Polyphenolic Composition of *Quercus suber* Cork from Different Spanish Provenances

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Polyphenolic composition was studied by HPLC and classical chemical methods in reproduction cork of *Quercus suber* from different Spanish provenances. The low molecular weight polyphenols (gallic, protocatechuic, vanillic, caffeic, ferulic, and ellagic acids; protocatechuic, vanillic, coniferyl, and sinapic aldehydes; and aesculetin and scopoletin) and the ellagitannins (roburins A and E, grandinin, vescalagin, and castalagin) were identified and quantified. Ellagic acid was the main component in the ether soluble fraction, and the group of hydrolyzable tannins, and among them castalagin, was the most abundant in the tannic extract in all the samples. Although there was an important variability among provenances, no significant differences were found in the total tannin content and in the individual content of each ellagitannin. However, gallic and caffeic acids and protocatechuic aldehyde provided the greatest discrimination among provenances.

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

INTRODUCTION

Studies on the polyphenolic composition of *Quercus suber* cork have shown the presence of different groups of components that can be extracted from cork in aqueous alcoholic solution, which are susceptible to migrating into wine after bottling and can modify the wine properties. Therefore, the polyphenols must be considered in the studies on cork−wine interactions.

In a previous work (Conde et al., 1997), we identified in reproduction cork the following low molecular weight polyphenols: gallic, protocatechuic, vanillic, caffeic, ferulic, and ellagic acids; protocatechuic, vanillic, coniferyl, and sinapic aldehydes; and aesculetin and scopoletin. Some of them revealed marked quantitative differences among trees (protocatechuic acid and aldehyde and vanillin) or concerning the industrial processing stages of the first transformation (coniferyl aldehyde, sinapic aldehyde, and ellagic acid).

Regarding the tannic composition of cork, the scarce studies refer only to global valuations of tannins (Pereira, 1979, 1988). The quantitative valuations of the different tannin groups underscored the small amount of tannins extracted by MeOH/H_2O, and the group of ellagitannins was the most representative of this polyphenolic extract (Cadahía et al., 1996).

We have identified in cork of *Quercus suber* (Cadahía et al., 1996) several ellagitannins with wide distribution in the wood and bark 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). These were roburins A and E, grandinin, vescalagin, and castalagin, besides other ellagitannins with related structures. Both the global content of different groups of tannins and the content of each of the ellagitannins varied notably among the different trees and even among the samples of the same tree.

The aim of this work is the study of the variability of the polyphenolic composition of Spanish cork from different provenances. The polyphenols were extracted from reproduction cork samples from three different Spanish production areas. The global valuation of the different groups of tannins and the quantification of the low molecular weight polyphenols and the main ellagitannins described in cork were carried out.

EXPERIMENTAL PROCEDURES

Samples. 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 1 includes these populations and their UTM coordinates and site conditions. Three to five trees were selected in each provenance, and some pieces of the planks of each tree were chosen to obtain similar commercial quality cork samples. To minimize the site effect, samples were selected within fixed limits for canopy (40−80%), stand density (40−100 ft/ha), phytoclimate, altitude, and substratum (see Table 1).

Standards. Reference compounds of low molecular weight polyphenols were purchased from Fluka Chemie AG, Buchs, Switzerland (gallic and caffeic acids, aesculetin, and scopoletin), Aldrich Chemie, Neu-Ulm, Germany (protocatechuic, vanillic, sinapic, and ferulic acids, syringaldehyde, and coniferaldehyde), Apin Chemicals Ltd., Nr Abindgon, Oxon, U.K. (ellagic acid), Merck, Darmstadt, Germany (vanillin), and Sigma Chemical Co., St. Louis, MO (protocatechuic aldehyde). 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., 1997). The suspension was filtered, and MeOH was removed by vacuum distillation. The resulting aqueous solution (solution I) was extracted with Et_2O (3 × 10 mL) and freeze-dried. The Et_2O extract was dried and

