

Separation and HPLC-MS Identification of Phenolic Antioxidants from Agricultural Residues: Almond Hulls and Grape Pomace

MÓNICA RUBILAR,[†] MANUEL PINELO,[‡] CAROLINA SHENE,[†] JORGE SINEIRO,[§] AND MARÍA JOSÉ NUÑEZ*^{·§}

Departamento de Ingeniería Química, Universidad de La Frontera, Av. Francisco Salazar, 01145 Temuco, Chile, BioProcess Engineering, Department of Chemical Engineering, Technical University of Denmark, Building 227-2800 Lyngby, Denmark, and Escuela Técnica Superior de Ingeniería E.T.S.E., Universidad de Santiago de Compostela, Av. Lope Gómez de Marzoa s/n. 15782, Santiago de Compostela, Spain

Almond hulls and grape pomace are residues abundantly generated by agricultural industries, which could be processed to obtain bioactive products. To this purpose, crude ethanol extracts from both agricultural byproducts were attained and subsequently fractionated in order to obtain an organic/water fraction (FOW). Extracts and fractions were analyzed for antioxidant power and their phenolic components tentatively identified by HPLC-MS. Chromatographic peaks of almond hull extracts showed the occurrence of hydroxybenzoic and cinnamic acid derivatives, with minor presence of flavan-3-ols (ECG, EGCG), whereas the FOW fraction offered the additional presence of epicatechin (EC) and glycosylated flavonols. In the composition for extracts of white and red grape pomace several of these compounds were also detected but basically consisted of glycosylated flavonols (quercetin, kaempferol). As a difference between both grape pomaces, myricetin glycoside was found in that from the red variety, whereas flavan-3-ols (EC, afzelechin) were only identified in white pomace. When their FOW fractions were analyzed, gallic acid and some hydroxybenzoic acids were additionally detected. Antioxidant activity was assessed by DPPH and TBARS assays. Almond hulls showed inhibition percentages lower than 50% in both assays, while the inhibition percentage ranged from 80% to 90% in pomace extracts. Red grape pomace extract was the most efficient antioxidant, with an EC₅₀ value of 0.91 g/L for TBARS and 0.20 g/L for DPPH. Even appearing as two quite different vegetal matrixes, the composition of phenolics in grape pomace and almond hulls is quite similar, the main difference being the major occurrence of flavonols in grape pomace. This fact could presumably explain the lower antiradical activity of hull extracts.

KEYWORDS: Agricultural byproduct; almond hull; grape pomace; phenols; HPLC-MS

INTRODUCTION

Residues from the processing of fruits and vegetables, traditionally considered as an environmental problem, are being increasingly recognized as sources for obtaining valuable products (1). To this regard, the recovery of phenolic compounds from industrial wastes is gaining considerable attention, especially ascribable to the antioxidant properties that these compounds exert (2). Food, pharmaceutical, and cosmetic industries are nowadays claiming for natural solutions to some of the customers' needs such as the use of natural colorants, texturizers, functional ingredients, or shelf life extenders. Phenols, natural

compounds able to satisfy to some extent these needs, have also physiological functions which can additionally result in benefit for human health. The capacity to scavenge free radicals, which were found to be responsible for lipid oxidation, is indeed one of the main reasons for food deterioration that phenols are able to partially prevent (3). Generation of free radicals also has a remarkable role in diseases such as cancer, atherosclerosis, inflammation, and aging (4–8).

Almond hulls, a barely studied residue from nuts and the dried fruits industry, have been reported to contain flavan-3-ol, hydroxybenzoic acids, and cinnamic acids (9, 10). The presence of flavonols or flavanons, which are phenol subcategories that some of the most active phenols belong to, has not been reported for almond hulls yet. Other byproducts of almond, such as skins and green shell, contain flavonol glycosides and phenolic acids (11, 12). Regarding wine byproducts, several authors have

* To whom correspondence should be addressed. Telephone: 34-81-563100ext 16792. Fax: 34-81-528050. E-mail: eqmajose@usc.es.

[†] Universidad de La Frontera.

[‡] Technical University of Denmark.

[§] Universidad de Santiago de Compostela.

reported these residues being sources rich in polyphenolic compounds (13–15). Especially for grape pomace, the recovery of phenols has so far been accomplished with several solvents, including ethanol, methanol, ethyl ether, and ethyl acetate. This latter was indeed found to be very suitable for the extraction of procyanidin oligomers, particularly used in the food industry (2).

This work will focus on the study of the composition and activity of almond hulls and grape pomace extracts (from Spanish and Chilean origin) using ethanol as an extractive solvent. The procedure first involved obtaining crude extracts and their subsequent fractionation in an extract soluble in both ethyl acetate and water (named as organic/water fraction or FOW). The composition of the resulting extracts was analyzed by HPLC-MS, while the antiradical activity of the extracts was assessed by using both the DPPH and TBARS assays. Being DPPH an assay with a broader application, TBARS was used for being more adequate to analyze the potential for lipid protection against oxidation (application to food preservation).

MATERIALS AND METHODS

Substrates. Grape residues (*Vitis vinifera*) from Garnatxa and Cabernet Sauvignon varieties were supplied by Bodegas Torres, Villafranca del Penedes, Barcelona, Spain, and Agrícola Valle Grande, Valle del Maipo, Chile, respectively. Almond hulls (*Prunus amygdalus*) were supplied by Borges S.A. Tárrega, Lleida, Spain. Pomaces were stored at $-20\text{ }^{\circ}\text{C}$ until use, not being subjected to any further pretreatment before extraction. Almond hulls were ground in an agate mortar, and only the particles smaller than 0.5 mm were used. Moisture content (almond hulls, $10.00 \pm 0.03\%$; Garnatxa grape pomace, $60.00 \pm 1.44\%$; Cabernet Sauvignon grape pomace, $55.56 \pm 0.79\%$) was assessed by maintaining the samples in a stove at $105\text{ }^{\circ}\text{C}$ until constant weight.

