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Antioxidant Phenols in Barley (*Hordeum vulgare* L.) Flour: Comparative Spectrophotometric Study among Extraction Methods of Free and Bound Phenolic Compounds

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Phenolic compounds are found in both free and bound forms in cereals. The majority is in the insoluble bound form, that is, bound to cell wall material, such as ferulic acid and its derivatives. The antioxidant properties of the phenolic compounds in grains are associated with the health benefits of grains and grain products. The extraction capacity of several solvent mixtures, for extracting free phenols from barley flours, and the possibility of employing a rapid automated solvent extraction method were studied. The extraction yield of each method was evaluated by correlating several spectrophotometric indices (absorption at 280, 320, and 370 nm and total phenolic compounds by the Folin−Ciocalteu method) with the antioxidant activities of the barley extracts (scavenging activity by the 2,2-diphenyl-1-picrylhydrazyl method). Interesting results were obtained when ethanol and acetone-based extraction mixtures were employed to extract free phenols. A comparison was made between alkaline and acid hydrolysis. The extraction yield of bound phenolic compounds increased when the digestion time for alkaline hydrolysis was prolonged.

KEYWORDS: Barley; phenolic compounds; antioxidant activity; spectrophotometric indices; extraction methods

INTRODUCTION

Cereals and their derivatives are the most important foods in the Mediterranean diet mainly because of the energy that they provide, due to their high carbohydrate content. However, in recent years, researchers have also begun to study their antioxidant profiles.

In fact, phenols are presumed to be responsible for the beneficial effects derived from the consumption of whole grains, fruits, and vegetables. Phenolic compounds have strong in vitro and in vivo antioxidant activities associated with their ability to scavenge free radicals, break radical chain reactions, and chelate metals. Moreover, a high phenol consumption has been correlated with a reduced risk of cardiovascular diseases and certain cancers (*1*, *2*).

Barley is a widely consumed cereal, because of its dietary and technological properties. In fact, barley meals and fractions are now gaining renewed interest as ingredients for the production of functional foods (pastas, baked products) (*3*, *4*), due to their concentration of bioactive compounds, such as β-glucans and tocols (*5*, *6*). Moreover, there are several classes of compounds in barley that have a phenolic structure, such as benzoic and cinnamic acid derivatives, proanthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds (*7*−*10*). Phenolic compounds in cereals are either in free or bound forms. Generally, the free phenolic compounds are proanthocyanidins or flavonoids, whereas the bound phenolic compounds are ester-linked to cell wall polymers and consist mainly of ferulic acid and its oxidatively coupled dimers (*11*−*15*).

A specific extraction methodology for obtaining free soluble phenols from cereals has not yet been found. Most research groups determine the amount of barley phenols after time-consuming extraction of the fine-powdered flour, using aqueous methanol, ethanol, and acetone (*16*−*23*) separately or mixed together to maximize the extraction yield. However, the total phenolic content of cereals has often been underestimated in the literature, because the content of bound phenolic compounds, usually found in significant quantities, was not determined. Most researchers determine bound phenols in cereal flours by alkaline hydrolysis. Generally, on the basis of digestion time, alkaline hydrolysis can be classified as short hydrolysis, when the digestion time ranges from 1 to 4−6 h, and long hydrolysis, when the digestion time lasts more than 16 h (*7*, *23*−*25*). Only a few studies have evaluated bound cereal phenols by acid hydrolysis (*26*, *27*). Moreover, automated pressurized solvent

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Table 1. Experimental Plan of the Conventional Solid/Liquid Extractions[a]

experiment name	EM cycle A	drying (N_2)	EM cycle B	EM cycle C	collect supernatant A to supernatant B or C
AcH_2O	s_3				
EtH_2O	s_1				
$EtAcH_2O$	s_4				
$MetH_2O$	s_2				
AcEt-N_2	s_3			s_1	A + C
EtAc-N_2	s_1			s_3	A + C
AcEt+N_2	s_3	yes	s_1		A + B
fract Ac and fract Et	s_3 (fract Ac)	yes	s_1 (fract Et)		supernatants A and B kept separated

[a] Abbreviations: EM, extraction mixture; s1, 4/1 ethanol/water (v/v); s2, 4/1 methanol/water (v/v); s3, 4/1 acetone/water (v/v); s4, 7/7/6 ethanol/acetone/water (v/v/v).

extraction may be an interesting alternative to the conventional time-consuming solid/liquid extraction method used for cereal phenols, since it is automated and rapid.

