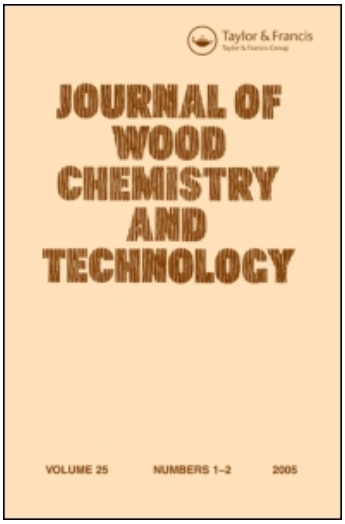

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CHEMICAL CHARACTERIZATION OF REPRODUCTION CORK FROM SPANISH *QUERCUS SUBER*

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ABSTRACT

The chemical composition of *Quercus suber* reproduction cork was studied in planks from three different trees at different stages of their industrial processing and in samples collected in seven locations in the three main Spanish production areas. Extracts in chloroform, methanol and water, neutral and acid fractions of waxes, suberin, lignin, holocellulose and pentosans, and polyphenols (low molecular weight polyphenols and tannins), were quantified. Suberin was the main component in all the samples, followed by lignin and holocellulose in lower concentrations. The most affected variables throughout the industrial processing were: lignin, chloroform and water extracts and the acid fraction of waxes. These variables did not allow one to distinguish the studied trees, which are differentiated by the percentages of methanol extracts, the tannic fraction of polyphenols, the free of suberin residue and the holocellulose content. Four variables were selected as those which provided the greatest discrimination among provenances: methanol extract, low molecular weight polyphenols, desuberinized residue and acid fraction of waxes. However, the studied populations can not be clearly distinguished by their chemical composition and no relationship was found between geographical proximity of their provenances and chemical resemblance.

INTRODUCTION

Cork is a secondary plant tissue produced from the cork cambium (phellogen) of *Quercus suber* L. Its cellular structure and chemical composition are responsible for some of its special properties, such as impermeability to liquids, elasticity and resilience, good heat and acoustic insulation and resistance to disease and to chemical and microbial attack[1-3].

The bark of the cork oak is stripped for the first time when the tree is 20-25 years old; afterwards, the stripping is carried out at intervals of 9 to 10 years. The first-generation cork is called virgin cork, and the subsequent layers, produced by regenerated phellogens, are designated reproduction cork[2-3].

Although the chemical composition of cork has been studied since the end of the XVIIIth century[4], the knowledge of the chemical characteristics of the raw material (cork planks) and its changes throughout the industrial processing is not yet complete[5], neither does a joint study of all the main components in Spanish cork from different production areas exist, wich includes comparable sampling and analytical conditions. As far as we know, a similar research was only made by Pereira[2] with Portuguese virgin cork and by Marcos de Lanuza[6], who studied the extraction with petroleum ether, sulphuric ether, ethanol and water on Spanish cork samples from different origins.

There is a extensive information on the yields of the successive extractions of cork with solvents of increasing polarity. Yields of 6.7% for $CHCl_3$, 4.4% for MeOH and 4.7% for H_2O[7], when the same extraction sequence to the one we performed was applied, or with different extractive sequence, 5.1-7.9% for lower polarity solvents (diethyl ether or dichlorometane, or 3.68-26.80% for petroleum ether and 1.78-4.00% for sulphuric ether, in a particular group of samples from Seville-Spain), 3.2-17.4%

for medium polarity solvent (ethanol) and 1.9-14.8% for more polar solvents (H_2O or acqueous Na_2SO_3)[2,6,8,9] were reported.

The main groups of components present in cork are: suberin, waxes, polyphenols, polysaccharides and lignin.

Suberin is the most important structural component of the walls of cork cells. Suberin contents of 33.2%-53.4%[2,7,9-19] were reported. This component is virtually insoluble in all solvents and therefore it must be depolymerised by hydrolysis into its monomers in order to carry out its chemical analysis. The hydrolysis product consists of ω-hydroxyacids, α,ω-dicarboxylic and epoxy acids and long-chain fatty acids and alcohols[7,21-26] and of phenolic components probably involved in the structure of suberin[26,27].

Waxes are associated with suberin in the secondary wall. They are a mixture of aliphatic hydrocarbons (n-alkanes, C_{16}-C_{34}) and alcohols (C_{20}-C_{26}, even numbers)[28,29], and triterpenes, mainly cerin and friedelin[5,30]. Global wax contents of 3.5-7.9% were given[2,7-9,31-33]. However, no data was found about the relative percentages of their neutral and acid fractions. The presence of a 0.6% of alkanoids and 0.7% of fatty alcohols in cork has been reported[28,29], but both groups of components are included in the neutral fraction, that consists also of an important quantity of triterpenic components. Moreover, a 45.8% of fatty acids in the waxes was described[33]. The fatty acids are the main components of the acid fraction, that also contains some very polar triterpenoids.