Extraction of Phenolic Compounds. Samples and ethanol were disposed in capped flasks and subsequently extracted in a G24 rotary shaker (New Brunswick Scientific Co. Inc., New Brunswick, NJ) at a constant stirring rate of 140 rpm. The extraction was performed at a solvent-to-solid ratio of 1:1 and $25\text{ }^{\circ}\text{C}$ during 90 min (16). Then, the extract was filtered and the solvent was evaporated in a Büchi Rotavapor R-114. Resultant crude (or raw) extracts (extract C) were freeze-dried, stored at $5\text{ }^{\circ}\text{C}$, and kept in a dark place until use. Polyphenols were quantified by using the Folin–Ciocalteu method (17).

Fractionation of Phenolics. Fractionation was done by the method described in Figure 1. Extract C was defatted with petroleum ether and the solvent decanted. The solid was first dried and then suspended in distilled water. After acetic acid addition, the monomeric and oligomeric components were extracted with ethyl acetate to obtain an organic fraction, O. Then, the solvent was evaporated from fraction O under vacuum and the pellet suspended in water. The remaining organic solvent was eliminated under vacuum, the resulting suspension being filtered through a porous plate. The pellet (soluble in ethanol) was washed with water, and the filtrates were pooled, centrifuged, decanted, and lyophilized to yield an OW fraction, which contained those soluble species in both ethyl acetate and water.

DPPH Assay. A DPPH radical-scavenging assay was employed as described by Brand-Williams et al. (18) to determine the hydrogen-donating ability of the redissolved extract. A volume of $980\text{ }\mu\text{L}$ of $6.1 \times 10^{-5}\text{ M}$ DPPH methanol solution was used. The reaction was started by the addition of $20\text{ }\mu\text{L}$ of extract. The bleaching of DPPH \cdot was followed at 515 nm at $25\text{ }^{\circ}\text{C}$ for 16 min. The inhibition percentage (IP) of the DPPH \cdot radical was calculated as follows:

$$\text{IP} = \frac{\text{absorbance}_{t=0\text{min}} - \text{absorbance}_{t=16\text{min}}}{\text{absorbance}_{t=0\text{min}}} \times 100 \quad (1)$$

Several dilutions were done when necessary, expressing the results as EC_{50} (defined as the extract concentration which achieves a decrease of DPPH absorbance to 50% of the initial value).

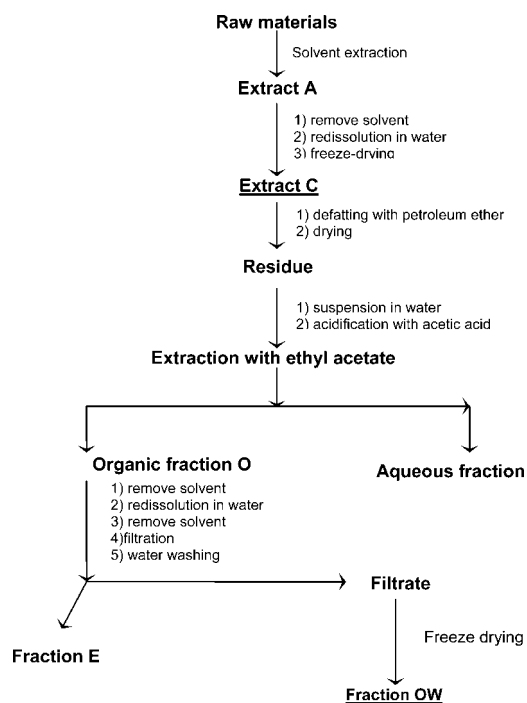


Figure 1. Fractionation scheme for different raw materials.

TBARS Assay. Freeze-dried samples of extract C (Figure 1) and FOWs were analyzed by TBARS assay (19) to measure their ability to inhibit lipid peroxidation at pH 7.4. A liposome system from egg lecithin (Sigma; purity >60%) was used to perform the assay, as described by Miyake et al. (20). The samples contained in a total volume of 1 mL were conducted in a PBS solution (phosphate buffer solution: 3.4 mM $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$, 0.15 M NaCl, pH 7.4); a 0.5 mg/mL solution of liposomes and $100\text{ }\mu\text{M}$ FeCl_3 were added. The concentration of extracts/fractions ranged from 0.1 to 10 g/L. The reaction was started by the addition of $100\text{ }\mu\text{M}$ ascorbate, and the reactive mixture was incubated at $37\text{ }^{\circ}\text{C}$ for 60 min, after adding 0.1 mL of 2% (w/v) butylated hydroxytoluene (BHT), 1 mL of 1% (w/v) thiobarbituric acid (TBA), and 1 mL of 2.8% (w/v) trichloroacetic acid. Solutions were heated in a water bath at $80\text{ }^{\circ}\text{C}$ for 20 min to promote the formation of a pink pigment product of the reaction from TBA with malondialdehyde [(MDA) $_2$ –TBA]. The chromogen was extracted into 2 mL of butanol-1-ol and the extent of peroxidation measured at 532 nm in the organic layer. The experiment was followed until stabilization, and the percentage of inhibition was calculated by an expression similar to eq 1. Results were also expressed as EC_{50} (g/L extract).

HPLC-MS Analysis. Crude extracts and their OW fractions were analyzed as described by Rubilar et al. (19). Samples were dissolved in methanol, filtered through a $0.45\text{ }\mu\text{m}$ nylon filter, and injected ($20\text{ }\mu\text{L}$) into an HPLC system. The reverse-phase HPLC apparatus with a PU-980 pump connected to a LG-1580-04 quaternary gradient unit, a JASCO UV-1575 UV-V detector, and a Rheodyne model 7725 loading sample injector with a $20\text{ }\mu\text{L}$ sample loop was used to determine the phenolic composition of the different fractions. The column ($250\text{ mm} \times 4.6\text{ mm}$) was a C_{18} Hypersil ODS ($5\text{ }\mu\text{m}$ particle size) (Supelco).