There appears to be little literature on the correlation of phenol content with antioxidant activity in barley. Therefore, the aim of this study was to evaluate the ability of several organic mixtures to selectively extract free and bound barley phenols. Moreover, automated pressurized liquid extraction was compared to solid/liquid extraction. The extraction yields were evaluated using spectrophotometric measurements: the classical colorimetric Folin−Ciocalteu method was used to determine total phenolic compounds, while four phenolic groups were quantified using specific UV spectrophotometric indices (hydroxycinnamic acids at 320 nm, flavonols at 370 nm, phenols at 280 nm, and *o*-diphenols at 370 nm, after reaction with molybdate). The antioxidant activity of the extracts was also verified by carrying out a 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. This study is the preliminary spectrophotometric step toward the further characterization of phenolic compounds in barley.

MATERIALS AND METHODS

Samples and Sample Preparation. Organic whole barley flour was purchased at a local market. Unless otherwise stated, all solvents were pro analysis grade.

Extraction of Free Phenolic Compounds. Unless otherwise stated, every extraction trial was replicated three times ($n = 3$). The extracts were stored at −18 °C until used.

Solid/Liquid Extraction. A 5 g sample of whole barley flour was extracted by sonication with 40 mL of an organic solvent/water extraction mixture for 10 min to extract the free phenolic compounds. The extraction mixtures were used as follows: s_1, 4/1 ethanol/water (v/v) (EtH_2O extract); s_2, 4/1 methanol/water (v/v) ($MetH_2O$ extract); and s_3, 4/1 acetone/water (v/v) (AcH_2O extract) (**Table 1**). To reach a compromise between alcoholic and acetone extractions, a 7/7/6 ethanol/acetone/water (v/v/v) mixture (s_4) was tested ($EtAcH_2O$ extract). After centrifugation at 1000*g* for 10 min, the supernatant was removed and the extraction was repeated once more. The supernatants were pooled, evaporated at 40 °C with a vacuum evaporator, and reconstituted with 5 mL of 99.7/0.3 water/formic acid (v/v) (extraction cycle A). The residual flour was either dried (extraction cycle B) or not dried (extraction cycle C) with nitrogen and then extracted two more times using a different organic mixture so as to maximize the extraction yield of the phenols. **Table 1** and **Figure 1** illustrate the experimental extraction procedures. To verify the selective extraction of the phenolic classes, both fractions were also, in one case, kept separate between the A and the B extraction cycles.

Pressurized Liquid Extraction. Model ASE 200 (Dionex, Idstein, Germany), an automated extraction system for pressurized liquid extraction, was used to extract phenols from barley flour. Two barley flour weight/Hydromatrix ratios (Dionex) were used as follows: 5 g of whole barley flour mixed with 3 g of Hydromatrix, and 2 g of whole barley flour mixed with 4 g of Hydromatrix, and 33 mL of extraction cells were used. Two 5 min static cycles at 20 MPa were used with a 4/1 ethanol/water (v/v) extraction mixture. The extraction temperatures were set at 60, 90, and 120 °C (**Table 2** shows the experimental plan and the sample abbreviation). The solvent flush was 60%, and the purging time was 60 s. The fractions extracted were evaporated at 40 °C with a vacuum evaporator and reconstituted to adequate proportions