Three groups of polyphenols were described in cork: lignin, an aromatic polymer, mainly constituted of phenylpropane units; low molecular weight polyphenols, present in very low concentration, and tannins, (condensed tannins or proanthocyanidins and hydrolyzable tannins or ellagitannins and gallotannins)[5,34,35]. Contents of lignin

between 17.5 and 34.36% were reported[2,6,31,36,37]. This wide range is connected with the influence of the depolymerization method on the quantitative evaluation of this compound (like insoluble lignin-Klason lignin- and acid-soluble lignin[2], organosolv lignin-like polymer[38],"milled cork lignin"[20,39]).Regarding the polyphenolic composition, the information is very sparse, as we described in previous works[34,35]. We only found references of 6%[31] and 7%[40] for tannins and other phenolic substances, without any explanation about the composition of these polyphenolic fractions. Our own results revealed 5.46% for the total polyphenolic extract and 1.15% for the ether fraction[35].

The group of polysaccharides of cork consists of cellulose, a β-D-glucopyranose polymer, and hemicelluloses, polymers of hexoses and pentoses. Both are associated to lignin in the cell wall[2,5]. The range of carbohydrate contents described is very wide: 12-30.2%[2,31,36] of holocellulose and 4.5%[10] of pentosans.

The reported data on the chemical composition of cork are very variable and, in some cases, contradictory. The differences are probably due to the geographical origin and the tree phenology, the way the sampling was carried out, the kind of cork analyzed (virgin or reproduction cork), the cork quality and, finally, the analytical methods performed.

The aim of this work is to contribute to the knowledge on the change of the chemical composition of reproduction cork from Spanish *Quercus suber*, throughout the industrial processing and its relationship with the geographical origin of the production area. In this paper, we describe the extraction and analysis of the different groups of cork components (suberin, waxes, lignin, hollocellulose, pentosans, low molecular weight polyphenols and tannins) and we study the variability of these compounds in some planks from three different trees throughout the industrial processing of first transformation, beginning with the stripping and ending with the plank shipment to the stopper factory and also the variability in planks from several trees grown in seven different Spanish locations.

TABLE 1
Chemical Composition (% Related to Dry Cork) of Reproduction Corks from *Quercus suber* at Different Stages of the Industrial Processing

	Stripping (a)		First rest (b)		Boiling + Open Air Rest (c1)		Boiling + Store-Room Rest (c2)		Total§	
	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
Chloroform extract	10.13	33	8.08	43	4.91	15	5.32	17	7.25	35
Methanol extract	5.34	36	4.86	20	4.50	28	5.17	22	4.99	28
Water extract	6.20	43	9.90	55	9.69	26	5.93	50	7.79	45
Waxes-Neutral fraction	3.75	51	4.46	31	3.63	33	3.65	36	3.86	39
Waxes-Acid fraction	2.51	46	1.61	60	1.80	43	0.96	17	1.75	49
Free of suberin residue	45.37	11	47.79	8	43.87	23	44.50	15	45.37	15
Suberin	66.13	5	63.16	8	54.29	13	63.96	16	62.17	11
Lignin	22.21	8	19.94	7	25.07	9	23.41	6	22.64	8
Holocellulose	16.59	20	19.68	25	27.86	26	21.58	47	21.15	32
Pentosans	7.16	12	6.77	11	6.96	16	6.41	8	6.84	12
Polyphenols (total ext.)	5.87	26	5.05	18	5.86	25	5.01	21	5.46	23
Polyphenols (ether f.)	1.20	40	1.21	15	1.04	31	1.14	32	1.15	31
Polyphenols (acqueous f.)	4.05	21	3.84	19	4.58	32	3.96	19	4.10	24

Average and coefficient of variation (CV) were calculated for 15 samples at the stripping stage, 12 after the first rest, 12 after boiling with open air rest and 13 after boiling with store-room rest.
§ Average and CV were calculated for the whole 52 samples.

RESULTS AND DISCUSSION

Tables 1-3 show the results of the chemical analyses, percentages of the extracts in chloroform ($CHCl_3$), methanol (MeOH) and water (H_2O) and of desuberinized residue, and contents of waxes, suberin, lignin, holocellulose, pentosans and polyphenols. Table 1 shows the average values of each variable for all samples, at each different processing stage. On the other hand, Table 2 give the average values of each variable for all samples, from each of the three trees of the processing study. Table 3 includes the average values of each variable for each population.

It can be inferred from these tables that the most important components of cork were suberin, lignin and holocellulose, and the most variable values, according to their coefficients of variation, were water extracts, the neutral and acid fraction of waxes and holocellulose.