The solvents used to develop the gradient were (A) 0.5% acetic acid Milli-Q water solution and (B) methanol. The solvent gradient in volumetric ratios of solvents A and B was as follows: 0–10 min, 95A/5B; 10–60 min, 50A/50B; 60–80 min, 30A/70B; 80–90 min, 95A/5B. Detection was carried out using 280 nm excitation. The flow rate was set to 0.7 mL/min. Three determinations were made on each extract obtained.

The equipment used for electrospray mass spectrometry in the positive ion mode was a HP-Serie1100-MSD. Conditions were as follows: nitrogen as the drying gas at 13 L/min and $350\text{ }^{\circ}\text{C}$, nebulizer pressure at 40 psig, and fragmentor voltage at 60 V.

Statistical Analysis. The results reported in this work are the average of at least three measurements, and the coefficients of variations,

Table 1. Antioxidant Power of Extracts/Fractions

	CUC	CUG	OWUC	OWUG	CAE
DPPH					
% inhibition	81 ± 3.2	70 ± 1.7	60 ± 3.1	56 ± 2.0	36 ± 0.3
EC ₅₀ (g/L)	0.20 ± 0.00	1.00 ± 0.01	0.28 ± 0.00	1.40 ± 0.02	
TBARS					
% inhibition	65 ± 4.1	62 ± 3.8	58 ± 2.0	50 ± 2.0	40 ± 1.3
EC ₅₀ (g/L)	0.91 ± 0.05	1.10 ± 0.08	1.40 ± 0.08	2.10 ± 0.09	

expressed as the percentage ratio between standard derivations (SD) and the mean values, were found to be ≤10 in all cases.

RESULTS AND DISCUSSION

Antioxidant Activity of Crude Extracts and FOW Fractions. The values of antioxidant activity of crude extracts and FOW fractions from both grape pomaces and almond hulls, obtained by DPPH and TBARS methods, are shown in **Table 1**. The raw extracts contained 200, 350, and 215 ppm of polyphenols, for white and red pomaces and almond hulls, respectively, while only 13%, 39%, and 45% of the phenolics contained in the extracts passed to the FOW fractions.

Grape pomace samples worked better as antiradical agents than as lipid peroxidation inhibitors, although high inhibition values were obtained by both assays. Red grape pomace (Cabernet Sauvignon) was found to be the extract with the highest antioxidant activity, attaining an EC₅₀ (DPPH) value of 0.20 g/L in the crude extract. FOWs showed a quite lower inhibition activity, particularly in the TBARS test, presumably ascribable to the more hydrophobic nature of their compounds. In agreement with the "polar paradox", hydrophobic compounds have less capacity to protect lipids from oxidation. Extracts from almond hulls showed lower values of inhibition, about 40%; that is why EC₅₀ values were not determined. Values of the percentage of inhibition shown in this work are in the same range of others in the literature, about 60–80%, although comparison is difficult because of the use of different conditions (solvent, temperature, etc.) (21). EC₅₀ values of 0.41 g/L were reported by other authors for the antioxidant capacity (DPPH) of red grape pomaces (22). The effect of extract concentration on TBARS inhibition percentage is plotted in **Figure 2**, suggesting that a value of 5 g/L of extract would be enough for complete lipid protection.

From the data shown in the chromatographic profiles (see ahead), it can be stated that the main difference among the

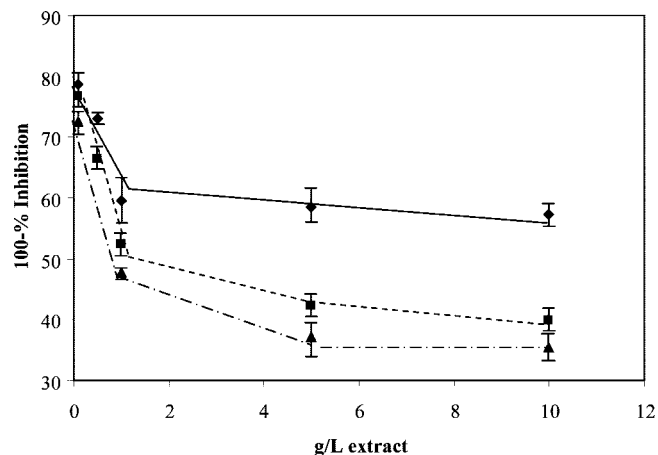


Figure 2. Values of peroxidation inhibition percentage for different phenolic concentrations of agricultural byproducts obtained by TBARS assay: (◆) almond hulls; (■) Garnatxa grape pomace; (▲) Cabernet Sauvignon grape pomace.

phenolic composition of the extracts from the studied materials was related to the flavonol content (the same extract concentration was employed for the different materials), which was indeed quite low in almond hull extract, increasing considerably in extracts of grape pomace.

Cabernet pomace extract, offering the best values as antioxidant, could also contain a significant amount of anthocyanins, not detected at the employed λ ; the presence of anthocyanic pigments is indeed commonly the main difference between the phenolic profile of white and red grapes. A qualitative assay in UV–vis spectrophotometry, following the methodology of Diaz et al. (23), at 520 nm, with malvidin and cyanidin as standards, showed in fact the occurrence of these substances in Cabernet Sauvignon extracts. Many previous works also confirm that malvidin, free or in glycosylated form, is predominant in red pomaces (24).

