatechuic, vanillic, coniferylic, and sinapic aldehydes, and aesculetin and scopoletin were identified and quantified. Ellagic acid was the main component in all of the samples, followed by the rest of the phenolic acids, which had very much lower concentrations. Four components, caffeic, ferulic, and protocatechuic acids and vanillin, were selected as those that provided the greatest differences among the trees studied. In relation to the four industrial processing stages considered, marked differences were observed between the first two stages, stripping and first rest, and the stages after boiling. In this case, the discriminant variables were coniferaldehyde, sinapaldehyde, and ellagic acid.

Keywords: *Quercus suber; cork; polyphenols; phenolic acids and aldehydes; coumarins; high-performance liquid chromatography*

INTRODUCTION

The use of cork stoppers made of reproduction bark of cork oak (*Quercus suber*) for the closure of wine bottles has been widely studied as far as the technological problems related to the physical properties of this material are concerned (Jung and Hamatscheck, 1992; Casey, 1994). Moreover, the affect of cork microorganisms on changes in wine flavors has been also evaluated (Sponholz and Muno, 1994).

Different components can be extracted from cork when it is macerated in acqueous−alcoholic solution. These cork components can migrate into wine after bottling and modify the wine properties. Some of them are volatile compounds, such as hydrocarbons, alcohols, acids, aldehydes, and ketones (Mazzoleni *et al.*, 1994), and they can be responsible for odors and flavors in wine (Boidron *et al.*, 1984; Rigaud *et al.*, 1984; Simpson, 1990; Valade *et al.*, 1993).

However, very little information is available on the phenolic composition of cork from *Q. suber*, although this group of components can also be extracted by hydroalcoholic solutions and by wine; thus they must be considered in the cork−wine interactions. Some studies have provided total contents of polymeric polyphenols, such as lignins and tannins (Pereira, 1979, 1988). Concerning low molecular weight polyphenols, ferulic, sinapic, and 4-hydroxy-2-methoxybenzoic acids and dihydroconiferol have been described as constituents of suberin, a characteristic structural polymer of the walls of cork cells (Zimmermann *et al.*, 1985; Agullo and Seoane, 1982; García-Vallejo *et al.*, 1997). But, to our knowledge, no information has been published on free low molecular weight polyphenols in cork.

With this work, we begin the study of the polyphenolic composition of cork and the valuation of its variability throughout industrial processing. The free low molecular weight polyphenols are analyzed in planks from reproduction cork of three different trees after each stage of the first industrial processing: from stripping at the stopper factory to the plank sending.

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EXPERIMENTAL PROCEDURE

Samples. Reprodution cork planks were collected from three trees (A, B, and C) grown in Constantina, located in the northern mountains of the Seville province (Spain): three planks from tree A and one plank from trees B and C. Three pieces (20 × 20 cm^2) from each plank were randomly selected immediately after each stage of the industrial processing was completed.

The following stages were considered:

(a) Stripping: separation of the cork plank from the tree stem.

(b) Rest or "maturation" stage: From the time of stripping until the boiling process, the cork planks remained piled in the field or in the factory, for 5 months.

(c1) After boiling and open air rest: The planks were boiled in water for 1 h in order to soften, clean, and disinfect them. After boiling, the planks were piled and dried in the open air for 2 weeks.

(c2) After boiling and storeroom rest: The boiling process was carried out as described before, but the 2 weeks' rest was inside a storeroom, where a high relative humidity (80−100%) atmosphere is maintained. During this period, microorganisms proliferate over the plank surface.

Standards. Reference compounds were purchased from Fluka Chimie AG (Buchs, Switzerland): gallic and caffeic acids, aesculetin, and scopoletin. Aldrich Chimie (Neu-Ulm, Germany): protocatechuic, vanillic, sinapic, and ferulic acids, syringaldehyde, and coniferaldehyde. Apin Chemicals Ltd. (Abingdon, Oxon, U.K.): ellagic acid. Merck (Darmstadt, Germany): vanillin. Sigma Chemical Company (St. Louis, MO, U.S.A.): protocatechuic aldehyde.