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Table 1. UTM Coordinates and Site Conditions of Each Spanish Provenance

region	province	site	coordinates	altitude	phytoclimate	substratum
Andalucía	Sevilla	Almaden de la Plata	29SQC601921	435	IV_4	granite
Andalucía	Cádiz	Los Barrios	30STF708225	286	IV_2	sandstone
Andalucía	Cádiz	Medina Sidonia	30STF587221	160	IV_2	sandstone
Extremadura	Cáceres	El Chaparral	29SPC978508	360	IV_4	slate
Extremadura	Badajoz	Jeréz de los Caballeros	29SPC857486	400	IV_4	sandstone
Cataluña	Gerona	Maçanet de Cabranys	31TDG795926	370	$VI(IV_4)$	granite
Cataluña	Gerona	Forallac	31TEG053394	260	$IV(VI)_2$	gneiss

Table 2. Extraction Yields and Total Phenol, Ellagitannin, and Proanthocyanidin Contents of Cork Extracts from Different Spanish Origins[a]

	1		2		3		4		5		6		7		global[b]	
	x	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD
ether fraction[c]	11.78	2.02	13.32	5.91	6.93	0.76	15.46	1.99	12.73	0.95	5.02	1.53	16.22	4.01	11.93	3.21
aqueous fraction[c]	22.54	13.91	39.48	12.86	34.25	5.29	64.00	10.80	51.67	12.59	31.93	4.27	10.03	1.90	35.52	9.94
total phenols[d]	6.78	2.80	9.96	1.75	9.30	0.58	9.36	5.20	9.42	3.96	7.78	1.51	5.40	0.70	8.20	3.02
ellagitannins[e]	2.03	1.25	2.12	0.81	2.28	0.51	1.92	0.67	2.86	0.92	1.98	0.94	1.66	0.41	2.07	0.81
proanthocyanidins[f]	0.20	0.05	0.47	0.18	0.30	0.04	0.22	0.09	0.36	0.22	0.22	0.01	0.16	0.04	0.27	0.14

[a] 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 (*x*) and standard deviation (STD) were calculated for five samples in Almadén de la Plata, Los Barrios, El Chaparral, and Forallac, for four samples in Medina Sidonia and Maçanet de Cabranys, and for three samples in Jerez de los Caballeros. [b] Average and STD were calculated for the whole 31 samples. [c] Milligrams per gram. [d] Expressed in milligrams of gallic acid per gram of dry cork; gallic acid molar absorbance is 22.3×10^3. [e] In milligrams of ellagic acid per gram of dry cork. [f] In milligrams of cyanidin per grams of dry cork.

redissolved in MeOH for HPLC analysis (Conde, 1994; Conde et al., 1995a,b, 1996, 1997; Fernández de Simón et al., 1996a,b). Solution I was used for the 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. The classical chemical evaluations were carried out following the Folin–Ciocalteu method for total phenols (Singleton and Rossi, 1965) and the method of oxidative hydrolysis in *n*-BuOH–HCl to anthocyanidins for proanthocyanidins (Porter et al., 1986). Ellagitannins were estimated by HPLC evaluation (Conde et al., 1995b) of the ellagic acid yielded after they were submitted to methanolysis in MeOH/HCl. Two hundred fifty microliters of solution I was freeze-dried, 1 mL of 6 N MeOH/HCl (9:1) was added, and the mixture was heated at 100 °C for 4 h (Cadahía et al., 1996).

Polyphenol Identification. Identifications were carried out by comparing the UV spectra and the chromatographic behaviors (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 used for elution: solvent A, MeOH/H_3PO_4 (999:1); and solvent B, H_2O/H_3PO_4 (999:1). Two different gradient profiles were used: 0–40 min (20–100% A), 40–45 min, 100% A (isocratic), for low molecular weight polyphenols analysis; and 0–40 min (0–10% A), 40–70 min (10–30% A), 70–90 min (30–100% A) (isocratic), for tannin analysis. 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).

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

Statistical Analysis. Data were analyzed using the BMDP package. Univariate analysis (BMDP P7D) and stepwise discriminant analysis (BMDP P7M) for the groups of low molecular weight polyphenols and 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 stepwise manner (Jennrich and Sampson, 1985). Both forward and backward selections of variables were possible; at each step, the variable that adds the highest degree of separation among 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 on the extraction yields with MeOH/H_2O (ether and aqueous fraction) and total phenol, proanthocyanidin, and ellagitannin contents in cork samples from the different Spanish provenances are shown in Table 2.