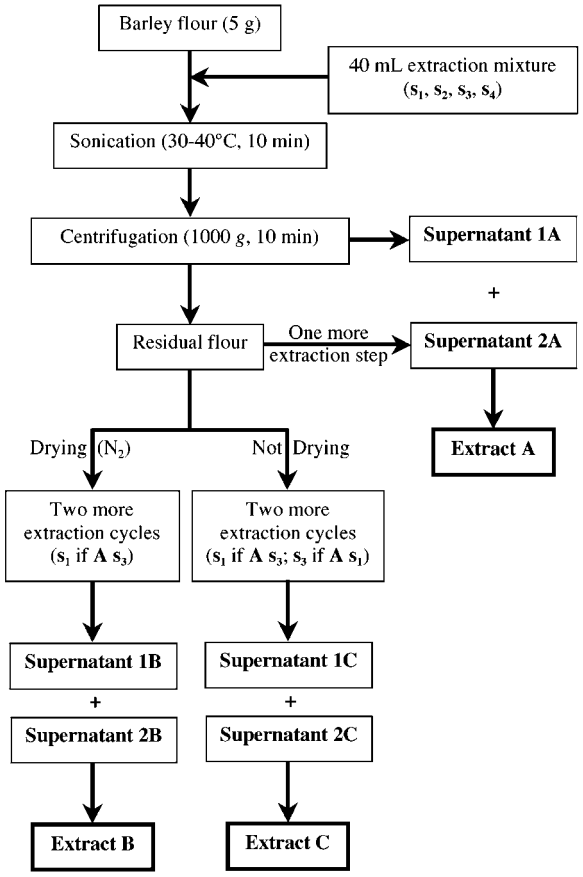

Figure 1. Experimental plan of the extraction of phenolic compounds from barley flour. When extract A is a 4/1 acetone/water (v/v) extract (s_3) and extract C is a 4/1 ethanol/water (v/v) extract (s_1), and they were collected, the sample was called AcEt-N_2. When extract A is a 4/1 ethanol/water (v/v) extract (s_1) and extract C is a 4/1 acetone/water (v/v) extract (s_3), and they were collected, the sample was called EtAc-N_2. When extract A is a 4/1 acetone/water (v/v) extract (s_3) and extract B is a 4/1 ethanol/water (v/v) extract (s_1), and they were collected, the sample was called AcEt+N_2. When extract A is a 4/1 acetone/water (v/v) extract (s_3) and extract B is a 4/1 ethanol/water (v/v) extract (s_1), and they were kept separated, the samples were called fract Ac and fract Et, respectively.

Table 2. Pressurized Liquid Extraction Conditions of the Experimental Plan[a]

experiment name	temperature (°C)	flour (g)	hydromatrix (g)
ASE60-5/3	60	5	3
ASE90-5/3	90	5	3
ASE120-5/3	120	5	3
ASE60-2/4	60	2	4
ASE90-2/4	90	2	4
ASE120-2/4	120	2	4

[a] Cycle time, 5 min (two cycles in static mode); solvent flush, 60%; pressure extraction, 20 MPa; extraction mixture, 4/1 ethanol/water (v/v), for all methods.

(5 and 2 mL for the 5 and 2 g samples, respectively) of 99.7/0.3 water/formic acid (v/v).

Extraction of Bound Phenolic Compounds. Unless otherwise stated, every extraction trial was replicated three times ($n = 3$). The extracts were stored at −18 °C until used.

Alkaline Hydrolysis. One gram of whole flour was digested with 100 mL of 2 M NaOH at room temperature at two digestion times (4 and 20 h) by shaking under nitrogen gas. The mixture was then brought to pH 2−3 by adding 10 M hydrochloric acid in a cooling ice bath and extracted with 500 mL of hexane to remove the lipids. The final solution was extracted five times with 100 mL of 1/1 diethyl ether/ethyl acetate (v/v). The organic fractions were pooled and evaporated to dryness. The phenolic compounds were reconstituted with 5 mL of 99.7/0.3 water/formic acid (v/v).

Acid Hydrolysis. One gram of whole flour was shaken with 6 mL of 96% ethanol and 30 mL of 25% hydrochloric acid at 65 °C for 30 min. Then, 10 mL of 96% ethanol and 50 mL of 1/1 diethyl ether/petroleum benzine 40−60 °C (v/v) were added to the digested samples. The organic fraction was discarded, and the residue was washed twice with 25 mL of 1/1 diethyl ether/petroleum benzin 40−60 °C (v/v). Last, the aqueous fraction was washed five times with 100 mL of 1/1 diethyl ether/ethyl acetate (v/v) to remove lipids. The organic fractions were pooled and evaporated to dryness. The phenolic compounds were reconstituted with 5 mL of 99.7/0.3 water/formic acid (v/v).

Spectrophotometric Determinations. The spectrophotometric analyses were performed using a UV-1601 spectrophotometer from Shimadzu (Duisburg, Germany) and were replicated three times for each extract or calibration point ($n = 3$).