Extraction. Cork samples, free of outer bark, were ground and sieved (0.5−1 mm particle size), and 2 g of the samples was extracted with 150 mL of MeOH−H_2O (80:20) at room temperature for 24 h. The suspension was filtered, and MeOH was removed by vacuum distillation. The aqueous solution (solution I) was extracted with Et_2O. The dried Et_2O extract was redissolved in MeOH and analyzed by HPLC (Conde, 1994; Conde *et al.*, 1995a,b, 1996; Fernández de Simón *et al.*, 1996a,b).

Total Phenol Contents. In solution I, total phenol contents were determined according to the method of Folin−Ciocalteu (Singleton and Rossi, 1965), using gallic acid as standard.

Polyphenol Identification. Identifications were carried out by comparing the UV spectra and the chromatographic behavior (HPLC) of the unknown compounds with those of

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Table 1. Extraction Yields and Total Phenol Contents of Cork Extracts from Trees A, B, and C of *Q. suber*

	A[a]		B[a]		C[a]		global[b]	
	average	CV	average	CV	average	CV	average	CV
total extracts (mg/g)	56.4	18	40.5	14	65.3	20	54.6	19
ether extracts (mg/g)	11.9	27	8.3	27	13.9	23	11.5	27
total phenols (mg/g)[c]	4.0	80	4.8	61	6.9	60	4.8	73

[a] Average and coefficient of variation (CV) were calculated for 29 samples in A, for 12 in B, and for 11 in C. [b] Average and CV were calculated for all 52 samples. [c] Expressed in gallic acid; gallic acid molar absorbance is 22.3×10^3.

Table 2. Extraction Yields and Total Phenol Contents of Extracts from Corks of *Q. suber* in Different Industrial Processing Stages

	stripping[a]		first rest[a]		after boiling−open air rest[a]		after boiling−storeroom rest[a]		global[b]	
	average	CV	average	CV	average	CV	average	CV	average	CV
total extracts (mg/g)	58.7	26	50.5	17	58.6	25	50.1	21	54.6	23
ether extracts (mg/g)	12.0	40	12.1	15	10.4	31	11.4	32	11.5	31
total phenols (mg/g)[c]	2.6	58	3.2	38	3.5	60	9.9	23	4.8	73

[a] Average and coefficient of variation (CV) were calculated for 15 samples in the stripping stage, for 12 in the first rest, for 12 after boiling with open air rest, and for 13 after boiling with storeroom rest. [b] Average and CV were calculated for all 52 samples. [c] Expressed in gallic acid; gallic acid molar absorbance is 22.3×10^3.

Table 3. HPLC Quantitative Evaluation of Low Molecular Weight Polyphenols (μg/g Related to Dry Cork) in the Ether Extracts of Cork from Trees A, B, and C of *Q. suber*

	A[a]		B[a]		C[a]		global[b]	
	average	CV	average	CV	average	CV	average	CV
gallic acid	22.1	45	12.6	72	15.2	91	18.3	59
protocatechuic acid	64.8	78	22.7	111	37.5	103	48.8	89
protocatechuic aldehyde	9.5	68	7.5	99	5.2	113	8.1	82
aesculetin	7.3	32	6.2	36	9.3	35	7.5	34
vanillic acid	23.1	34	31.1	47	33.7	37	27.4	39
caffeic acid	9.0	73	11.6	57	19.8	58	12.1	66
vanillin	15.5	96	16.9	109	16.8	167	16.1	120
scopoletin	15.7	34	8.7	84	9.5	88	12.7	52
ferulic acid	7.4	65	18.5	82	10.6	52	10.7	79
coniferaldehyde	11.4	45	12.5	64	9.6	60	11.2	54
sinapaldehyde[c]	5.0	43	4.4	78	3.7	68	4.5	56
ellagic acid	223.0	60	162.5	69	307.0	68	228.4	66

[a] Average and coefficient of variation (CV) were calculated for 29 samples in A, for 12 in B, and for 11 in C. [b] Average and CV were calculated for all 52 samples. [c] Expressed as sinapic acid.

standards and literature data. Some identifications were confirmed by GC−MS.

