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Free and bound phenolic compounds in barley (*Hordeum vulgare* L.) flours Evaluation of the extraction capability of different solvent mixtures and pressurized liquid methods by micellar electrokinetic chromatography and spectrophotometry

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Abstract

Phenolic compounds exist in free and bound forms in cereals. The efficiency, reliability and suitability of recovering free phenolic compounds from barley by conventional, solid–liquid and pressurized solvent extractions, using different mixtures and methods, were tested. The extraction recovery of bound phenolics was evaluated using alkaline and acid hydrolyses. This study illustrates a rapid application of micellar electrokinetic chromatography for the analysis of free and bound phenolic compounds in barley samples. After developing a capillary electrophoresis optimization plan, barley phenols were analyzed within 5.5 min, using a buffer containing 20 mM sodium tetraborate, 10 mM sodium dodecyl sulfate and 5 mM KH_2PO_4 (pH 9), a 40 cm × 50 μm capillary, 30 kV and 30 °C. The selectivity of the extraction methods in recovering phenolic classes was evaluated by capillary electrophoresis and compared with spectrophotometric measurements. Electropherograms of free phenolic extracts showed flavan-3-ol compounds, proanthocyanidins and hydrolysable tannins. Aqueous acetone and aqueous ethanol solvents extracted the highest amount of catechins and hydrolysable tannins, respectively. The extraction yield of bound phenolic compounds (especially hydroxycinnamic acids) increased when the digestion time for alkaline hydrolysis was prolonged.

Keywords: Micellar electrokinetic chromatography; Barley; Free radical scavenging activity; Phenolic compounds; Extraction methods

1. Introduction

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. Increased consumption of phenolic compounds has been correlated with a reduced risk of cardiovascular diseases and certain cancers [1,2].

Barley grains are widely consumed due to their positive dietary and technological properties, while barley meals and fractions are used in the production of functional foods (pastas, baked products) [3,4], because they contain bioactive compounds such as β-glucans and tocols [5,6]. Furthermore, a wide range of antioxidant compounds with a phenolic structure has been found in barley, such as benzoic and cinnamic acid derivatives, proanthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds [7–9]. Phenolic compounds are found in both free and bound form in cereals. Generally, the free phenolic

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compounds are proanthocyanidins or flavonoids, while the bound phenolic compounds are ester-linked to cell-wall polymers, ferulic acid and its dehydrodimer derivatives being the major phenolic compound present [10–15].

Most studies in literature determine the amount of free and bound phenols in cereals (after their extraction from finely-ground flour), by spectrophotometric analysis, high-performance liquid chromatography (HPLC) [7,16–20], capillary gas chromatography (cGC) [11,21], or gas–liquid chromatography (GLC) [22,23]. Conventional HPLC analysis of phenolic compounds in cereals is time-consuming, while capillary electrophoresis might represent a good compromise between the analysis time and the characterization of the phenolic compounds in cereals. There are as yet no explicit references regarding the separation of barley phenols through capillary electrophoresis, so it could be interesting to evaluate whether this analytic technique is suitable for quantifying and characterizing phenolic compounds in cereals [24–26].

Most previous studies, concerning the extraction of free phenolic compounds from cereals have used various aqueous solutions of methanol, ethanol and acetone [22,27–33]. Therefore, a universal methodology for extracting free soluble phenols from cereals has not yet been established. Moreover, these studies require long extraction times and/or the use of finely powdered samples in order to ensure the highest extraction yield of phenolic compounds from cereal flour. However, an exhaustive composition of phenolic compounds cannot be obtained by evaluating free soluble phenolic compounds alone, since cereals also contain a significant amount of bound phenolic compounds.

Most researchers determine the bound phenolic compounds in cereal flours using alkaline hydrolysis, divided into rapid hydrolysis (from 1 to 4–6 h) and long hydrolysis, when the digestion time is more than 16 h [7,22,34,35]. Only few reports have evaluated the recovery of the phenolic compounds of cereals using acid hydrolysis [16,17].