Regarding the ether fraction, associated with the low molecular weight polyphenolic components, two populations, Medina Sidonia and Maçanet de Cabranys, showed the lowest values, the opposite from El Chaparral and Forallac with the maxima values of ether extractives. The aqueous fraction contained the polymeric polyphenols, and its values indicate a much higher contribution of the tannins to the MeOH/H_2O extractives compared with that of low molecular weight polyphenols. The only exception was the Forallac population, with 16.22 mg/g of ether fraction and 10.03 mg/g of aqueous fraction. The highest values of aqueous fraction were derived from El Chaparral and Jerez de los Caballeros, and the lowest value was from Forallac. The more notable significant differences were found between El Chaparral and Forallac.

Referring to the total phenol, ellagitannin, and proanthocyanidin contents, it can be seen in Table 2 that the group of ellagitannins was more abundant than proanthocyanidins in the tannin extract of all the samples studied and also presented a high contribution to the total phenol content. The samples have shown an important variability in tannin contents, particularly in total phenols and ellagitannins, as can be deduced from their high dispersity. The highest total phenol contents concerned were for samples from Los Barrios,

Table 3. HPLC Quantitative Evaluation of Low Molecular Weight Polyphenols (Micrograms per Gram, Related to Dry Cork) in the Extracts of *Q. suber* Cork from Different Spanish Provenances[a]

	1		2		3		4		5		6		7		global[b]	
	x	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD
gallic acid	3	1	21	7	10	3	25	4	19	4	7	4	3	6	12	5
protocatechuic acid	6	7	65	31	8	9	126	57	75	1	17	13	8	17	44	28
protocatechuic aldehyde	5	5	22	6	7	4	10	3	10	2	8	2	3	5	9	4
aesculetin	3	2	9	2	6	2	8	2	9	5	5	2	3	1	6	2
vanillic acid	6	2	40	18	44	23	25	10	18	3	57	21	13	9	28	14
caffeic acid	1	1	10	8	18	9	3	3	2	1	22	9	4	3	8	6
vanillin	7	7	28	19	11	6	24	10	21	7	18	13	16	8	18	11
scopoletin	6	4	16	6	4	5	16	3	16	9	0	0	1	1	8	4
ferulic acid	3	2	21	19	38	21	7	5	4	1	50	16	22	12	20	13
coniferaldehyde	15	14	22	5	15	4	19	3	19	8	14	2	20	6	18	7
sinapaldehyde[c]	3	2	0	0	0	0	3	2	3	2	0	0	0	0	1	1
ellagic acid	111	51	313	68	156	123	327	79	272	97	47	47	115	21	192	72

[a] 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 (*x*) and standard deviation (STD) were calculated for five samples in Almadén de la Plata, Los Barrios, El Chaparral, and Forallac, for four samples in Medina Sidonia and Maçanet de Cabranys, and for three samples in Jerez de los Caballeros. [b] Average and STD were calculated for the whole 31 samples. [c] Expressed as sinapic acid.

Table 4. HPLC Quantitative Evaluation of Ellagitannins (Micrograms per Gram, Related to Dry Cork) in the Extracts of *Q. suber* Cork from Different Spanish Provenances[a]

	1		2		3		4		5		6		7		global[b]	
	x	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD	*x*	STD
roburin A	30	23	75	61	72	46	75	59	46	29	49	37	16	7	51	44
grandinin	63	38	248	180	238	137	309	327	149	69	150	107	47	13	172	177
vescalagin	31	46	56	47	69	47	60	81	74	72	124	170	14	16	58	77
roburin E	53	47	145	129	74	37	83	37	74	35	82	59	32	26	78	68
castalagin	181	221	709	286	619	214	506	682	443	256	443	285	82	68	418	383

[a] 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 (*x*) and standard deviation (STD) were calculated for five samples in Almadén de la Plata, Los Barrios, El Chaparral, and Forallac, for four samples in Medina Sidonia and Maçanet de Cabranys, and for three samples in Jerez de los Caballeros. [b] Average and STD were calculated for the whole 31 samples.

Medina Sidonia, El Chaparral, and Jerez de los Caballeros (average between 9.30 and 9.96 mg/g). However, there were significant differences only between Cádiz provenances, Los Barrios or Medina Sidonia, and Forallac, this one from Gerona. Neither of these populations from Gerona showed common behavior, since there were no significant differences with Maçanet de Cabranys, also from the Gerona province, when compared with the other provenances. Regarding the ellagitannin contents, no significant differences were found among the origins. For proanthocyanidin contents, significant differences were found only between Medina Sidonia and Forallac provenances.