HPLC. HPLC analyses were carried out with a chromatograph equipped with a diode array detector. The column used was a Hypersil ODS (200 × 4 mm i.d.), protected with a precolumn of the same material. Two solvents were employed for elution, A [MeOH−H_3PO_4 (999:1)] and B [H_2O−H_3PO_4 (999:1)]. The gradient profile was 0−40 min, 20−100% A; 40−45 min, 100% A (isocratic). The flow rate was 1 mL min^{-1}, and the temperature of the chromatographic oven was 30 °C. Detection was carried out at 325 nm with a bandwidth of 150 nm (Conde *et al.*, 1995b).

GC−MS. A gas chromatograph fitted with an EI-mass selective detector and a capillary column (methylsilicone, 12 m × 0.22 mm i.d., 0.33 μm film thickness) was used. Carrier gas was He at 1 mL min^{-1}. Oven temperature was 75−325 °C at 10 °C min^{-1}. Injector and detector temperatures were 300 and 335 °C, respectively (Conde, 1994).

Quantitative Determination of Polyphenolic Compounds. Quantitative determinations were made using the external standard method with the available commercial standards, except for sinapaldehyde which was expressed as sinapic acid.

Statistical Analysis. Data were analyzed using the BMDP package. Univariate analysis (BMDP P7D) and stepwise discriminant analysis (BMDP P7M) were carried out. Average values, standard deviations, and coefficients of variation were calculated, by univariate analysis, using a single-variable model. The pairwise *T*-test was also carried out in order to determine the significance levels of the differences of all the variables grouped by stages or trees. In stepwise discriminant analysis, the variables used in computing the linear classification functions are chosen in a stepwise manner (Jennrich and Sampson, 1985). Both forward and backward selection of variables was possible; at each step, the variable that adds the most of separation of the groups is entered into (or the variable that adds the least is removed from) the discriminant function. The graphical representation of the projections of the points on the two principal canonical axes indicates the statistical distances among the groups.

RESULTS AND DISCUSSION

The results of the extraction yields and total phenol contents are shown in Tables 1 and 2 for the cork extracts classified according to trees A, B, and C and according to the different industrial processing stages, respectively. It must be considered that MeOH−H_2O (80:20) extracts (total extracts) and ether extracts consist not only of phenolic compounds but also of some of the main low polar components of cork, such as waxes, partially extracted with MeOH−H_2O (80:20). In fact, when we assayed the cleaning of a cork sample with $CHCl_3$ or petroleum ether (24 h, at room temperature) before MeOH−H_2O extraction, the MeOH−H_2O extracts were significantly smaller (4.11% and 4.19%, respectively) than those obtained for the same sample without cleaning (9.77%). However, the HPLC analysis of the $CHCl_3$ and petroleum ether extracts revealed that they contained significant amounts of the low molecular weight polyphenols. In addition, the waxes can be clearly distinguished, because they are eluted at retention times much higher than those of phenolic com-

