Changes in Tannic Composition of Reproduction Cork *Quercus suber* throughout Industrial Processing

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Tannic composition was studied in reproduction cork samples from three different trees of Spanish *Quercus suber* and at different industrial processing stages. The ellagitannins, roburins A and E, grandinin, vescalagin, and castalagin, were identified and quantified by HPLC. Global evaluations of tannins were also carried out, using classical chemical methods. The group of hydrolyzable tannins was the most abundant in the tannic extract in all samples; among them, castalagin was the main component, followed by vescalagin, grandinin, roburin E, and, to a much lesser extent, roburin A. The changes in tannic composition throughout the industrial processing are mainly related to the boiling process and are more pronounced in total phenol and proanthocyanidin contents than in individual ellagitannins content. Vescalagin and roburins A and E were selected as those variables that provided the greatest discrimination among stages. Important differences in the ellagitannin contents were observed among the trees studied, all of the ellagitannins being discriminant variables in this case.

Keywords: *Quercus suber; cork; tannins; ellagitannins; proanthocyanidins; polyphenols; high-performance liquid chromatography*

INTRODUCTION

The enological interest of studying the composition of cork, from the point of view of the in-bottle evolution of wine, led us to develop several works to recognize the different groups of polyphenolic components present in cork. Thus, we have identified in *Quercus suber* cork (Cadahía et al., 1996) several molecular structures of ellagitannins with a wide distribution in wood of species of the genus *Quercus* (Mayer et al., 1967, 1969, 1971; Nonaka et al., 1989; Scalbert et al., 1989; Hervé du Penhoat et al., 1991). There were roburins A and E, grandinin, vescalagin, and castalagin, besides other ellagitannins with related structures. We have equally developed a parallel research on the identification of the low molecular weight polyphenols of reproduction cork (Conde et al., 1997a) and on the changes that this group of components underwent throughout the industrial processing of cork. Moreover, we have carried out a joint characterization of tannins and low molecular weight polyphenols in cork samples from different Spanish provenances (Conde et al., 1997b).

This work continues the research on the polyphenolic composition of cork. Here we report the analysis of the tannin composition of planks from reproduction cork of three different trees after each stage of the first industrial processing: from the stripping to the stopper factory.

EXPERIMENTAL PROCEDURES

Samples. Reproduction cork planks were collected from three trees (A−C) grown in Constantina, located in the northern mountains of the Seville province (Spain): three planks from tree A and 1 plank from both trees B and C. Three pieces (20 × 20 cm) were picked randomly from each plank, just after each stage of the industrial processing.

The following stages were considered: (a) stripping (separation of the cork plank from the tree stem); (b) rest or "maturation" stage (from stripping until the boiling process, cork planks remained piled in the field or in the factory for 5 months); (c1) boiling and open air rest [after planks were placed in boiling water (100 °C) for 1 h, they were piled and dried in the open air for 2 weeks]; (c2) boiling and storeroom rest [boiling process was carried out as described before, but the 2 week rest was carried out inside a storeroom, where a high relative humidity atmosphere (80−100%) was maintained, and during this period, microorganisms proliferate over the planks' surface].

Standards. Standards of vescalagin, castalagin, roburins A and E, and grandinin were kindly provided by Dr. A. Scalbert.

Extraction. Cork samples, free of outer bark, were ground and sieved (0.5−1 mm particle size) and 2 g was extracted with 150 mL of MeOH/H_2O (80:20) at room temperature for 24 h (Conde et al., 1997a,b). The suspension was filtered, and MeOH was removed by vacuum distillation. The aqueous solution (solution I) was extracted with Et_2O and freeze-dried. Solution I was used for quantitative analysis of total phenols, proanthocyanidins, and ellagitannins, whereas the lyophilized material was used for HPLC qualitative and quantitative analyses (Cadahía et al., 1997a−c).

Analytical Methods. Classical chemical evaluations were carried out following the methods of Folin−Ciocalteu for total phenols (Singleton and Rossi, 1965) and the method of autoxidation in *n*-BuOH.HCl to anthocyanidins for proanthocyanidins (Porter et al., 1986). Ellagitannins were estimated by HPLC evaluation (Conde et al., 1995) of the ellagic acid yielded after they were submitted to methanolysis in MeOH/HCl. Two hudred and fifty microliters of solution I was freeze-dried, 1 mL of 6 NMeOH/HCl (9:1, v/v) was added, and the mixture was heated at 100 °C for 4 h (Cadahía et al., 1996).