Determination of TPC. The TPC of the extracts was determined with the Folin−Ciocalteu spectrophotometric method (*28*). Briefly, 100 μL of each extract was shaken for 1 min with 500 μL of Folin−Ciocalteu reagent (Merck, Darmstadt, Germany) and 6 mL of distilled water. After the mixture was shaken, 2 mL of 15% Na_2CO_3 was added and the mixture was shaken once again for 0.5 min. Finally, the solution was brought up to 10 mL by adding distilled water. After 2 h, the absorbance at 750 nm (25 °C) was evaluated using glass cuvettes. The TPC was assessed by plotting the gallic acid calibration curve (from 1 to 1500 μg/mL). The equation of the gallic acid calibration curve was $A = 1.0559c + 0.0178$, and the correlation coefficient was $r^2 = 0.999$.

PI. The PI was obtained by spectrophotometric measurement (*29*, *30*). Briefly, 50 μL of each extract was brought up to 5 mL with 99.7/0.3 water/formic acid (v/v). The solution was shaken, and the absorbance was evaluated at 280 nm (25 °C) using quartz cuvettes. A gallic acid calibration curve (from 1 to 2000 μg/mL) was plotted to assess the PI. The equation of the gallic acid calibration curve was $A = 0.4142c + 0.0017$, and the correlation coefficient was $r^2 = 0.999$.

FI. The FI was calculated according to the Maillard et al. method (*30*), with some modifications. Two hundred microliters of each extract was diluted with 10 mL of methanol, and the absorbance was evaluated at 320 nm (25 °C) using quartz cuvettes. A quercetin calibration curve (from 1 to 1000 μg/mL) was plotted to assess the FI. The equation of the quercetin calibration curve was $A = 0.6346c + 0.0033$, and the correlation coefficient was $r^2 = 0.999$.

HI. The HI was calculated according to the Maillard et al. method (*30*), with some modifications. Two hundred microliters of each extract was diluted with 10 mL of methanol, and the absorbance was evaluated at 320 nm (25 °C) using quartz cuvettes. A ferulic acid calibration curve (1−1000 μg/mL) was plotted to assess the HI. The equation of the ferulic acid calibration curve was $A = 0.8974c - 0.0119$, and the correlation coefficient was $r^2 = 0.996$.

ODI. The spectrometric determination of *o*-diphenols by Mateos et al. (*31*) was slightly modified. Briefly, 250 μL of each extract was added to 2.25 mL of a 1/1 methanol/water (v/v) mixture; 2 mL of this solution was shaken vigorously with 0.5 mL of a 5% sodium molybdate dihydrate solution (in 1/1 methanol/water, v/v). After 15 min, the absorbance was measured at 370 nm, in a quartz cuvette, at 25 °C. A gallic acid calibration curve was plotted (from 1 to 2000 μg/mL) to assess the ODI. The equation of the gallic acid calibration curve was $A = 1.0673c + 0.0363$, and the correlation coefficient was $r^2 = 0.999$.

Evaluation of the TEAC of the Extracts. To determine the TEAC of the extracts, the DPPH radical scavenging method was performed according to the Parejo et al. and Brand-Williams et al. methods (*32*, *33*), with some modifications. A 100 μL sample of each extract was added to 2.9 mL of 100 μM DPPH (Sigma, St. Louis, MO) solution in 80/20 methanol/water (v/v). A decrease in absorbance was determined at 517 nm in the 0−30 min range (at 25 °C). One hundred microliters of 99.7/0.3 water/formic acid (v/v) added to 2.9 mL of 80/20 methanol/water (v/v) was used to zero the spectrometer. The initial DPPH concentration (101.465 μM, C_{DPPH}) in the reaction medium was calculated from the DPPH calibration curve with the equation: $A_{517nm} = 0.010\ C_{DPPH} + 0.055$ ($r^2 = 0.999$). A Trolox calibration curve was plotted to assess the antioxidant activity. The equation of the Trolox calibration curve was $A = 0.0270c + 0.0008$, the correlation coefficient was $r^2 = 0.999$, and the results were expressed as μmol of Trolox equivalent/100 g of flour.

Statistical Analysis. The results reported in this study are the averages of three repetitions ($n = 3$), unless otherwise stated. Tukey's honest significant difference multiple comparison (one-way analysis of variance) and Pearson's linear correlations, both at $p < 0.05$, were evaluated using Statistica 6.0 (2001, StatSoft, Tulsa, OK).