The literature offers examples of natural phenolics applied to the food field (25), where fractions enriched in phenolic acids, anthocyanins, flavonols, and proanthocyanidins were proved on food lipid oxidation. The flavonol fraction exhibited the greatest inhibitory effect and was also the most effective fraction in inhibiting thiobarbituric acid reactive substance (TBARS) formation. In red grape pomaces, this role is played by anthocyanins, which the flavonols converted into during ripening. Then, flavonols and anthocyanins could be mainly responsible for the behavior of agricultural residues on inhibition of peroxidation, which has a great value for a subsequent application of grape pomace extracts to the food field.

Composition of Crude Extracts and OW Fractions.

Ethanol extracts from all materials were fractionated with a successive series of solvents as described in the Materials and Methods section, in order to obtain the organic/water fraction (FOW). HPLC-MS chromatograms showed the presence of phenolic compounds, and the identification of the peaks was made on the basis of the data obtained and for comparison with standards and literature. Retention times, spectral characteristics (λ_{\max}), and m/z in positive ions are presented for the respective compounds in **Tables 2–7**, where the corresponding peaks from **Figures 3–6** are identified. Spectral characteristics (λ_{\max}) were obtained from the samples where the signals of the respective compounds were most intense and pure; m/z with masses in parentheses refers to the assumed structural units that rendered the experimentally found fragments. From the positive ion mass spectra of the peaks the formation of sodium adducts with the phenolic compounds studied can be inferred. Sodium is commonly present in vegetal materials, making then possible this kind of association (26, 27). The sugar moieties linked to the phenol compounds present in both plant matrixes were the following: glucose or galactose (162 mass units), rhamnose (146), xylose or arabinose (132), malonate (86), and glucose or galactose malonate (248), all of them naturally occurring in plants.

Almond Hulls. Chromatograms of crude extracts (CAE) and the OW fraction (OWAE) evidenced the presence of 13 and 14 different phenolic compounds, respectively (**Figure 3**). Tentative identification of chromatographic peaks is shown in **Tables 2 and 3**. In the crude extract, peaks 1, 2, and 7 were identified as derivatives of hydroxybenzoic acids. Their UV–vis spectra, with a maximum absorbance around 260 and 294 nm, had a very similar pattern to the protocatechuic acid spectrum, showing a maximum at 260 and 295 nm. The presence of greater mass units in the benzoic acid can be interpreted as attached saccharide structures; peak 2, for instance, was identified as hydroxymethoxybenzoic acid glycosylated (vanilloylhexose).

Table 2. Identification of Phenolic Species Contained in an Ethanol Extract of Almond Hulls (Crude Extract)

peak	retention time (min)	λ_{\max} (nm)	m/z	positive ion (m/z) fragments	identification
1	22.525	258, 294	137, 339, 394		hydroxybenzoic acid derivative
2	26.048	254, 288	353, 169	353 (168 + 162 + 23)	vanilloylhexose
3	26.519	280, 312	213, 219		cinnamic acid derivative
4	32.138	278	459, 289, 137	(458 + 1)	flavan-3-ol (EGCG)
5	33.542	290	169	(168 + 132 + 162 + 1)	NI ^a
6	34.191	278	291, 185		flavan-3-ol catechin
7	37.291	260, 292	169, 165, 422	(168 + 231 + 23)	hydroxybenzoic acid derivative
8	38.723	278, 304	197, 157		cinnamic acid derivative
9	40.851	280, 310	153, 207, 441		cinnamic acid derivative
10	44.329	306	183, 155		NI ^a
11	64.423	254, 352	317	(316 + 162 + 23) 501	flavonol glycoside (rhamnetin or isorhamnetin)
12	83.166	314	291, 289, 307, 309, 605, 763		galloylated polymeric flavan-3-ols, linked to hexosides
13	83.399	322	307–289		polymeric flavan-3-ol

^a Nonidentified.**Table 3.** Identification of Phenolic Species Contained in an Ethanol Extract OW Fraction of Almond Hulls

peak	retention time (min)	λ_{\max} (nm)	m/z	positive ion (m/z) fragments	identification
1	25.127	260, 292	155, 171, 353		hydroxybenzoic acid derivative
2	28.936	278, 306	213, 195		cinnamic acid derivative
3	30.825	280, 304	139, 111		cinnamic acid derivative
4	34.735	274	291, 137	(290 + 1)	flavan-3-ol with $m/z = 137$
5	36.423	280	291	(290 + 1)	flavan-3-ol (EC)
6	40.578	260, 292	169, 163, 359		hydroxybenzoic acid derivative
7	41.640	274, 302	197, 157		cinnamic acid derivative
8	44.242	280, 308	153, 125, 227		cinnamic acid derivative
9	47.381	310	153, 155, 183		NI ^a
10	49.248	278, 306	167, 151		cinnamic acid derivative
11	58.991	260, 362	303	(302 + 177 + 23) 501, 177 (146 + 31)	methoxyhesperetin/quercetin rhamnoside
12	61.440	266, 360	303, 287	(302 + 162 + 23) 487, (286 + 45) 331	flavonoid mixture
13	65.801	266, 350	287	(286 + 162 + 23) 471	flavonol/flavanon/flavon glycosylated
14	66.343	256, 360	317	(316 + 162 + 23) 501	glycosylated rhamnetin

^a Nonidentified.**Table 4.** Identification of Phenolic Species Contained in an Ethanol Extract of Garnatxa Grape Pomace (Raw Extract)

peak	retention time (min)	λ_{\max} (nm)	m/z	positive ion (m/z) fragments	identification
1	36.149		291		flavan-3-ol (EC or EGC)
2	36.978	270	265, 2491		NI ^a
3	44.604		275, 291	(274 + 1)	flavan-3-ol (afzelechin)
4	59.653	258, 354	303	(302 + 146 + 31 + 23) 502	quercetin rhamnoside
5	61.736		303	(302 + 162 + 23) 487	quercetin glycoside or galactoside
6	64.983		287	(286 + 162 + 23) 471	vitexin/kaempferol galactoside
7	66.318		287	(286 + 162 + 23) 471	luteolin/ kaempferol glycoside