Comparing the average values and the coefficients of variation of our results with those reported in other publications, it can be observed that, in some cases, they were

TABLE 2
Chemical Composition (% Related to Dry Cork) of Reproduction Cork from Trees A, B and C of *Quercus suber*.

	Tree A		Tree B		Tree C		Total§	
	Average	CV	Average	CV	Average	CV	Average	CV
Chloroform extract	8.14	45	4.67	21	7.71	31	7.25	42
Methanol extract	5.20	17	3.41	21	6.17	25	4.99	21
Water extract	6.77	42	9.08	54	9.09	51	7.79	49
Waxes-Neutral fraction	3.57	29	3.67	47	4.85	41	3.86	37
Waxes-Acid fraction	1.85	58	1.62	51	1.63	68	1.75	59
Free of suberin residue	43.66	15	51.78	7	42.86	11	45.37	13
Suberin	64.43	13	58.51	13	60.22	10	62.17	12
Lignin	22.91	13	21.83	6	22.84	9	22.64	11
Holocellulose	19.03	41	26.05	24	21.42	34	21.15	35
Pentosans	7.03	13	6.79	6	6.36	14	6.84	12
Polyphenols (total ext.)	5.64	18	4.05	14	6.53	20	5.46	19
Polyphenols (ether fr.)	1.19	27	0.83	27	1.39	23	1.15	27
Polyphenols (acqueous fr.)	4.06	13	3.15	11	5.24	24	4.10	17

Average and coefficient of variation (CV) were calculated for 29 samples of A tree, 12 of B tree and 11 of C tree. § Average and CV were calculated for the whole 52 samples.

very similar. Regarding the yields of the successive solvent extractions of the cork samples, the variability found in other studies can be explained considering not only the differences in solvent polarity and sample characteristics, but in the extraction conditions of each case, as reflected by the results of Pes and Lissia[32] about cork extraction with organic solvents.

The average values concerning waxes, lignin, holocellulose and pentosans are clearly included in the range described in study above. It must be explained that our values of lignin contents (22.64%, 22.70%) are very similar to those of insoluble lignin in reproduction cork (21.8%), which was estimated in the same conditions[2]. Moreover, the high percentage of pentosans in our samples allows us to conclude that the role of hemicelluloses, especially xylans, compared with that of cellulose, seems to be more important in cork than in other lignocellulose materials, as was previously proved by Pereira[2].

The suberin contents obtained are higher than those found in the literature. As we showed in a previous work[26], the comparison among suberin contents obtained in

TABLE 3
Chemical Composition (% Related to Dry Cork) of *Quercus suber* Reproduction Corks from Different Spanish Provenances

	1		2		3		4		5		6		7		Total§	
	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
Chloroform extract	6.45	14	7.69	14	11.05	44	6.51	13	7.70	28	9.49	37	7.07	22	7.87	31
Methanol extract	4.10	35	4.57	27	3.44	12	8.23	11	6.38	23	3.46	5	3.22	18	4.75	21
Water extract	7.04	21	5.37	48	5.28	67	6.09	19	6.87	8	3.52	22	6.36	34	5.81	35
Waxes-Neutral fraction	4.50	50	4.77	58	8.57	56	7.11	65	3.59	36	5.08	31	6.48	28	5.80	53
Waxes-Acid fraction	0.90	24	1.02	79	2.02	81	1.49	19	1.38	45	2.94	30	2.41	19	1.71	46
Free of suberin residue	54.03	11	52.40	16	57.54	12	51.11	11	48.06	20	35.85	20	61.14	7	51.97	13
Suberin	63.88	14	67.59	19	70.29	9	58.76	25	52.92	38	57.67	20	53.64	19	60.97	20
Lignin	21.99	7	22.26	10	21.48	7	22.03	10	23.28	15	23.69	1	24.32	11	22.70	9
Holocellulose	23.53	36	22.88	26	15.31	37	24.00	64	34.34	59	31.21	36	32.21	35	25.88	44
Pentosans	6.22	11	5.89	2	5.63	2	5.91	11	6.52	6	5.78	3	5.72	3	5.94	7
Polyphenols (total ext.)	5.94	22	4.60	23	3.52	26	7.89	26	5.27	15	2.76	36	2.54	17	4.70	26
Polyphenols (ether f.)	1.18	17	1.33	44	0.69	11	1.55	13	1.27	7	0.50	31	1.62	25	1.19	27
Polyphenols (acqueous f.)	2.25	62	3.95	36	3.43	15	6.40	17	5.17	24	3.19	13	1.03	19	3.55	28