RESULTS AND DISCUSSION

Overview of the Extraction Methods Used for Free and Bound Phenolic Compounds in Barley Flour. Several extraction experiments, based on different mixtures and methods, were carried out in order to verify the efficiency, reliability, and convenience of extracting phenolic compounds from barley. Moreover, the selectivity of each solvent and method in extracting certain phenolic groups was evaluated. For the purpose, several spectrophotometric determinations were made. The Folin−Ciocalteu method and absorption at 280 nm provided the TPC, while the specific UV spectrophotometric indices enabled the quantification of the three main phenolic groups: flavonols, hydroxycinnamic acids, and *o*-diphenols, which were identified at 370, 320, and 370 nm (after reaction with molybdate), respectively. Moreover, a spectrophotometric evaluation was made of the antioxidant capacity of each extract by carrying out the DPPH test. Therefore, a spectrophotometric investigation was carried out in order to identify the best extraction method for barley phenols and constitutes the preliminary step toward further identification and characterization of the phenols extracted with the proposed methods (*34*).

As reported in the Materials and Methods, the first extractions were made using different mixtures: s_1, 4/1 ethanol/water (v/v); s_2, 4/1 methanol/water (v/v); and s_3, 4/1 acetone/water (v/v). Higher extraction yields for all of the phenolic groups were realized with the alcoholic mixtures, such as s_1, 4/1 ethanol/water (v/v), and s_2, 4/1 methanol/water (v/v), while the specific extraction of flavan-3-ols and proanthocyanidins, such as procyanidins and prodelphinidins, can be achieved using acetone-based mixtures (*8*, *9*, *34*).

With regard to automated pressurized liquid extraction, the ASE 200 system was used to evaluate whether the extraction steps could be automated, which would lower the overall extraction time and the amount of organic solvents required. Therefore, the extraction yield of phenols obtained through ASE was compared to those obtained from conventional solid/liquid methods. The 4/1 ethanol/water (v/v) mixture (s_1) was chosen to extract the whole phenolic pattern. The total experimental time, which included 5 min of extraction time (static mode) and the time allocated to preparing the sample cell, was comparable to that of conventional solid/liquid extraction methods.

Bound barley phenols were extracted by soft acid and alkaline hydrolyses. Generally, alkaline hydrolysis is the method most used for extracting bound phenols (*7*, *23*−*25*), probably because room temperature conditions are required to release the phenolic

Table 3. Spectrophotometric Indices of Barley Extracts[a]

extraction method	TPC	PI	ODI	FI[c]	HI[d]	TEAC[e]
AcH_2O	0.68 ± 0.09	0.30 ± 0.09	0.66 ± 0.03	0.02 ± 0.00	0.06 ± 0.00	421.07
EtH_2O	0.38 ± 0.02	0.34 ± 0.01	0.56 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	122.11
$EtAcH_2O$	0.32 ± 0.07	0.37 ± 0.03	0.74 ± 0.05	0.02 ± 0.00	0.05 ± 0.01	116.69
$MetH_2O$	0.29 ± 0.04	0.29 ± 0.04	1.08 ± 0.04	0.01 ± 0.01	0.03 ± 0.02	103.87
AcEt-N_2	0.37 ± 0.08	0.25 ± 0.05	0.87 ± 0.02	0.01 ± 0.01	0.03 ± 0.01	227.60
EtAc-N_2	0.42 ± 0.01	0.41 ± 0.06	0.71 ± 0.03	0.02 ± 0.00	0.06 ± 0.02	154.40
AcEt+N_2[f]	0.25 ± 0.14	0.41 ± 0.11	1.10 ± 0.29	0.01 ± 0.01	0.07 ± 0.02	164.87
fract Ac[f]	0.65 ± 0.21	0.39 ± 0.09	0.72 ± 0.28	0.02 ± 0.01	0.05 ± 0.01	383.24
fract Et[f]	0.13 ± 0.04	0.17 ± 0.02	0.64 ± 0.22	0.01 ± 0.00	0.02 ± 0.00	25.01
ASE60-5/3	0.24 ± 0.05	0.28 ± 0.06	0.65 ± 0.09	0.02 ± 0.01	0.03 ± 0.01	94.51
ASE90-5/3	0.20 ± 0.06	0.30 ± 0.04	0.60 ± 0.04	0.02 ± 0.01	0.03 ± 0.01	73.07
ASE120-5/3	0.18 ± 0.09	0.26 ± 0.06	0.48 ± 0.05	0.01 ± 0.01	0.03 ± 0.00	62.96
ASE60-2/4	0.32 ± 0.03	0.39 ± 0.04	0.82 ± 0.18	0.03 ± 0.01	0.01 ± 0.01	97.96
ASE90-2/4	0.30 ± 0.06	0.37 ± 0.04	0.71 ± 0.04	0.03 ± 0.01	0.02 ± 0.00	90.07
ASE120-2/4	0.22 ± 0.07	0.37 ± 0.11	0.69 ± 0.24	0.06 ± 0.01	0.07 ± 0.02	43.24
soft acid hydrolysis[f]	0.49 ± 0.14	6.85 ± 1.65	1.19 ± 0.33	0.04 ± 0.03	0.24 ± 0.07	426.74[g]
alkaline hydrolysis 4 h[f]	0.27 ± 0.15	0.56 ± 0.14	0.59 ± 0.19	0.19 ± 0.11	0.26 ± 0.15	20.08
alkaline hydrolysis 20 h	0.24 ± 0.21	1.10 ± 0.05	0.43 ± 0.15	0.42 ± 0.06	1.43 ± 0.05	133.70