Table 4. HPLC Quantitative Evaluation of Low Molecular Weight Polyphenols (μg/g Related to Dry Cork) in the Ether Extracts from Corks of *Q. suber* in Different Industrial Processing Stages

	stripping[a]		first rest[a]		after boiling−open air rest[a]		after boiling−storeroom rest[a]		global[b]	
	average	CV	average	CV	average	CV	average	CV	average	CV
gallic acid	13.2	55	9.9	59	28.4	38	22.6	49	18.3	49
protocatechuic acid	23.3	100	12.9	113	81.4	63	81.1	50	48.8	71
protocatechuic aldehyde	2.8	118	2.8	104	12.8	41	14.5	27	8.1	49
aesculetin	7.0	27	7.3	55	9.1	18	6.8	36	7.5	35
vanillic acid	30.1	28	31.0	40	19.7	21	28.0	57	27.4	40
caffeic acid	13.8	64	15.0	80	3.6	53	15.3	29	12.1	65
vanillin	2.0	131	3.5	116	29.3	49	32.0	67	16.1	81
scopoletin	10.5	77	9.3	71	18.1	17	13.3	55	12.7	53
ferulic acid	10.0	69	9.2	65	6.1	37	17.2	85	10.7	82
coniferaldehyde	6.3	28	6.4	27	15.3	21	17.6	28	11.2	28
sinapaldehyde[c]	5.0	22	4.3	16	7.4	21	1.5	155	4.5	34
ellagic acid	235.6	41	82.5	55	285.0	67	302.6	53	228.4	59

[a] Average and coefficient of variation (CV) were calculated for 15 samples in the stripping stage, for 12 in the first rest, for 12 after boiling with open air rest, and for 13 after boiling with storeroom rest. [b] Average and CV were calculated for all 52 samples of the different industrial processing stages considered. [c] Expressed as sinapic acid.

Table 5. Significance Levels of Pairwise *T*-Tests of All the Components Grouped by Stages and Trees (Variances Are Not Assumed To Be Equal)[a]

		component											
factor	group	gallic acid	protocatechuic acid	protocatechuic aldehyde	aesculetin	vanillic acid	caffeic acid	vanillin	scopoletin	ferulic acid	conifer-aldehyde	sinap-aldehyde	ellagic acid
stage	a vs b												***
	a vs c1	**	*	***	*	**	**	***	*		***	**	
	a vs c2		**	***				**			***	**	
	b vs c1	***	**	***		–	*	***	**		***	***	*
	b vs c2	*	***	***				**			***	**	**
	c1 vs c2				–		***					***	
tree	A vs B	*	**						*	–			
	A vs C					*	*		–				
	B vs C				*								

[a] a = stripping; b = first rest; c1 = after boiling with open air rest; c2 = after boiling with storeroom rest. *** 0.1% significance; ** 1% significance; * 5% significance; −10% significance; no symbol indicates >10% significance.

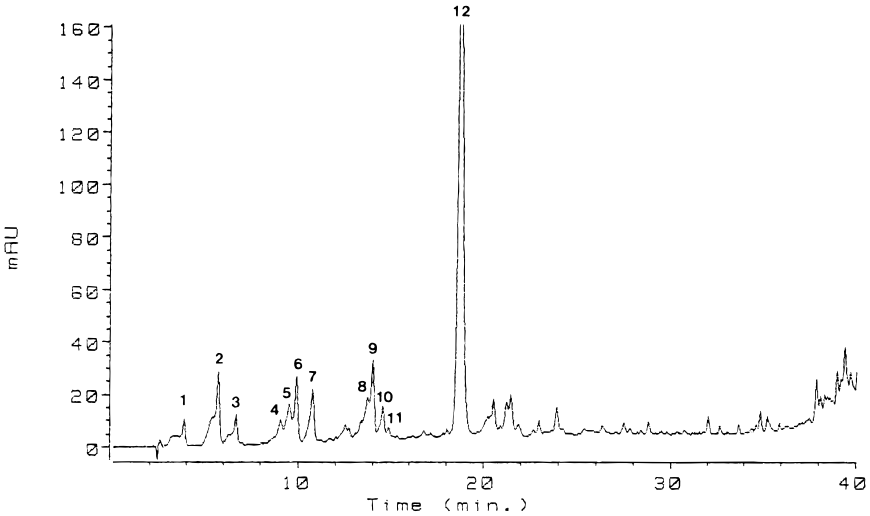

Figure 1. HPLC chromatogram of the ether extracts from *Quercus suber* cork. Detection at 325 ± 75 nm. 1, Gallic acid; 2, protocatechuic acid; 3, protocatechuic aldehyde; 4, aesculetin; 5, vanillic acid; 6, caffeic acid; 7, vanillin; 8, scopoletin; 9, ferulic acid; 10, coniferaldehyde; 11, sinapaldehyde; 12, ellagic acid.

pounds. Therefore, we did not clean the waxes off before the MeOH−H_2O extraction.