^a Nonidentified.**Table 5.** Identification of Phenolic Species Contained in an Ethanol Extract OW Fraction of Garnatxa Grape Pomace

peak	retention time (min)	λ_{\max} (nm)	m/z	positive ion (m/z) fragments	identification
1	11.182	270			gallic acid derivative
2	13.128	272			gallic acid derivative
3	14.978	270			gallic acid derivative
4	25.862	260, 296			hydroxybenzoic acid derivative
5	35.342	256	291, 101		NI ^a
6	37.062	280	291		flavan-3-ol
7	41.262	262, 290			hydroxybenzoic acid derivative
8	45.429	278	291		flavan-3-ol
9	60.026	256, 354	303	(302 + 146 + 31 + 23) 502	quercetin rhamnoside
10	62.432	256, 296, 354	303	(302 + 162 + 23) 487	quercetin glycoside/galactoside
11	63.162	258	284		NI ^a
12	65.757	260, 358	287	(286 + 162 + 23) 471	vitexin
13	66.890	266, 350	287	(286 + 162 + 23) 471	kaempferol/luteolin glycoside

^a Nonidentified.

Peaks 3, 8, and 9 had a similar type of UV–vis spectrum among them, with maximum absorbances at 280 and 310 nm, presumably corresponding to cinnamic acid derivatives. Peaks 4 and 6, with a maximal absorbance at 278 nm, were identified as

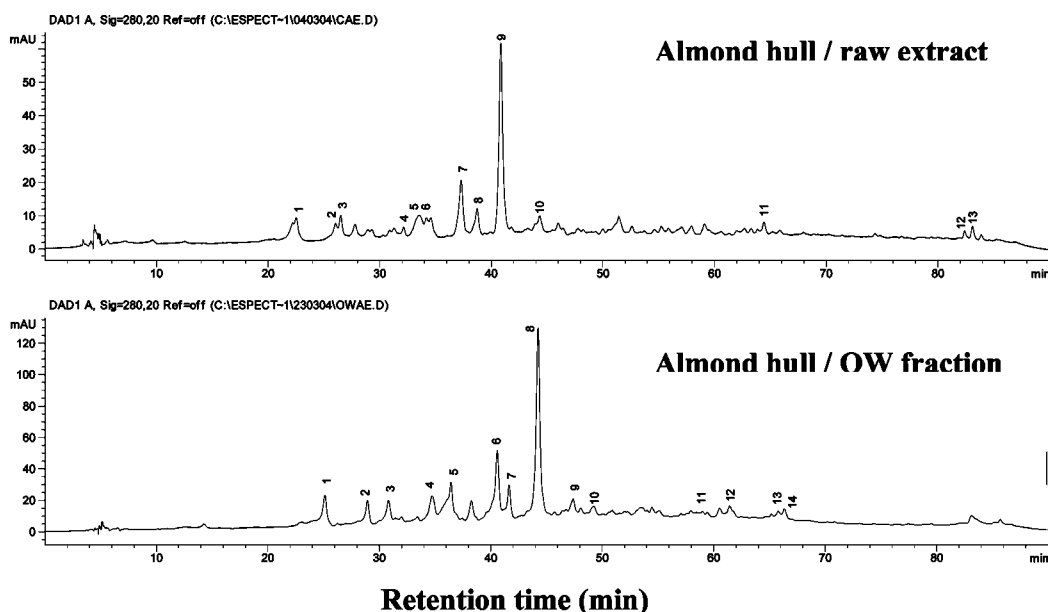
flavan-3-ols. The m/z 459 of peak 4 indicates the presence in the extract of epigallocatechin gallate (EGCG), while the m/z of peak 6 corresponded to epicatechin gallate (ECG). The literature reports similar spectra for EGCG and ECG, which

Table 6. Identification of Phenolic Species Contained in an Ethanol Extract of Cabernet Sauvignon Grape Pomace (Raw Extract)

peak	retention time (min)	λ_{\max} (nm)	m/z	positive ion (m/z) fragments	identification
1	43.823	276	155, 199		NI ^a
2	56.659		319	(318 + 162 + 23) 503	miricetyn glycoside
3	59.741	256, 358	303	(302 + 176 + 1) 479, (302 + 176 + 23) 501	quercetin glucuronide
4	61.751	256, 358	303	(302 + 162 + 23) 487	quercetin + quercetin glycoside/galactoside
5	66.229	266, 350?	287	(286 + 162 + 23) 471	kaempferol glycoside/galactoside
6	66.689	254, 356	317	(316 + 31) 347, (347 + 162 + 23) 531, (316 + 162 + 23) 501	metoxi (iso)rhamnetin and (iso)rhamnetin glycosides or galactosides

^a Nonidentified.**Table 7.** Identification of Phenolic Species Contained in an Ethanol Extract OW Fraction of Cabernet Sauvignon Grape Pomace

peak	retention time (min)	λ_{\max} (nm)	m/z	positive ion (m/z) fragments	identification
1	12.276	270			gallic acid derivative
2	12.678	270			gallic acid derivative
3	13.913	272			gallic acid derivative
4	24.735	258, 294	219, 189		hydroxybenzoic acid derivative
5	31.601	274	141, 185, 167		NI ^a
6	40.394	260, 292	179, 169, 182		hydroxybenzoic acid derivative
7	44.138	274	155, 199, 140		NI ^a
8	46.810	272	171, 199, 127		NI ^a
9	59.390	258, 354	479, 303	(302 + 176 + 1) 479, (302 + 176 + 23) 501	quercetin glucuronide
10	61.476	256, 355	303	(302 + 162 + 23) 487	quercetin + quercetin glycoside or galactoside
11	61.98	256, 355	303	(302 + 162 + 23) 487	quercetin glycoside or galactoside
12	62.60	266	303	303, 441, 605, 763	quercetin malonylglycoside
13	64.76	254, 366	287, 303, 319	(471 + 133 + 1) 605, (286 + 162 + 23) 471, (318 + 146 + 1) 465	mixed glycosides and xylosides of quercetin and myricetin
14	66.589	254, 358	317	(316 + 31) 347, (347 + 162 + 23) 531, (316 + 162 + 23) 501	metoxi (iso)rhamnetin and (iso)rhamnetin glycosides or galactosides
15	71.72	256, 370	303	(302 + 248 + 23) 573	quercetin malonylglycoside