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Table 1. Total Phenol, Ellagitannin, and Proanthocyanidin Contents of *Q. suber* Cork Extracts in Different Industrial Processing Stages

	stripping (a)[d]		first rest (b)[d]		boiling + open air rest (c1)[d]		boiling + storeroom rest (c2)[d]		total[e]	
	av	CV	av	CV	av	CV	av	CV	av	CV
total phenols[a]	2.6	58	3.2	38	3.5	60	9.8	23	4.8	73
ellagitannins[b]	1.7	32	3.0	17	3.2	26	2.6	18	2.6	34
proanthocyanidins[c]	1.1	21	1.2	19	0.7	17	0.3	30	0.8	47

[a] Expressed in milligrams of gallic acid per gram of dry cork; gallic acid molar absorbance was 22.3×10^3. [b] In milligrams of ellagic acid per gram of dry cork. [c] In milligrams of cyanidin per gram of dry cork. [d] Average and coefficient of variation (CV) were calculated for 15 samples in the stripping stage, for 12 samples in the first rest and after boiling with open air rest, and for 13 samples after boiling with storeroom rest. [e] Average and CV were calculated for all 52 samples.

Table 2. Total Phenol, Ellagitannin, and Proanthocyanidin Contents of Cork Extracts from Trees A–C of *Q. suber*

	tree A[d]		tree B[d]		tree C[d]		total[e]	
	av	CV	av	CV	av	CV	av	CV
total phenols[a]	4.0	80	4.8	61	6.9	60	4.8	73
ellagitannins[b]	2.2	35	2.9	27	3.1	27	2.6	34
proanthocyanidins[c]	0.9	45	0.7	53	0.8	43	0.8	47

[a] Expressed in milligrmas of gallic acid per gram of dry cork; gallic acid molar absorbance was 22.3×10^3. [b] In milligrams of ellagic acid per gram of dry cork. [c] In milligrams of cyanidin per gram of dry cork. [d] Average and coefficient of variation (CV) were calculated for 29 samples for tree A, for 12 samples for tree B, and for 11 samples for tree C. [e] Average and CV were calculated for all 52 samples.

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

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, v/v); B, H_2O/H_3PO_4 (999:1, v/v). The gradient profile used was as follows: 0–40 min, 0–10% A; 40–70 min, 10–30% A; 70–90 min, 30–100% A. 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 (Cadahía et al., 1997c).

Quantitative Determination of Tannic Compounds. Quantitative determinations were made using the external standard method, with the available standards.

Statistical Analysis. Data were analyzed using the BMDP package. Univariate analysis (BMDP P7D) and stepwise discriminant analysis (BMDP P7M) for the groups of ellagitannins were carried out. Average values and coefficients of variation were calculated by univariate analysis, using a single-variable model. The pairwise *t* test was also carried out to determine the significance levels of the differences of all the variables grouped by provenances. 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 selections of variables were possible; at each step, the variable that adds the most 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

Tables 1 and 2 show the results on total phenol, proanthocyanidin, and ellagitannin contents for cork samples at each of the industrial processing stages and for samples of each of the trees studied, respectively.

Regarding the data obtained at the different stages of the industrial processing, an extremely important and significant increase of total phenol contents is observed after boiling with storeroom rest (c2). In contrast, proanthocyanidin contents showed a significant decrease in the two rest periods after boiling, more pronounced than when the rest elapsed in the storeroom. These results can be explained by a partial degradation of lignins and/or tannins (Scalbert, 1991; Artaud and Odier, 1993) produced by the microorganisms that grew on the cork surface during the storeroom rest in high-humidity conditions. This partial degradation would result in an increase of the quantity of free hydroxyl groups susceptible to react with the Folin–Ciocalteu reactive.

Considering the results for each of the studied trees, the practical absence of significant differences among them and the high coefficients of variation of total phenol contents, ellagitannins, and proanthocyanidins should be emphasized.

The study of the tannin composition throughout the different stages of the industrial processing was focused on the identification and evaluation of ellagitannins, since this group of tannins presented the highest levels in the previous analysis of polyphenol contents (Tables 1 and 2). The HPLC analysis confirmed that the hydrolyzable tannins or ellagitannins were the most abundant soluble tannins in cork. Two groups of hydrolyzable tannins were detected: the first one comprises roburins A and E, grandinin, vescalagin, and castalagin, among other ellagitannins with related structures; and the second one includes ellagic acid derivatives with higher HPLC retention times and a UV spectrum similar to that of ellagic acid, both of which were in very much lower proportion.