As can be seen in Table 1, the total extract values allow us to differentiate trees C, A, and B. However, although the average values of the ether extracts decreased in the same way that those of total extracts did (C > A > B), there were no significant differences between ether extract values for A and C, but both were different from the values for B. The average value of total phenol contents in cork extracts of C was again the highest, but we must emphasize the important coefficients of variation of this variable in all the trees and, therefore, the absence of significant differences among them. Considering the different industrial processing stages (Table 2), there were significant differences neither in MeOH−H_2O extracts nor in ether extracts. In relation to total phenol contents, there was a tendency for the average values to increase throughout the industrial processing stages, smooth at the first three stages but very radical at the storeroom rest stage.

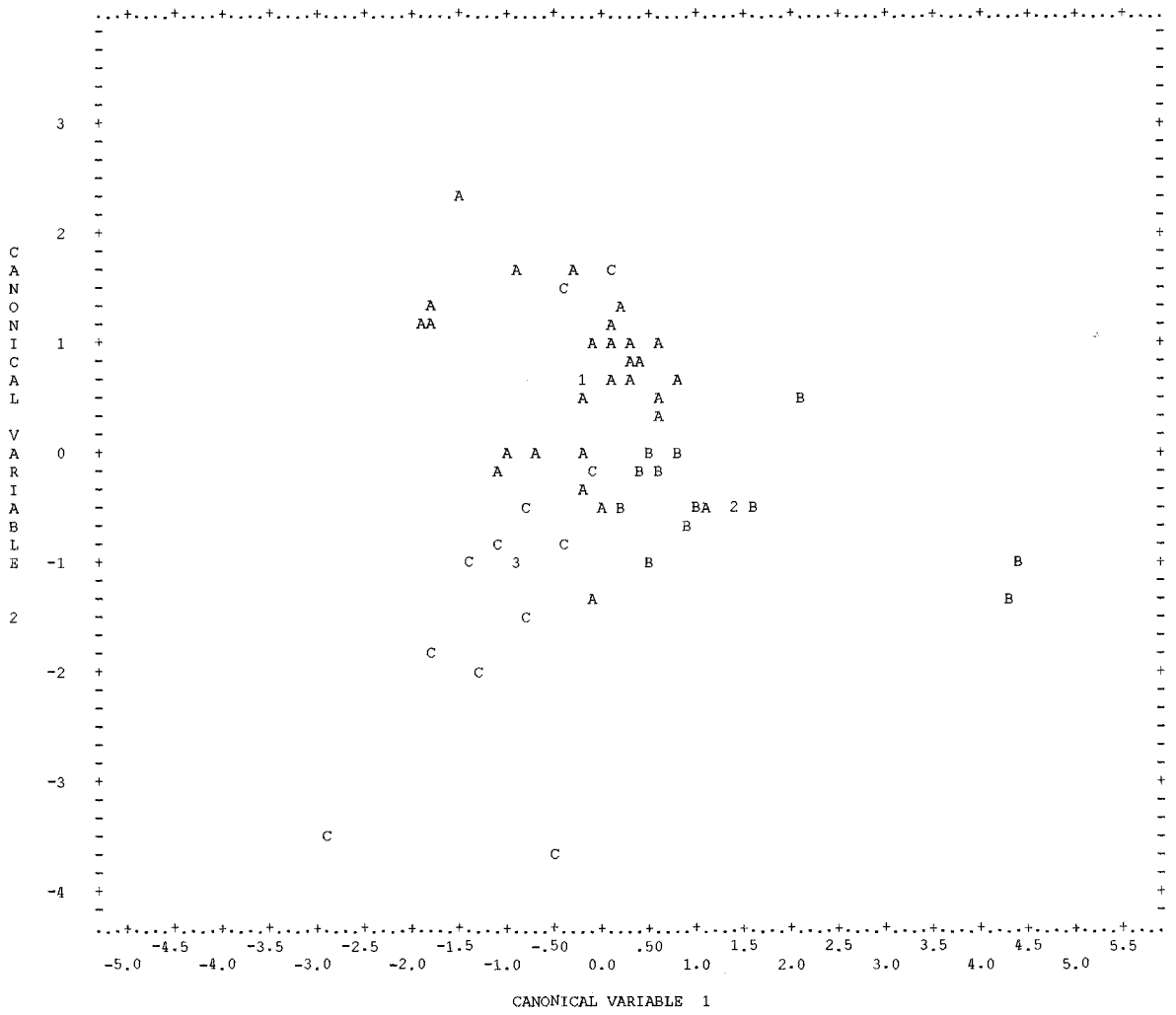

Figure 2. Stepwise discriminant analysis of low molecular polyphenols. Projections of the points of each tree on the two principal canonical axes. 1, 2, and 3 refer to the group centroids of trees A, B, and C, respectively.

There were no significant differences among the first three stages, however, but only the samples at c2 stage showed strong differences respect to those of the other three processing stages. Because this increase was not assocciated with the total extracts, it could be explained if possible partial degradation of lignins and tannins by the microorganisms that grew on the cork surface during the storeroom rest was considered. In this way, the breaking of the intramolecular linkage of these polymers would result in an increase of the quantity of free hydroxyl groups susceptible to reaction with the Folin−Ciocalteu reagent.