^a Nonidentified.**Figure 3.** Chromatograms corresponding to an ethanol crude extract from almond hulls (CAE) and its OW fraction (OWAE).

show spectra with maximal absorbances at 275, 279 nm and 276.5, 280 nm, respectively (28). Peak 11, with m/z ratio 317, coincided with the spectrum of (iso)rhamnetin, a flavonol glycoside. Peaks 12 and 13 probably correspond with polymeric procyanidins whose fragmentation originated compounds tentatively identified as epicatechin gallate linked to hexose (605) or a procyanidin dimer linked to hexose (763). Some of the mentioned compounds were detected in almonds by other authors (29), while others such as vanilloylhexose are rather unusual in almonds, although it has been often detected in berries (27).

Soluble substances in both water and ethyl acetate remain in the OW fraction of almond hulls (Figure 3). A different profile was found, but mass spectra showed the presence of the same type of compounds in crude extracts and FOW. For instance, peaks denoted as “1” and “6” corresponded with peaks “1CAE” and “7CAE” found in the crude extract. They shared the same UV-vis spectral pattern with a peak of absorbance at 260, 290 nm, thus being classified as derivatives of hydroxybenzoic acids. Peaks 2, 3, 7, 8, and 10 were identified as cinnamic acid derivatives. Peaks 2, 7, and 8 corresponded to peaks 3, 8, and 9 in CAE, respectively. Peaks 4 and 5 of OWAE were classified

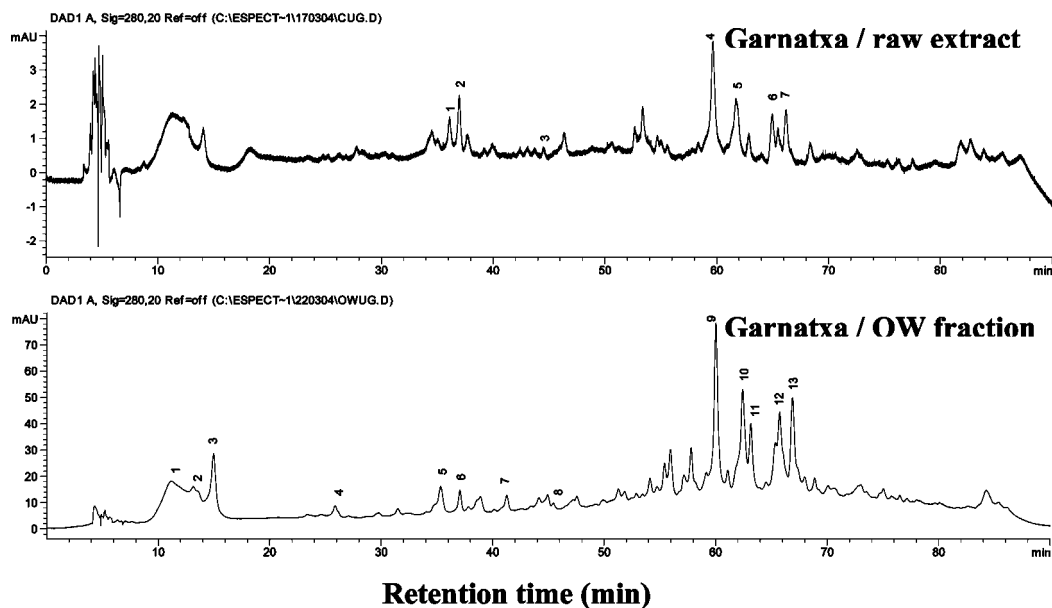


Figure 4. Chromatograms corresponding to an ethanol crude extract from Garnatxa grape pomace (CUG) and its OW fraction (OWUG).