1. Almadén de la Plata; 2. Los Barrios; 3. Medina Sidonia; 4. El Chaparral; 5. Jerez de los Caballeros; 6. Maçanet de Cabranys; 7. Forallac. Average and coefficient of variation (CV) were calculated for 5 samples in Almadén de la Plata, Los Barrios, El Chaparral and Forallac, 4 samples in Medina Sidonia and Maçanet de Cabranys and 3 samples in Jerez de los Caballeros.
§ Average and CV were calculated for the whole 31 samples.

different depolymerization and analysis conditions is difficult and these results can only be precisely confronted when the work conditions are the same. We have calculated this percentage from the $CHCl_3$ extract of the depolymerization mixture obtained by methanolysis of cork free of extracts (see Experimental). The $CHCl_3$ extract was dried under very mild conditions, in order to avoid oxidation or degradation of its components, and the pasty nature of this extract made it difficult to eliminate completely the solvent in the rotary evaporator. We carried out drastic drying conditions on a group of model samples in order to estimate the solvent contents of the $CHCl_3$ extract dried in the rotary evaporator. The solvent contents reached values of 40%, which implies an overvaluation of suberin contents by about 20%. Taking into account this overvaluation, our values would come into ranges described in other studies.

Regarding polyphenolic composition, the global values of other publications are not significantly different from ours. As can be observed, the tannin fraction (aqueous fraction) was much more important than the low molecular weight polyphenols fraction (ether fraction) in the polyphenolic extract (4.10% or 3.55% versus 1.15% or 1.19%). Moreover, it must be considered that MeOH-H_2O (80:20) extractives (total extractives) and ether extractives 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) and transferred to the ether fraction.

The performance of the pairwise *T*-test of all the variables, grouped by stages or trees and by provenances, gave the significance levels shown in Tables 4 and 5.

Considering the chemical composition of samples in the different stages of industrial processing (Tables 1 and 4), there were no significant differences between stripping and first rest stages, except in lignin content, slightly lower in the samples taken just after the first rest. Moreover, there was a decreasing trend in the average values of $CHCl_3$ extract (waxes), throughout the industrial processing, low in the two

TABLE 4

Significance Levels of Pairwise *T*-Test of all the Components Grouped by Stages and Trees (variances are not assumed to be equal).

	a *versus*		b *versus*		c1 *versus*	A *versus*		B *versus*
	b	c1	c2	c1	c2	c2	B	C	C
Chloroform extract		***	***	-			***		**
Methanol extract							***		***
Water extract		*				*			
Waxes-Neutral fraction									
Waxes-Acid fraction			***			*			
Free of suberin residue							***		***
Suberin		***		*		-			
Lignin	**	*		***	***				
Holocellulose		**		*			*		
Pentosans			-						
Polyphenols (total ext.)							***		***
Polyphenols (ether fr.)							***		***
Polyphenols (acqueous fr.)							***	•	***

a=Stripping; b= first rest; c1= boiling followed by open air rest; c2= boiling followed by store-room rest
*** 0.1% significance; ** 1% significance; * 5% significance; - 10% significance; no symbol indicates >10% significance

first stages, but much greater after the stages of boiling, followed by resting in the open air (c1) or in store-room (c2), with very significant differences among stripping and the two last stages. This behaviour was also observed in the acid fraction of waxes, with very important differences between the values obtained after the stripping and the c2 stage. The decrease observed in the content of suberin after the boiling process, when it was followed by resting in the open air, can be underlined. But no significant differences were found when the rest look place in store-room. Since suberin is responsible for some of the special properties of cork[1,2] (impermeability, elasticity, insulation, resistance to the chemical and microbial attack), the decrease of its concentration would suppose a partial loss of these properties. Therefore, the store-room rest is recommended instead of the open air rest in order to preserve the cork quality. The main differences among the samples taken after the first rest stage ("maturation") and the two after boiling stages are due to the contents of lignin. However, the two last stages can hardly be distinguished by their chemical composition, because only slightly significant differences were obtained.

TABLE 5
Significance Levels of Pairwise *T*-Test of all the Components Grouped by Provenances (variances are not assumed to be equal).

	1 *versus*						2 *versus*					3 *versus*				4 *versus*			5 *versus*		6 *versus*
	2	3	4	5	6	7	3	4	5	6	7	4	5	6	7	5	6	7	6	7	7
Chloroform extract																					
Methanol extract			*					*				**					**	***			
Water extract					-														*		
Waxes-Neutral fraction																					
Waxes-Acid fraction						*															
Free of suberin residue														-							-
Suberin																					
Lignin																					
Holocellulose																					
Pentosans																					
Polyphenols (total ext.)					-	-											-	-			
Polyphenols (ether fr.)		-			*							**	*				***		*		*
Polyphenols (acqueous fr.)			*									*			*		*	**			*

1. Almadén de la Plata; 2. Los Barrios; 3. Medina Sidonia; 4. El Chaparral; 5. Jerez de los Caballeros; 6. Maçanet de Cabranys; 7. Forallac.
*** 0.1% significance; ** 1% significance; * 5% significance; - 10% significance; no symbol indicates >10% significance

There was also an important variability among samples at each of the stages, especially significant in the case of the waxes acid fraction in the three first stages, the waxes neutral fraction in the stripping stage, the water extracts in the b and c2 stages and the holocellulose contents in c2.