In the ether fractions of the cork extracts, the HPLC analysis revealed the presence of the following components: gallic, protocatechuic, vanillic, caffeic, ferulic, and ellagic acids; protocatechuic, vanillic, coniferilic, and sinapic aldehydes and the coumarins, aesculetin and scopoletin. As mentioned above, ferulic acid is the only component that has been previously described as constituent of the suberin polymer. However, in woods of other *Quercus* species, it has been described the presence of these phenolic acids and aldehydes and coumarins (Black *et al.*, 1953; Pearl *et al.*, 1958; Guymon and Crowell, 1968; Chen, 1970; Seikel *et al.*, 1971; Joseph and Marche, 1972; Miller *et al.*, 1992; Fernández de Simón *et al.*, 1996a,b), with the exception of protocatechuic aldehyde. Moreover, siringic acid and aldehyde, also present in woods of other *Quercus* species, were not detected in the samples of cork that were analyzed in this investigation.

The HPLC quantitative evaluations of the low molecular weight polyphenols in the ether soluble fractions of the cork samples are gathered in Tables 3 and 4 for trees A, B, and C and for the different industrial processing stages, respectively. Table 5 includes the results of the significance levels of pairwise *T*-test of all the components grouped by stage or by tree. Components are arranged according to their retention times in the chromatogram (Figure 1).

Phenolic acids were the most abundant low molecular weight polyphenols in cork. Considering the global average values, the main component of this group was ellagic acid, followed in decreasing order of abundance by protocatechuic, vanillic, and gallic acids. Both cinnamic acids (ferulic and caffeic) showed lower concentrations. Protocatechuic and ferulic acids presented the highest global coefficients of variation, which means that their concentrations were the most variable among those components identified in the ether soluble fractions of cork extracts. Benzoic and cinnamic aldehydes and coumarins were minor components, and the coefficients of variation of vanillin and protocatechuic aldehyde (mainly when samples are grouped by tree) were extremely high.