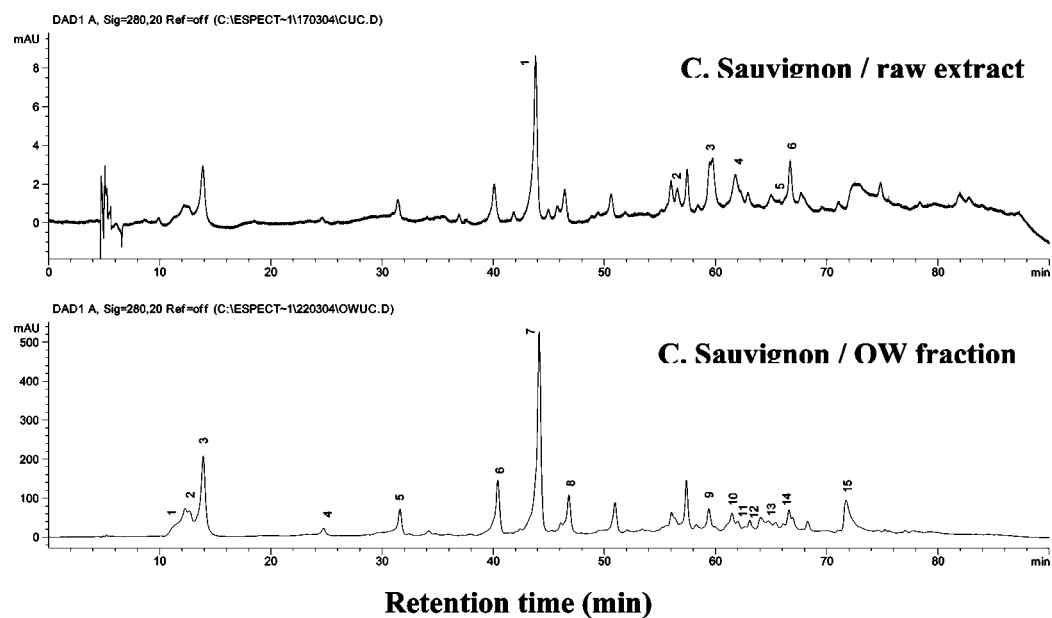


Figure 5. Chromatograms corresponding to an ethanol crude extract from Cabernet Sauvignon grape pomace (CUC) and its OW fraction (OWUC).

as flavan-3-ols (catechin and epicatechin derivatives). Peak 4 appeared mixed with an unknown compound, recognized by the change of the UV profile along the peak evolution (not shown), which gave a fragment at m/z 137. Finally, peaks 11, 12, 13, and 14 of OWAE were identified through the characteristics masses, UV-vis spectra, and sugar moieties: peak 11 (mass 303) could be a flavonol quercetin or flavanone hesperetin attached to 146 (rhamnose) and 23 (sodium adduct). As indicated in **Table 3**, peak 12 is a mixture of the m/z 302 fragment (quercetin or hesperetin) glycosylated to hexose and a compound with 286 mass (kaempferol, luteolin, or isosakuranetin). Peak 13 seems to be quercetin or hesperetin again, glycosylated to hexose. The last peak, 14, was identified as (iso)rhamnetin attached to hexose again (fragment m/z 316).

To sum up, it can be said that phenolic acids occur to a greater extent than flavonoids in almond hulls. The presence of flavonoids as flavanols or flavanones in almond hulls had not yet been reported until now, even when they were reported to be present in other parts of almonds as skins and green shell

(12). As indicated above, several derivatives of cinnamic acids were found, although chlorogenic acid was not identified as such. Almond hull is therefore a rich source of phenolic acids, particularly cinnamic and hydroxybenzoic acids. Up to now, the known constituents of almond hulls were basically catechin, protocatechuic acid, ursolic acid, chlorogenic acid, and their isomers (9, 10). Flavanols were the predominant flavonoids in this material. After fractionation, however, not only new monomers and oligomers of flavanols were found but a major presence of phenolic acids in the OW fraction were also detected (**Figure 3**).

Grape Pomace. Two varieties were studied, Garnatxa of Spanish origin and Cabernet Sauvignon from Chile. Both residues of winemaking are a mixture of skins, stems, and seeds, although the presence of seeds in our material was low enough.

Garnatxa Grape Pomace. **Figure 4** shows chromatograms corresponding to an ethanol crude extract (CUG) and its OW fraction (OWUG). As can be observed, 7 peaks were tentatively

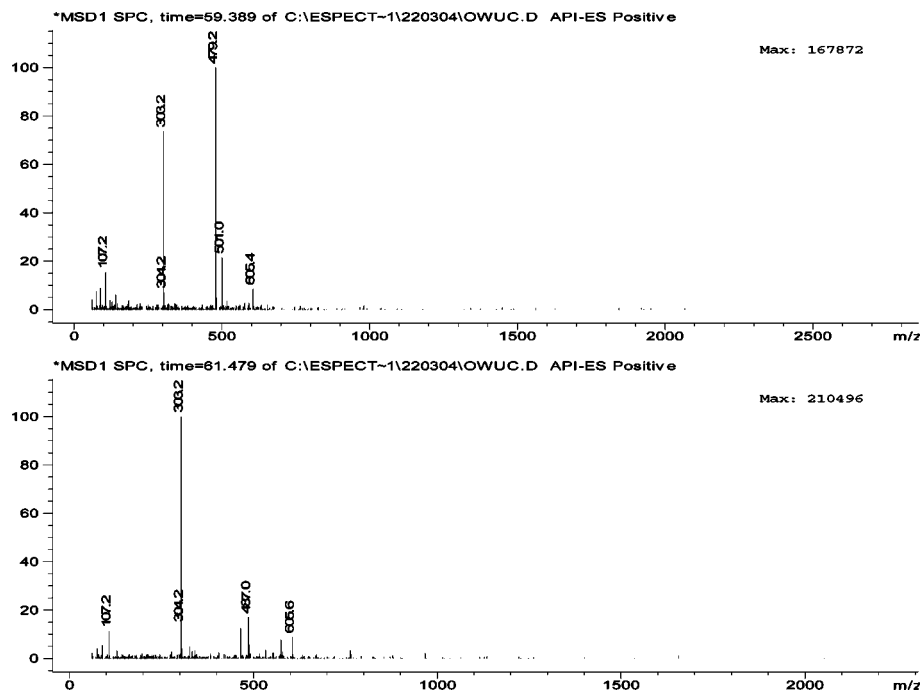


Figure 6. Mass spectra of peaks 9 (59.390) and 10 (61.476) of OWUC.

identified in the extract and 13 in the OW fraction. They are listed in **Tables 4** and **5**. Peaks 1 and 3 in the raw extract presumably contained catechin and epicatechin, with m/z 291 as the most frequent fragment. Peak 3 includes a more abundant fragment, with m/z 275, which could be the flavan-3-ol afzelechin, similar to other compounds which propylargininidins derive from (30). Sometimes, in relation to the elution order of flavonol glycosides, mass unit 162 elutes before than mass 146 (31). However, the methoxyl group in peak 4, attached to mass 146, changes this order and elutes before mass 162. The same case was observed with almond hulls, peaks 11 and 12 OWAE. Peaks 4 and 5 in the CUG chromatogram were identified as quercetin glycosides; this flavonol have already been reported (32) as present in white grape pomace. In the mass spectra of peaks denoted as 6 and 7 CUG, it appeared the fragment with m/z ratio 287 identified the first as vitexin and the second, not conclusively, as luteolin glycoside or kempferol glycoside. Regarding these compounds, Alonso et al. (32) and Pinelo et al. (33) have reported that myricetin glycoside predominates in red grapes, whereas the presence of vitexin is frequent in white grapes, especially in skins.