In the stepwise discriminant analysis among stages, six variables were selected, step by step, as those providing the greatest discrimination among stages: lignin, chloroform extracts (waxes), desuberinized residue, water extracts, acid fraction of waxes and total polyphenols fraction, and the resulting mathematical model explained the 100% of the total dispersion in three canonical functions. Figure 1 is a graphical representation of the projections of the points of each group on the plane defined by the two principal canonical axes, originated at the end of the statistical process, that represent 93% of the total dispersion. Although the group centroids are separated in this figure, the sets of points of the four stages were partially overlapped.

Considering the differences in composition among trees (Tables 2 and 3), it can be emphasized that A and C trees can not be distinguished, but B tree presents very significant differences to A and C trees. These differences were shown in the $CHCl_3$ and MeOH extracts, the contents of all the polyphenols fraction, related to the MeOH extracts, and the free of suberin residue. In addition, there were important differences in the composition of the different samples from each tree (Table 2). This concentration variability was greater for the water extracts and the acid fraction of waxes.

The graphical representation of the results of the discriminant analysis, considering the different trees (Figure 2), allows us also to distinguish B samples from the A and C samples. The sets of points of A and C overlapped, but were different from those of B. In this case, the variables selected by the discriminant analysis were the aqueous fraction of polyphenols, methanol extracts, pentosans, desuberinized residue and holocellulose. It must be said that only the desuberinized residue was selected in both

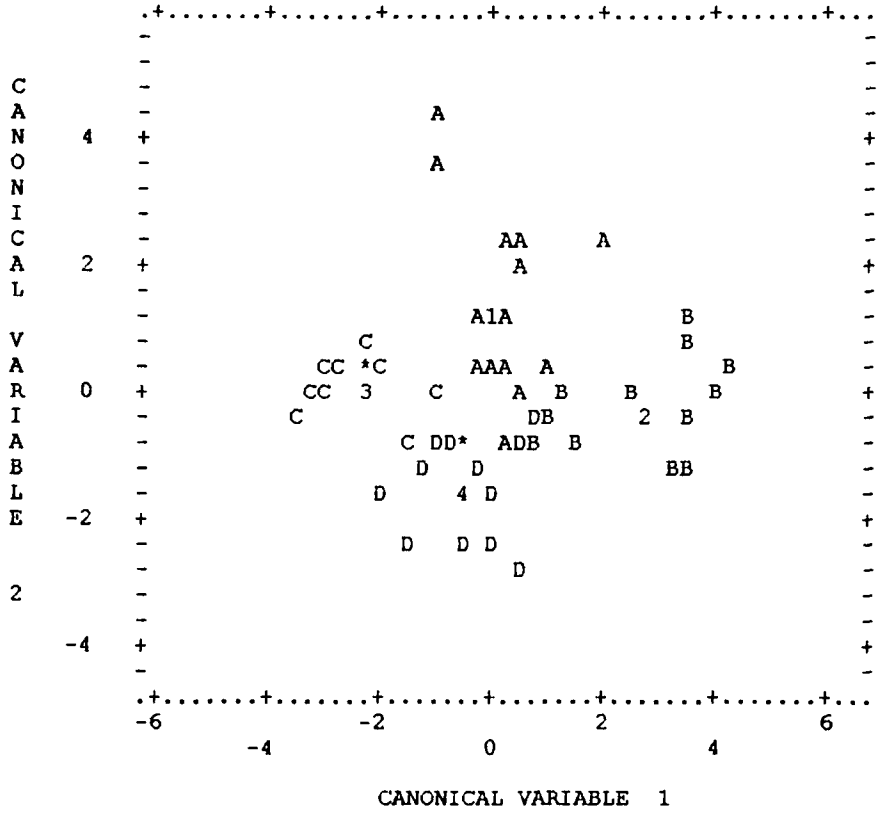

FIGURE 1. Stepwise discriminant analysis of the contents of each group of components. Projections of the points of each industrial processing stages on the two principal canonical axes. A = Stripping (a); B = First rest (b); C = Boiling followed by open air rest (c1); D = Boiling followed by store-room rest (c2). 1, 2, 3 and 4 are the group centroids for each stage, respectively.

discriminant analysis: trees and processing stages. The mathematical model also accounted for 100% of the total dispersion, explained in only two canonical functions. Therefore, Figure 2 is an accurate graphical representation of the statistical separation of the samples from the different trees.