[a] Values are expressed as averages ± standard deviation ($n = 3$). [b] Expressed as mg gallic acid/g flour. [c] Expressed as mg quercetin/g flour. [d] Expressed as mg ferulic acid/g flour. [e] Expressed as μmol Trolox equivalents/100 g flour. [f] Average value from six repetitions. [g] Average value from three repetitions.

compounds bound to the cell wall polysaccharides or associated to the starchy endosperm. On the other hand, acid hydrolysis requires higher temperatures, which might cause the degradation of the phenolic compounds. Usually, alkaline hydrolysis needs longer digestion times than acid hydrolysis. To verify the relationship between the digestion time and the release of bound phenols, two digestion times, 4 and 20 h, were compared.

Spectrophotometric Analysis of Free Phenols from Barley Flour Extracts. The spectrophotometric results are given in **Table 3**. The Folin−Ciocalteau method measured the total phenolic compounds expressed as gallic acid. The highest extraction yield for total phenolic compounds was obtained when the barley flour was extracted with 4/1 acetone/water (v/v) as a first extraction blend. In fact, the TPC value of the AcH_2O and fract Ac samples (0.68 ± 0.09 and 0.65 ± 0.21 mg gallic acid/g flour, respectively) was about double that of most extracts (from 0.13 ± 0.04, fract Et, to 0.38 ± 0.02, EtH_2O, mg gallic acid/g flour) and significantly different at $p < 0.05$. The only sample that was not significantly different ($p < 0.05$) from the AcH_2O and fract Ac samples was EtAc-N_2 (0.42 ± 0.01 mg gallic acid/g flour).

The PI represents the absorption of an adequate dilution of barley extracts at 280 nm. The PI enables the determination of the TPC of the barley extracts, since phenols exhibited an absorption maximum at 280 nm due to the hydroxyphenyl group. Riberau-Gayon (*29*) and Maillard et al. (*30*) have determined phenolic compounds at 280 nm in wine, barley, and malt, respectively. Generally, ethanol and acetone-based extracts (EtAc-N_2, AcEt+N_2, fract Ac, $EtAcH_2O$, and EtH_2O) and ASE extracts (2/4 flour/Hydromatrix ratio) gave higher phenol indices (from 0.34 ± 0.01 to 0.41 ± 0.11 mg gallic acid/g flour) than other extracts (from 0.17 ± 0.02 to 0.30 ± 0.09 mg/g flour), even though significant differences were not found at $p < 0.05$. The lowest significant absorption ($p < 0.05$), at 280 nm (0.17 ± 0.02 mg gallic acid /g flour), was found in the fract Et extract.

The ODI enables the quantification of the antioxidant compounds that have an *o*-diphenolic structure. The AcEt+N_2 extract had the highest significant ODI value (1.10 ± 0.29 mg gallic acid/g flour, $p < 0.05$), while the other samples had similar ODI results.