The results of the HPLC quantitative evaluation of the identified ellagitannins are shown in Tables 3 and 4 for cork samples arranged according to processing stages or to trees, respectively; the ellagitannins are arranged according to their retention time in the chromatogram. Castalagin was the main component in all samples, followed by vescalagin, grandinin, roburin E, and, to a much lower extent, roburin A. However, the contents of these compounds varied considerably among samples. Our results can be related with those of Mosedale et al. (1996), who found a high degree of variation in the ellagitannin concentration among individual trees, even trees grown under very constant conditions, indicating that this property is under strong genetic control. This concentration variability was more pronounced among samples of each industrial processing stage than among samples of each of the trees. Moreover, a decrease in the variability within each stage after boiling was not observed. Therefore, an important

Table 3. HPLC Quantitative Evaluation of Ellagitannins (Micrograms per Gram of Dry Cork) in the Extracts of *Q. suber* Cork in Different Industrial Processing Stages

	stripping (a)[a]		first rest (b)[a]		boiling + open air rest (c1)[a]		boiling + storeroom rest (c2)[a]		total[b]	
	av	CV	av	CV	av	CV	av	CV	av	CV
roburin A	46	55	51	42	59	65	54	42	52	53
grandinin	345	67	326	32	221	67	283	80	297	64
vescalagin	560	59	685	25	397	71	366	53	514	52
roburin E	117	57	114	30	108	81	137	56	119	58
castalagin	907	33	964	14	661	62	789	42	834	37

[a] Average and coefficient of variation (CV) were calculated for 15 samples in the stripping stage, for 12 samples in the first rest and after boiling with open air rest, and for 13 samples after boiling with storeroom rest. [b] Average and CV were calculated for all 52 samples.

Table 4. HPLC Quantitative Evaluation of Ellagitannins (Micrograms per gram of Dry Cork) in the Extracts of *Q. suber* Cork from Trees A−C

	tree A[a]		tree B[a]		tree C[a]		total[b]	
	av	CV	av	CV	av	CV	av	CV
roburin A	38	38	86	28	52	46	52	37
grandinin	184	38	373	28	510	49	297	46
vescalagin	389	57	747	33	588	55	514	49
roburin E	81	30	144	35	192	48	119	43
castalagin	743	38	929	18	975	47	834	37

[a] Average and coefficient of variation (CV) were calculated for 29 samples for tree A, for 12 samples for tree B, and for 11 samples for tree C. [b] Average and CV were calculated for all 52 samples.

Table 5. Significance Levels of Pairwise *t* Test of All the Ellagitannins Grouped by Stages and Trees (Variances Are Not Assumed To Be Equal)

		component[b]				
factor	group[a]	roburin A	grandinin	vescalagin	roburin E	castalagin
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, boiling followed by open air rest; c2, boiling followed by storeroom rest. [b] ***, 0.1% significance; **, 1% significance; *, 5% significance; −, 10% significance; no symbol indicates >10% significance.

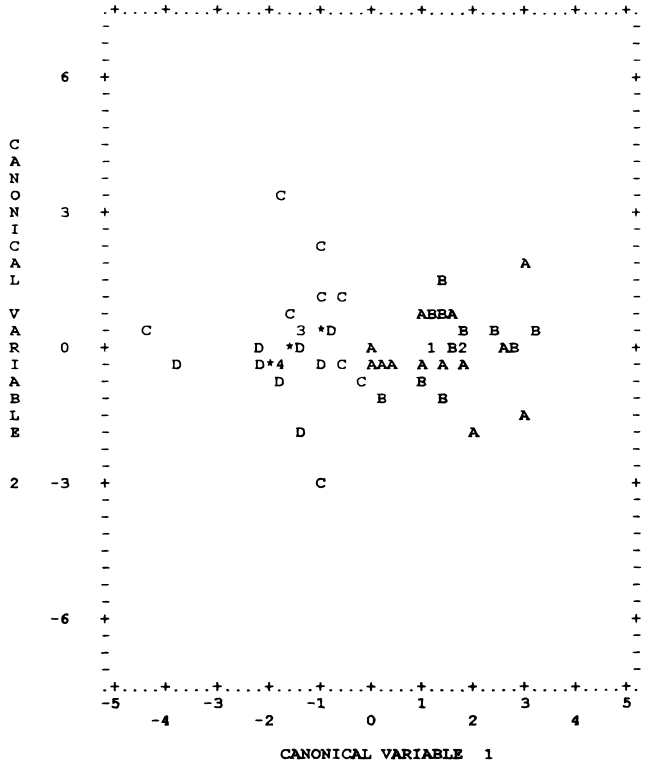

Figure 1. Stepwise discriminant analysis of ellagitannins as shown by projections of the points of each industrial processing stage on the two principal canonical axes: A, stripping; B, first rest; C, boiling followed by open air rest; D, boiling followed by storeroom rest. 1, 2, 3, and 4 are the group centroids for each stage, respectively. Eigenvalue for discriminant function 1 was 2.54 and for discriminant function 2 was 0.07. Standarized coefficients for discriminant functions 1 and 2: roburin A, −1.74, 0.94; vescalagin, 2.47, 0.11; roburin E, −0.88, −1.13, respectively.

cross-contamination of the planks during the boiling process did not occur, because it would have implicated a homogenization of the material.