Considering the differences in composition among trees, it can be pointed out that the only components which did not present significant differences among trees are the four phenolic aldehydes analyzed: protocatechuic aldehyde, vanillin, coniferaldehyde, and sinapaldehyde, and ellagic acid (Table 5). The remaining

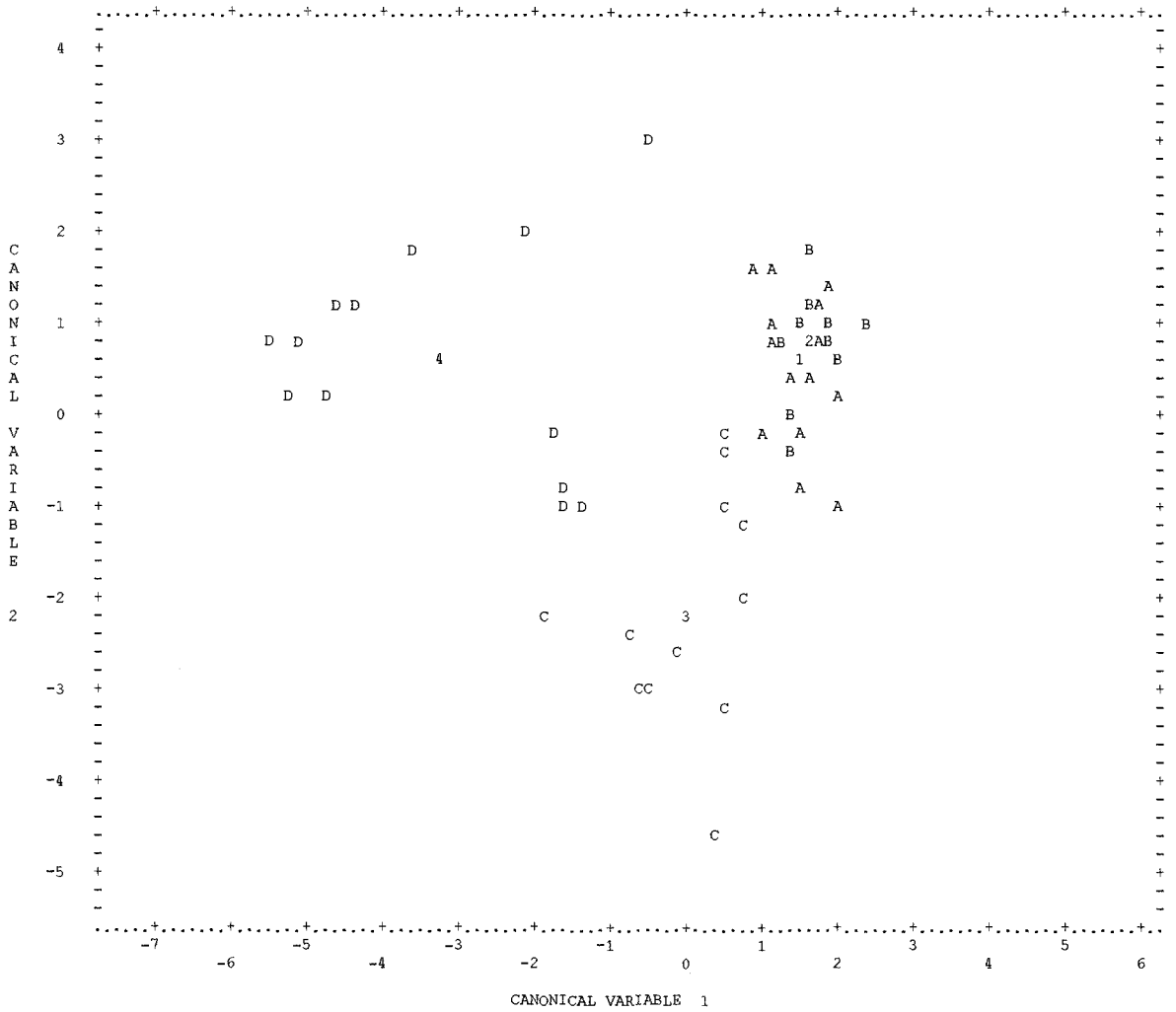

Figure 3. Stepwise discriminant analysis of low molecular polyphenols. Projections of the points of each industrial processing stages on the two principal canonical axes. A, stripping; B, first rest; C, after boiling with open air rest; D, after boiling with storeroom rest. 1, 2, 3, and 4 are the group centroids for each stage, respectively.