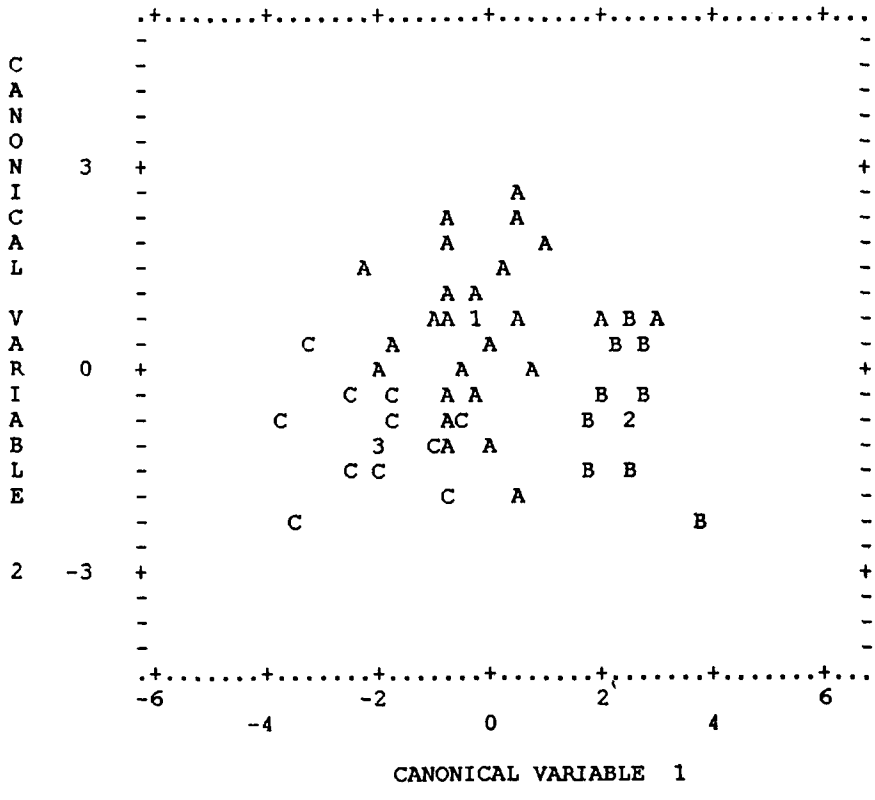

FIGURE 2. Stepwise discriminant analysis of the contents of each group of components. Projections of the points of each tree on the two principal canonical axes. 1, 2, 3 are the group centroids of A, B and C trees, respectively.

Considering the chemical composition of samples from different populations (Tables 3 and 5), it can be observed that some variables, chloroform extract, waxes neutral fraction, free of suberin residue, suberin, lignin, holocellulose and pentosans did not show significant differences among provenances. The more pronounced significant differences were found among the population 3 *versus* 4 and 4 *versus* 6 and *versus* 7. Moreover, it is important that these differences, when present, are not

associated with structural components (suberin, lignin, holocellulose), but with minor components (methanol extract and, therefore, polyphenols), whouse concentrations are more dependent on the growth conditions. In fact, the studies of Marcos de Lanuza[6] revealed the influence of the North or South orientation of the sample in the tree, the age of the tree, the distance of the sample from the base of the tree and the situation of the sample in the stem or in the branch in the extract contents of cork. The differences found in our results are also very similar to those obtained by Pereira[2] on virgin cork, which showed that the content of extractives and of polysaccharides varies among locations, but suberin and lignin contens are not significantly different.

No clear relationship was found between geographical proximity of the provenances and chemical similarity. For example, although there are logical resemblances among populations of close provenances, 2 and 3, both from the Cádiz province, and 4 and 5, both from the Extremadura region, there are no differences among populations from very distant geographical origins, such as 2, from the Cádiz province and 6 and 7, both from the Gerona province, or 5 from the Badajoz province and 7. Moreover, 3 shows a similar significance level of differences with 5, a relatively close provenance, and 6 and 7, both very far apart.

There was also an important variability among samples of each population, especially pronounced for the acid fraction of waxes in 2 and 3, the neutral fraction of waxes in 1, 2, 3 and 4, the water extract in 3, holocellulose in 4 and 5 and the acqueous fraction of polyphenols in 1. This between-tree variability is in accordance with previous studies about cork composition[2,6,26,34,35]. The populations from the Gerona province (6 and 7) seem to be less variable than the others. This lower variability might perhaps be due to the fact that the intervals of years among strippings are higher in the Gerona province.