As can be concluded from the results presented above, the values of the variables, total extractives, ether and aqueous fractions, and total phenol, ellagitannin, and proanthocyanidin contents did not present a common behavior related to the geographical provenances of the populations.

As we have described in a previous work (Conde et al., 1997), in the ether fractions of the cork extracts, the following low molecular weight polyphenols were identified: gallic, protocatechuic, vanillic, caffeic, ferulic, and ellagic acids; protocatechuic, vanillic, coniferylic, and sinapic aldehydes; and the coumarins aesculetin and scopoletin. The presence of these phenolic acids and aldehydes and coumarins has been also described in the woods of other *Quercus* species (Black et al., 1953; Pearl et al., 1957; Guymon and Crowell, 1968; Chen, 1970; Seikel et al., 1971; Joseph and Marché, 1972; Miller et al., 1992; Fernández de Simón et al., 1996a,b), with the exception of protocatechuic aldehyde. Moreover, syringic acid and aldehyde, also present in woods of other *Quercus* species, were not detected in the samples of cork analyzed.

The HPLC analysis of the tannic extracts from cork samples of different origins shows, as main components, the ellagitannis roburins A and E, grandinin, vescalagin, and castalagin. They have been previously described in cork of *Q. suber* among other ellagitannins with related structures and other ellagic acid derivatives (Cadahía et al., 1996) and, as we have mentioned before, they are widely distributed in the wood and bark 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)

The HPLC quantitative evaluations of the low molecular weight polyphenols in the ether soluble fractions and of the ellagitannins in the aqueous soluble fractions of the cork samples are shown in Tables 3 and 4. Components are arranged according to their retention times in the chromatograms.

Phenolic acids were the most abundant low molecular weight polyphenols in cork. Considering the global average values (Table 3), the main component of this group was ellagic acid, followed in decreasing order of abundance by protocatechuic, vanillic, ferulic, and, finally, gallic and caffeic acids, with lower concentrations. The concentrations of caffeic, protocatechuic, and ferulic acids were the most variable among those of the acids identified in the ether soluble fractions of cork extracts. Benzoic and cinnamic aldehydes and coumarins were minor components, with sinapaldehyde being both the least abundant and most variable.

Table 5 includes the results of the significance levels of the pairwise *t*-test of all the low molecular weight

Table 5. Significance Levels of Pairwise *t*-Test of All the Components Grouped by Provenances (Variances Are Not Assumed To Be Equal)[a,b]

	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
gallic acid	–		**								*	*					**	**		–	
protocatechuic acid				***									*						–	*	
protocatechuic aldehyde	*										*										
aesculetin	–										**							*			
vanillic acid																					
caffeic acid																					
vanillin																					
scopoletin			*							–	–						**	**			
ferulic acid																					
coniferaldehyde																					
sinapaldehyde																					
ellagic acid	*		*							**	*						**	–			

[a] 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. [b] ***, 0.1% significance; **, 1% significance; *, 5% significance; –, 10% significance; no symbol indicates >10% significance.

polyphenols grouped by populations from different geographic provenances. Regarding the differences in composition among populations, it can be deduced that six components, vanillic, caffeic, and ferulic acids, vanillin, coniferyl aldehyde, and sinapic aldehyde, did not present significant differences among populations. Moreover, the remaining components did not present pronounced significant differences, with the exception of the differences in the concentrations of protocatechuic acid between Almadén de la Plata and Jerez de los Caballeros provenances. The populations that showed a higher number of significant differences were Almadén de la Plata versus El Chaparral, Los Barrios versus Forallac, and El Chaparral versus Maçanet de Cabranys and versus Forallac. However, they did not show a unique pattern of differences, and the components implicated were not the same in all cases.

Moreover, there were important differences in the low molecular weight polyphenolic composition when the different samples of each individual population (high STD, Table 3) were considered. This concentration variability was particularly pronounced in Forallac samples for gallic and protocatechuic acids and scopoletin.