Table 5 includes the results of the significance levels of pairwise *t* test of all the ellagitannins grouped by stages of the industrial processing or trees. Regarding the changes in the composition of ellagitannins during the different stages, no drastic effects were observed. In the pairwise *t* test, significant differences were obtained only from the contents of one ellagitannin, vescalagin, that presented significantly different concentrations between the stage of first rest (b) and the stage after boiling followed by storeroom rest (c2). However, the stepwise discriminant analysis selected roburins A and E, in addition to vescalagin, as those that provided the greatest discriminantion among the processing stages. The resulting mathematical model accounted for 100% of the total dispersion, explained in three canonical functions. Figure 1 is a graphical representation of this statistical analysis, which shows the projections of the points of each group on the two principal canonical axes, originated at the end of the statistical process, which represented a cumulative proportion of 99.8% of the total dispersion. The sets of points of stages a and b (A and B in Figure 1, respectively) were almost completely overlapped, and, moreover, there was a less pronounced distinction among these stages and those after boiling (C and D in the Figure 1). This distribution of the set of points was similar to that obtained for the low molecular weight polyphenol composition in these industrial processing stages (Conde et al., 1997a). On the other hand, the humidity conditions during the rest stage after boiling did not show an important effect.

The results of the pairwise *t*-test considering the tree factor revealed that the only ellagitannin that did not show significant differences among trees is castalagin (Table 5). Moreover, the discriminant analysis among trees, whose graphical representation is shown in Figure

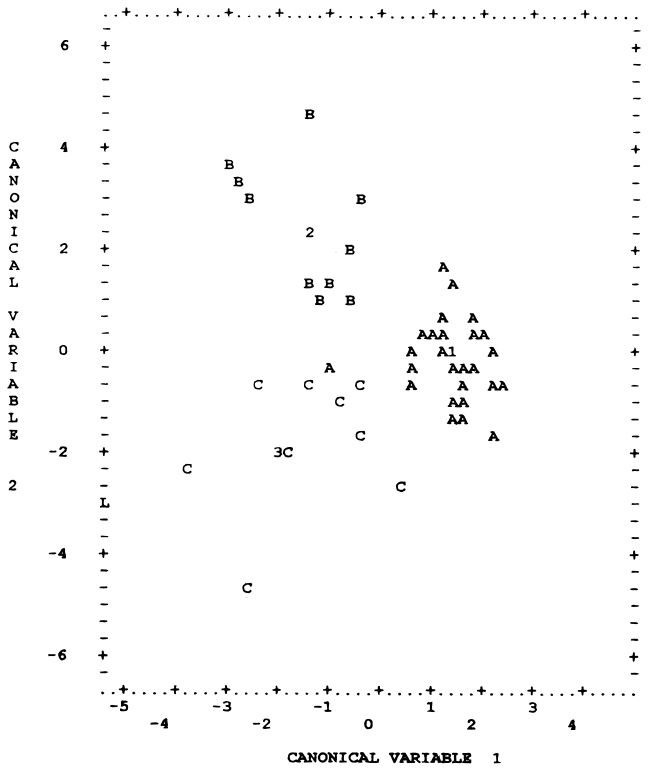

Figure 2. Stepwise discriminant analysis of ellagitannins as shown by projections of the points of each tree on the two principal canonical axes. 1, 2, and 3 are the group centroids of trees A, B, and C trees, respectively. Eigenvalue for discriminant function 1 was 2.54 and for discriminant function 2 was 2.10. Standarized coefficients for discriminant functions 1 and 2: roburin A, −0.29, 1.01; grandinin, −1.00, −1.41; vescalagin, −0.59, 1.53; roburin E, −0.82, −0.13; castalagin, 1.94, −0.90, respectively.

2, has selected all of the ellagitannins as discriminant variables. The mathematical model also accounted for 100% of the total dispersion, explained in two canonical functions. In Figure 2, a clear grouping of the points of the samples according to the tree from which they proceed is observed.

It can be concluded that the tannic composition of *Q. suber* reproduction cork shows diverse changes throughout the industrial processing, being more pronounced in the total phenol and proanthocyanidin contents than in the individual ellagitannin contents. These changes are mainly related to the boiling process, and the effect of the humidity conditions in the second rest is not as notable as it happens in the low molecular weight polyphenol contents (Conde et al., 1997a). However, the important differences in the ellagitannin contents observed in the studied trees cannot be ignored. The great concentrations of these compounds in the acqueous alcoholic extracts of cork make them important from an enological point of view. The different ellagitannis were solubilized in wine and spirits (Moutonnet et al., 1992; Viriot et al., 1993). Their oxidizing potential (Vivas and Glories, 1993, 1996) and their taste properties (Pocock et al., 1994) give them a real role in wine quality (Singleton, 1995).

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; MeOH, methanol; Et_2O, diethyl ether; *n*-BuOH, *n*-butanol.

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