In the stepwise discriminant analysis among provenances, four variables were selected, step by step, as those providing the greatest discrimination among

provenances: methanol extract, ether polyphenols fraction, desuberinized residue and waxes acid fraction, and the resulting mathematical model explained 100% of the total dispersion in four canonical functions. Figure 3 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, that represented a cumulative proportion of 92% of the total dispersion. Only the sets of points of 6 and 3 were clearly separated from the others. Moreover, the group of points of 7 did not overlap with those of 4 and 5.

As can be deduced from the above results, the industrial processing of first transformation causes several chemical changes in cork. The first rest stage only produces a decrease in the lignin percentage. The more pronounced changes were observed after the boiling process followed by a resting period in the open air or inside a store-room. In both cases, the boiling process seems to produce structural changes in cell wall of cork, mainly showed in a variation of the wax contents, but also in the suberin and lignin percentages. The variation of the wax content was associated with a decrease in their acid fraction in the stage after boiling with store-room rest. The samples submitted to a rest in the open air after boiling showed also a decrease in the suberin and an increase in the holocellulose contents. It is possible that the structural disposition of the cell wall resulting after this stage made the access of the methanolysis reactive to the suberin polymer difficult, obtaining, therefore, lower contents of suberin, and subsequently, higher values of holocellulose. These two chemical changes can be related to a loss of the special physical properties of cork and, therefore, of the final cork quality. Moreover, the differences in chemical composition among the trees studied and among the samples from seven different Spanish production areas are not associated with structural components, but with minor components, whose concentration is more dependent on climatic conditions, tree phenology and distance of the sample from the base of the tree. These minor components must be considered in the most important use of cork, wine closure, and in-bottle evolution of wine.

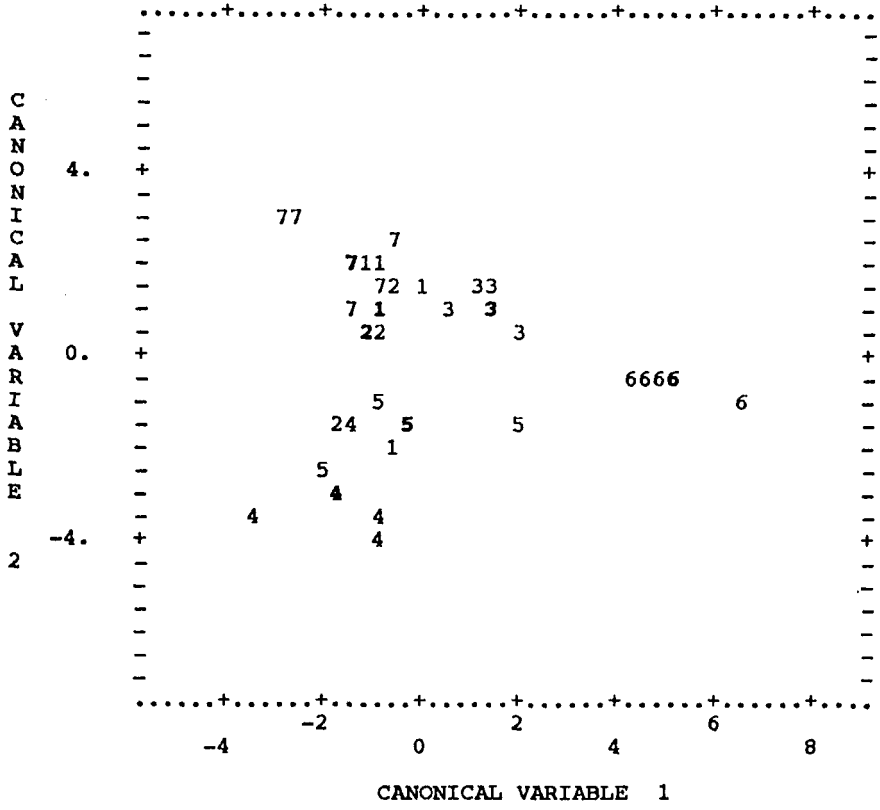

FIGURE 3. Stepwise discriminant analysis of contents of each group of components. Projections of the points of each provenances on the two principal canonical axes. 1 = Almadén de la Plata; 2 = Los Barrios; 3 = Medina Sidonia; 4 = El Chaparral; 5 = Jerez de los Caballeros; 6 = Maçanet de Cabranys; 7 = Forallac;. **1, 2, 3, 4, 5, 6** and **7** are the group centroids for each provenance, respectively.