The list of phenolic compounds contained in FOW from Garnatxa grape pomace is reported in **Table 5**. Peaks 1, 2, and 3 of OWUG were classified as gallic acid derivatives; their UV-vis spectra have a maximum absorbance between 270 and 272 nm, and gallic acid has a characteristic peak of 272, 270 nm. Peaks 4 and 7 of OWUG were attributed to hydroxybenzoic acid derivatives, whereas peaks 6 and 8 of OWUG were identified as oligomeric flavanols because of high molecular weights in the mass spectrum and the characteristic profile of flavan-3-ol in the UV spectrum.

Although some peaks were found in the crude extract and OW fraction, as a result of the fractionation, it can be noticed the enrichment in phenolic acids and flavan-3-ols of fraction OW.

Cabernet Sauvignon Grape Pomace. Chromatograms corresponding to the ethanol crude extract from Cabernet grape pomace (CUC) and its OW fraction (OWUC) are shown in **Figure 5**. In this work, as the same HPLC methodology was

applied to all samples, no peaks for anthocyanin compounds were detected. The use of a specific HPLC chromatography does not imply that the mass of anthocyanins cannot appear in the mass spectra, so special care was taken with m/z fragments that could correspond to anthocyanins. The UV profile was decisive to suggest the presence of a flavonol or anthocyanin when the mass of the main fragment coincided (for example, m/z 303 is compatible with quercetin or delphinidin). A simultaneously recorded spectrum at 550 nm did not show peaks of anthocyanins, as expected. Six peaks were tentatively identified in the raw extract (**Table 6**). They corresponded with flavonol glycosides: myricetin, quercetin, kaempferol, and methoxy (iso)rhamnetin glycoside. Several authors also identified these compounds in red grapes, as rhamnetin and quercetin glucoside and galactoside (34, 35). Peak 2 in the crude grape cabernet (CUC) was identified as myricetin glucoside, which eluted before quercetin glycoside (peaks 3 and 4). The first quercetin derivative was identified as quercetin glucuronide, which was reported by Kammerer et al. (36) as the major one of the quercetin derivatives in grapes. Peak 4 was linked to hexose (m/z 162), having been found previously in red grape skin. Peaks 5 and 6 presumably pointed to the presence of kaempferol glycoside and (iso)rhamnetin glycoside, respectively, in the sample. The OW chromatogram revealed 15 peaks of phenolic compounds, listed in **Table 7**. As well as in Garnatxa FOW, it was found to be enriched with phenolic acids and flavan-3-ols, whereas the crude extract mainly contained flavonol glycosides. Peaks 4 and 6 of OWUC were identified as hydroxybenzoic acid derivatives, also found in the raw extract of almond hulls. Peaks 9, 10, 11, 12, and 15 OWUC corresponded to flavonoids, the main fragment being m/z 303 (peak 9 showed the typical UV spectrum of quercetin and most of the mass fragments corresponded well to a glucuronide derivative). From the different ratio between the amounts of m/z 303 and m/z 487 fragments, it can be deduced that peak 10 corresponded to quercetin and peak 11 to quercetin hexosides. In the analysis of the raw extract, it was not possible to detect these compounds as individuals, but as a mixture (**Table 6**). Mass spectra of peaks 9 and 10 are presented in **Figure 6**. Peak 12 was very small,

having an UV spectrum clearly different from the ones of the surrounding peaks. The low signal made the identification difficult. Peaks 13 and 14 of OWUC were flavonols with m/z 287, 319, and 317. The first one was a mixture between kaempferol glycoside and myricetin glycoside. Peak 14, showing a predominant m/z 317, could be the flavonol (iso)rhamnetin glycoside, with a maximum absorbance at 254 and 358 nm. The spectrum of peak 15, almost identical to that of quercetin, and the finding of a main mass fragment m/z 573 suggest the presence of a malonyl glycoside derivative from quercetin, which is not very common in grapes but often detected in vegetables such as onions and mulberry leaves (37, 38).

In summary, flavan-3-ol, quercetin glycosides, vitexin, and kaempferol/luteolin glycosides were identified in extracts from Garnatxa grape pomace, additionally appearing gallic acid and hydroxybenzoic acid derivatives in FOW. Cabernet Sauvignon grape pomace, a red variety, contained gallic acid derivatives and flavonols, mainly quercetin and myricetin glycosides, the last one being a typical flavonol found in red varieties. The elution order of phenolic compounds was found to be similar to that obtained by other authors (32). From these results it can be said that flavonols are especially a remarkable group in relation to the total of phenolic compounds found in this residue. The low amount of flavan-3-ols detected in the grape pomaces fits with the results reported by Revilla and Ryan (39) in white and red grape skins. The content of flavanols is known to be conditioned not only by the type of grape but also by climatic and soil factors linked to the grape origin, which can make different phenolic profiles of two berries belonging to the same variety.

ABBREVIATIONS USED

EC, epicatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; CAE, crude almond extract; CUC, crude extract from Cabernet; CUG, crude extract from Garnatxa; OWAE, OW almond fraction; OWUC, OW Cabernet fraction; OWUG, OW Garnatxa fraction.

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