The absorbances at 370 and 320 nm were used to evaluate FI (expressed as mg quercetin/g flour) and HI (expressed as mg ferulic acid/g flour) contents, respectively. Interestingly, the highest significant FI values ($p < 0.05$) were found for the ASE extracts that had a 2/4 flour/Hydromatrix ratio. The highest FI was found in the ASE120-2/4 sample (0.06 ± 0.01 mg quercetin/g flour), whereas fract Et had the lowest significant FI value (0.01 ± 0.00 mg quercetin/g flour, $p < 0.05$). With regard to the HI, AcEt+N_2 and ASE120-2/4 had the highest significant values (0.07 ± 0.02 mg quercetin/g flour, $p < 0.05$), whereas fract Et, ASE60-2/4, and ASE90-2/4 extracts had the lowest HI values (lower than 0.02 mg quercetin/g flour, $p < 0.05$).

To assay the antioxidant activities of the extracts, as radical scavenging capacity (expressed as TEAC), the spectrophotometric DPPH method of Parejo et al. (*32*) and Brand-Williams et al. (*33*) was carried out with some modifications. Because interfering compounds might be extracted during the extraction steps, which could affect the previous spectrophotometric indices, the TEAC spectrophotometric measurement provided accurate information on the compounds that had authentic antioxidant properties. As **Table 3** reports, the AcH_2O and fract Ac samples showed the highest significant TEAC values (421.07 and 383.24 μmol Trolox equivalents/100 g flour, respectively, at $p < 0.05$ level), which were from two to four times greater than the other samples. The lowest antioxidant activity was found in the fract Et sample (25.01 μmol Trolox equivalents/100 g flour, $p < 0.05$), which was about 15 times lower than that of fract Ac.

Generally, the TEAC and TPC values of the ASE samples were lower than those for traditional solid/liquid extraction. This result did not always correspond to other spectrophotometric indices (PI, ODI, FI, and HI), which, in most cases, gave similar or higher results for the ASE extracts than for the other samples. Such behavior might be explained by the extraction principle of the automated method. In fact, both the diffusivity coefficient and the extraction power of a liquid extraction mixture increase when high pressure and high temperatures are applied. The increased extraction power allows a higher extraction yield for both the analytes and the interfering compounds, highly correlated to the sample matrix. Therefore, the nonphenolic compounds extracted with the ASE procedure, such as simple carbohydrates, may have interfered with the spectrophotometric indices (PI, ODI, HI, and FI), while they did not interfere with the measurement of the scavenging activity, since it was based on a specific phenolic structure reaction. Moreover, increasing

the temperature of the ASE extractions decreased the TPC, ODI, and TEAC values, probably due to the degradation of the phenolic compounds at higher temperatures.

Positive Pearson's linear correlations between TEAC and TPC ($r^2 = 0.876$, $p < 0.0001$) and between TEAC and PI ($r^2 = 0.361$, $p < 0.01$) were found, while relationships between TEAC and ODI, FI, and HI were not detected. Positive Pearson's linear correlations between TPC and PI ($r^2 = 0.459$, $p < 0.0001$) were also found. Therefore, the sum of the free phenolic compounds (detected by using the Folin−Ciocalteu method and absorption at 280 nm) showed the highest significant effect on the TEAC rather than on a specific phenolic group.

Moreover, the highest extraction yields of phenols, expressed as TPC and TEAC, were produced when the barley flour was extracted with a 4/1 acetone/water (v/v) as a first (without drying of the residual flour) or single extraction blend. The TPC (by the Folin−Ciocalteu method) and TEAC (by DPPH reaction) can be reliably used to obtain precise information on the extraction yield of barley phenols. Interesting extraction yields were also produced when ethanol-based mixtures were used as a first or a single extraction blend. However, pressurized liquid extraction (using 4/1 ethanol/water, v/v) did not provide suitable extraction yields of free phenolic compounds from barley flour.