phenolic acids and coumarins did not show a unique pattern of relative concentrations in the three trees. In addition, there were important differences in polyphenolic composition when the different samples of each individual tree were considered (Table 3). This concentration variability was especially pronounced for protocatechuic acid and aldehyde, vanillin, and ferulic acid, with coefficients of variation even over 100. In the stepwise discriminant analysis among trees, considering the contents of these low molecular weight polyphenols to be variables, four components were selected as those that provided the greatest discrimination among trees, caffeic acid, ferulic acid, protocatechuic acid, and vanillin, and the mathematical model that resulted explained 100% of the total dispersion. Figure 2 is a graphical representation of the projections of the points of each group on the two principal canonical axes, originated at the end of the statistical process. Group centroids were clearly separated in this figure, but the three sets of points overlapped, although those of B were the most distant.

In relation to the variation of the low molecular weight polyphenol contents throughout the industrial processing stages that were considered (Table 4), important differences were detected between the two first stages, stripping and first rest, on one side, and the two later stages, after boiling−open air rest and after boiling−storeroom rest, on the other. The two first stages showed significant differences only in the contents of ellagic acid, while the two latter showed differences in the contents of caffeic acid and sinapaldehyde (Table 5). The main components were ellagic acid > vanillic acid > protocatechuic acid, in stripping and first rest (stages a and b), and ellagic acid > protocatechuic acid > vanillin, in the two types of rest after boiling (stages c1 and c2). Moreover, in these two latter stages, there was a significant increase in the average contents of ellagic, protocatechuic, and gallic acids and protocatechuic aldehyde, vanillin, and coniferaldehyde. After boiling, there was a surprising decrease in vanillic, caffeic, and ferulic acids in the open air rest, if compared with values obtained after the storeroom rest, but these differences were only significant in the case of caffeic acid. There was also an important variability among samples at each one of the stages, very marked for protocatechuic acid and aldehyde and vanillin in the two first stages and for sinapaldehyde after boiling with storeroom rest. The graphical representation of the results of the discriminant analysis, considering the different stages (Figure 3), confirms that explained above. The set of points of stripping and first rest (stages a and b) were completely overlapped and, also, separated from those of each of the stages c1 and c2. Distances between centroids are much greater here than in Figure 2. In this case, the variables selected were coniferaldehyde, sinapaldehyde, and ellagic acid. It is to be stated that none of these variables was

selected when analyzing the tree factor. The mathematical model also accounted for the 100% of the total dispersion.

The results indicate that *Q. suber* cork contains a variety of low molecular weight phenolic components, most of them described in wine. The content variability of these compounds observed in samples analyzed was very high, which is in agreement with previous studies on polyphenolic composition of wood and bark from other *Quercus* species, because the age of the tree and the distance of the samples from the base of the tree can also influence the composition of the extractable polyphenols (Fernández de Simón *et al*., 1996a,b). Moreover, the interactions of these aromatic components with wine can be very complex. For some components, such as vanillin, these interactions have been studied, but for other components, whose concentrations in cork are high and affected by the industrial processing (ellagic, gallic, and protocatechuic acids, protocatechuic aldehyde, and coniferaldehyde) the interactions are unknown. So, the knowledge of the polyphenolic composition of the cork should be considered when improving the industrial processing of cork and studying the cork−wine relationship and the in-bottle wine evolution.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; GC−MS, gas chromatography−mass spectrometry; MeOH, methanol.

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