EXPERIMENTAL

Samples

a) Industrial processing study:

Reproduction cork planks were collected from 3 trees (A, B and C) of *Quercus suber* grown in Constantina, located in Northern mountains of the Seville province (Spain): 3 planks from A tree, 1 plank from B tree and 1 plank from C tree. Three pieces (20 x 20 cm) were picked out 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 the stripping until the boiling process, the cork planks remained piled in the field or in the factory, for 5 months.

c1) Boiling and open air rest: The planks were introduced in boiling water for 1 h. After boiling, the planks were piled in the open air for two weeks, to lose the water absorbed in the previous treatment.

c2) Boiling and store-room rest: The boiling process was carried out as described before, but the two weeks rest was carried out inside a store-room, where a high relative humidity (80-100%) atmosphere is maintained. During this period, a microorganisms proliferation takes place over the plank surface.

b) Provenances study:

Reproduction cork samples were collected from trees grown in seven different localities of the three most important production areas in Spain: Andalucía, Extremadura and Cataluña. Table 6 includes these populations and their UTM

TABLE 6
Sample provenances and their UTM Coordinates

Region	Province	Locality(*)	Coordinates
Andalucía	Sevilla	Almadén de la Plata(1)	29SQC601921
Andalucía	Cádiz	Los Barrios(2)	30STF708225
Andalucía	Cádiz	Medina Sidonia(3)	30STF587221
Extremadura	Cáceres	El Chaparral(4)	29SPC978508
Extremadura	Badajoz	Jeréz de los Caballeros(5)	29SPC857486
Cataluña	Gerona	Maçanet de Cabranys(6)	31TDG795926
Cataluña	Gerona	Forallac(7)	31TEG053394

(*) Identification number in the text

coordinates. 3-5 trees were selected in each locality and pieces of the planks from each tree were chosen in order to obtain similar commercial quality cork samples.

Sample Extraction

The cork samples free of outer corkbark were ground and sieved. The powdered cork (2 g, 0.5-1mm particle size) was successively and exhaustively extracted in Soxhlet with $CHCl_3$ (8 h), MeOH (8 h) and H_2O (16 h). $CHCl_3$ removes waxy materials and MeOH and H_2O extract free phenolic compounds with different molecular weights and polarities which could be wrongly associated with suberin structure.

Evaluation and Saponification of Cork Waxes

The $CHCl_3$ extract was dried under vacuum. Wax contents were determined from the weight of the dried $CHCl_3$ extract. The dried $CHCl_3$ extract was submitted to

saponification with 50 mL 0.5N KOH in EtOH boiling the mixture for 1 h under reflux. After adding 50 ml H_2O, the saponification mixture was extracted with petroleum ether (3 x 15 mL). The ether petroleum fraction (neutral components fraction) was dried over anh. Na_2SO_4 and the solvent was distilled under vacuum.

The remaining aqueous solution was acidified with 12M HCl and extracted again with petroleum ether (3 x 15 mL). This second petroleum ether fraction, mainly composed of acid wax components, was also dried over anh. Na_2SO_4 and the solvent was distilled.

Depolymerization and Isolation of Suberin Constituents

The cork samples free of extractives (1 g) were depolymerized by boiling under reflux for 18 h with 50 mL 0.5M NaOMe in dry MeOH in the presence of 2% (V/V) dry methyl acetate. Methyl acetate was used as a hydroxide scavenger to prevent the hydrolysis of the methyl esters formed[1,23,26,41].

The depolymerization mixture was filtered, acidified to pH 6 with 6N H_2SO_4 and dried under vacuum. After the addition of 50 mL H_2O to the residue, the suspension was extracted with $CHCl_3$ (4 x 20 mL). The combined $CHCl_3$ extracts were dried over anh. Na_2SO_4 and the solvent was removed by vacuum distillation.

Lignin Determination

Lignin contents were estimated in the cork free of extractives and suberin, in order to eliminate all the materials prone to condensation by a sulfuric acid treatment, which would contribute to the Klason residue[20]. The Tappi-T-13 wd-74 method was employed.

Holocellulose Determination

Holocellulose contents were also estimated in the cork free of extractives and suberin, by delignification with sodium clorite and acetic acid, following the Wise-Murphy method[42].

Pentosans Determination

The method used for pentosans determination was based on their HCl hydrolysis to furfural, and the valuation of furfural by reaction with orcinol-HCl, following the Tappi-223 os-78 standard.

Extraction and Fractionation of Polyphenols

Ground and sieved cork samples (2 g, 0.5-1mm particle size) were extracted with 150 mL 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 was extracted with Et_2O. The dried Et_2O extract contained the low molecular weight polyphenols fraction[35]. The remaining aqueous solution was freeze-dried and the residue obtained was considered to be tannin fraction[34].

Statistical Analysis

Data were analyzed using the BMDP package. Univariate analysis (BMDP P7D) and stepwise discriminant analysis (BMDP P7M) were carried out. In univariate analysis, average, standard deviation and coefficient of variation were calculated, 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 stepwise manner[43]. 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.

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