Spectrophotometric Analysis of Bound Phenolic Compounds from Barley Flour Extracts. With regard to alkaline hydrolysis, the effect of the digestion time on the extraction capacity of phenolic compounds was evaluated. The 20 h alkaline hydrolysis showed higher significant values ($p < 0.05$) for the HI, FI, PI, and TEAC as compared to the 4 h hydrolysis (TEAC, 133.70 μmol Trolox equivalents/100 g flour; HI, 1.43 $\pm$ 0.05 mg ferulic acid/g flour; FI, 0.42 $\pm$ 0.06 mg quercetin/g flour; and PI, 1.10 $\pm$ 0.05 mg gallic acid/g flour), while no significant differences were detected for TPC and ODI (**Table 3**). As can be seen, increasing the alkaline hydrolysis digestion time from 4 to 20 h clearly increased the HI and TEAC. Therefore, longer digestion times led to higher phenolic extraction yields (*7*, *23*−*25*), and the bound phenols were found to be mainly hydroxycinnamic acids (*11*−*15*).

Acid hydrolysis showed higher significant TPC, PI, ODI (0.49 $\pm$ 0.14, 6.85 $\pm$ 1.65, and 1.19 $\pm$ 0.33 mg gallic acid/g flour, respectively), and TEAC values (426.74 μmol Trolox equivalents/100 g flour) and less significant HI and FI values than alkaline hydrolysis at both 4 and 20 h ($p < 0.05$). Therefore, higher amounts of hydroxycinnamic acids and flavonols were extracted when the alkaline hydrolysis digestion time was prolonged, while higher extraction yields of the more generic phenolic compounds, presenting considerable antioxidant activity, were produced through soft acid hydrolysis. In fact, because acid hydrolysis produced TPC, ODI, and PI indices two, six, and three times higher, respectively, than alkaline hydrolysis at 20 h and, similarly, a TEAC value about three times higher, this could suggest that the phenolic compounds extracted by soft acid hydrolysis had a greater antiradical capacity than the hydroxycinnamic acids extracted by prolonged alkaline hydrolysis (the 20 h alkaline hydrolysis HI was about six times higher than the acid hydrolysis HI), probably as a result of their chemical properties. Moreover, as **Table 3** shows, when the hydrolysis digestion time was increased, this improved the overall precision of the analysis.

In conclusion, as reported in this study, aqueous acetone and aqueous ethanol-based extraction mixtures led to higher extraction yields of free phenols, mainly in terms of total phenolic compounds. In fact, a TPC value (0.68 $\pm$ 0.09 mg gallic acid/g flour, $p < 0.05$) double that of the extracts was achieved when 4/1 acetone/water (v/v) was used as the extraction blend. The Folin−Ciocalteu spectrophotometric assay used to evaluate total phenolic compounds and the DPPH method might be employed as precise and accurate indices to assess the amount of phenolic barley compounds presenting antioxidant activity. In fact, a positive correlation between these two indices was found ($r^2 = 0.876$, $p < 0.001$), with the AcH_2O having the highest TEAC value (421.07 μmol Trolox equivalents/100 g flour, $p < 0.05$). The automated pressurized liquid procedure could be used to extract free phenols from barley flour, which produces lower extraction yields, while bound phenols can only be extracted by prolonged hydrolysis. In fact, some classes of phenolic compounds bound to cell wall material can be selectively extracted through hydrolysis. Prolonged alkaline hydrolysis seems to be a reliable method for extracting hydroxycinnamic acids, while acid hydrolysis allows higher extraction yields of generic phenols, presenting considerable antioxidant activities. Therefore, complementary information on the bound phenolic pattern of barley can be obtained from both hydrolysis methods.

Interestingly, no correlations between the spectrophotometric and the antioxidant results of free and bound phenolic compounds were recorded, probably because of the different spectrophotometric response factors and antiradical properties of the phenols belonging to the free (catechins and proanthocyanidins) and bound (hydroxycinnamic acids) classes, as reported by several authors (*7*−*15*, *34*). As this work reports, to carry out a precise investigation on the antioxidant activity in barley, both free and bound phenolic compounds must be extracted in order to provide a complete antioxidant profile. Last, the analytical characterization of free and bound phenolic compounds, extracted using different methods, will be thoroughly investigated in further studies.

ABBREVIATIONS USED

TPC, total phenol content; PI, phenol index; ODI, *o*-diphenol index; FI, flavonol index; HI, hydroxycinnamic index; TEAC, Trolox equivalent antioxidant capacity.

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