In the stepwise discriminant analysis among provenances, considering the contents of these low molecular weight polyphenols as variables, three components were selected as those that provided the greatest discrimination among populations: gallic and caffeic acids and protocatechuic aldehyde. The mathematical model resulting accounted for 100% of the total dispersion, explained in three canonical functions. Figure 1 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 91% of the total dispersion. The sets of points of Almadén de la Plata, Medina Sidonia, Maçanet de Cabranys, and Forallac (1, 3, 6, and 7, respectively) were completely overlapped and separated from those of Los Barrios (2), on the one hand, and, on the other, from those of El Chaparral and Jerez de los Caballeros (4 and 5, both from Extremadura region), also overlapped. As happens with the extractive values and the tannin contents, there is no relationship between the geographical nearness of the populations and the chemical similarity in the low molecular weight polyphenol composition. Only the Extremadura populations have shown a particular behavior.

The HPLC quantitative evaluation of the ellagitannins (Table 4) revealed that the most abundant ellagitannin in all provenances was castalagin, with a mean concentration of 383 μg/g referred to dry cork. It must be considered that these results would have changed depending on the extraction solvent used, due to the tendency of these ellagitannins to undergo degradation and polymerization reactions in some solvents. Castalagin maximum levels are shown in Los Barrios, Medina Sidonia, and El Chaparral, with concentrations of 709, 619, and 506 μg/g, respectively. Grandinin was the second compound with a mean concentration of 172 μg/g. However, the important variability (high STD) of these variables in the different samples studied must be pointed out. 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 when grown under very constant conditions, indicating that this property is under strong genetic control.

Concerning the comparison of the contents of each one of these ellagitannins among provenances, the results of the significance levels of pairwise *t*-test showed that

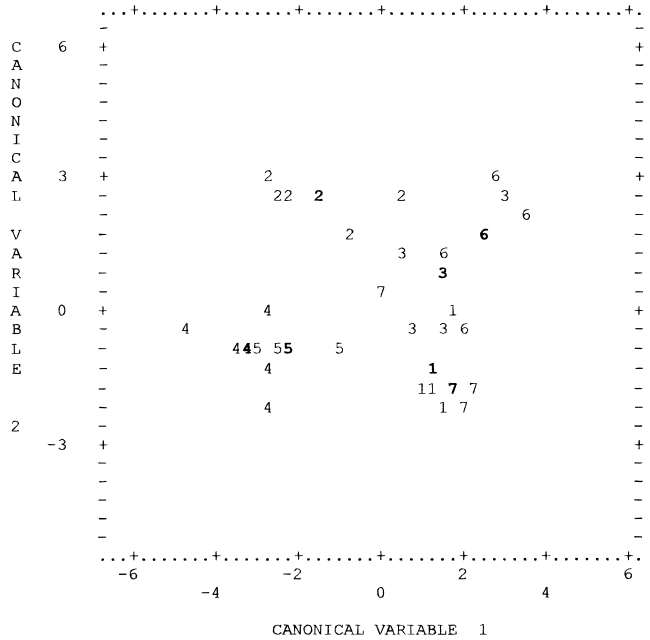

Figure 1. Stepwise discriminant analysis of low molecular polyphenols; projections of the points of each provenance on the two principal canonical axes: 1, Almadén de la Plata; 2, Los Barrios; 3, Medina Sidonia; 4, El Chaparral; 5, Jerez de los Caballeros; 6, Maçanet de Cabranys; 7, Forallac. **1**–**7** are the group centroids for each population, respectively.

none of the ellagitannins analyzed present significant differences among origins.

Moreover, the multivariate discriminant analysis carried out with these ellagitannins as variables, considering the different origins studied, revealed that none of these variables is discriminant among provenances and it is not possible to group the cork samples according to the origin of the populations.

From the above results, it can be concluded that *Q. suber* reproduction cork is characterized by a variety of low molecular weight phenols and important levels of tannins, particularly ellagitannins, that can be involved in the cork−wine interactions. The levels of each of these components present an important intra- and interpopulational variability, which is in accordance with previous studies on the polyphenolic composition of the wood and bark from other *Quercus* species, because the age of the tree and the distance of the sample from the base of the tree can also influence the composition of the extractable polyphenols (Fernández de Simón et al., 1996a,b) and also because the ellagitannin content seems to be under strong genetic control (Mosedale et al., 1996). There are no significant differences among the samples from different origins in the ellagitannin concentrations, which are important components of the polyphenolic extract. However, three minor components (gallic and caffeic acids and protocatechuic aldehyde) have been selected as those that provided the greatest discrimination among populations. Moreover, we have not found a relationship between geographical nearness of the populations and chemical similarity in the polyphenolic composition.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; MeOH, methanol.

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