Moreover, an automated system of pressurized solvent extraction may be an interesting alternative to the conventional time-consuming solid–liquid extraction method for extracting phenols from flours, since it is automated and rapid.

Therefore, the aim of this work was to evaluate the yield and selectivity of different extraction methods for both free and bound phenolic compounds of barley. Furthermore, an automated system of pressurized liquid extraction (PLE) was compared to conventional solid–liquid extraction. The extraction yield was assayed, correlating several spectrophotometric measurements with the free radical scavenging activity of extracts (FRSA, using the 2-diphenyl-1-picrylhydrazyl (DPPH) assay) and the optimized micellar electrokinetic chromatography quantification. The classical colorimetric Folin–Ciocalteu method and absorption at 280 nm were used to evaluate the extraction yield of total phenolic compounds, while three main groups of phenolic compounds were quantified using specific UV spectrophotometric indices: flavonols at 370 nm, hydroxycinnamic acids at 320 nm, and *o*-diphenols at 370 nm (after reaction with molybdate). Moreover, the capability of different solvent systems to extract free and bound barley phenols and the suitability of the optimized micellar electrokinetic chromatographic (MEKC) method for quantification and characterization of barley phenolic compounds were also discussed. To our knowledge, this study is the first in-depth and extensive attempt to quantify the complete pattern of phenolic compounds in barley by capillary electrophoresis.

2. Experimental

2.1. Samples, reagents and materials

Organic wholemeal barley flour was purchased in a local market. Unless otherwise stated, all solvents were pro-analysis grade and from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), KH_2PO_4, and water for HPCE were from Fluka (Buchs, Switzerland). Procyanidin B1 and B2 were from Extrasynthese (Genay, France); prodelphinidin B3, (+)-catechin, (−)-epicatechin, *trans*-ferulic acid, *trans*-*p*-coumaric, cinnamic acid, gallic acid and quercetin were from Sigma–Aldrich (St. Louis, MO, USA).

Unless otherwise stated, every extraction trial on the phenolic compounds was replicated three times ($n = 3$). The extracts were stored at −18 °C before use.

2.2. Extraction of free phenolic compounds

2.2.1. Solid–liquid extraction

In order to collect the free phenolic compounds, 5 g of wholemeal barley flour were extracted by sonicating the flour with 40 mL of various organic solvent/water extraction mixtures for 10 min. The following extraction mixtures were used: s_1, ethanol–water (4:1, v/v) (EtH_2O extract), s_2, methanol–water (4:1, v/v) ($MetH_2O$ extract), s_3, acetone–water (4:1, v/v) (AcH_2O extract). After centrifugation at 1000 g for 10 min, the supernatant was removed and extraction was repeated once again. Supernatants were pooled, evaporated at 40 °C with a vacuum evaporator and reconstituted with 5 mL of water–formic acid (99.7:0.3, v/v) (extraction cycle A). In order to maximize the phenolic compounds' extraction yield, the residual flour from the first two extraction steps was either dried (extraction cycle B), or not dried (extraction cycle C) with nitrogen and extracted two more times using a different organic mixture (see Table 1 and Fig. 1 for the experimental extraction plan and sample explanation). In order to verify the selective recovery of phenolic classes, the extraction fractions were also, in one case, kept separate between the A and B extraction cycles.

2.2.2. Pressurized liquid extraction

An ASE 200 Model (Dionex, Germany), an automated extraction system for pressurized liquid extraction, was used to

Table 1
Experimental plan of conventional solid–liquid and pressurized liquid extractions

Experiment name	Conventional solid–liquid extraction				
	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	–	–	–	–
$MetH_2O$	s_2	–	–	–	–
EtAc-N_2	s_1	–	–	s_3	A + C
Fract Ac and Fract Et	s_3 (Fract Ac)	Yes	s_1 (Fract Et)	–	Supernatants A and B kept separated
Experiment name	Automated PLE				
	Temperature (°C)	Flour (g)	Hydromatrix (g)		
PLE60-2/4	60	2	4		
PLE90-2/4	90	2	4		
PLE120-2/4	120	2	4		

Abbreviations: EM, extraction mixture; s_1, ethanol–water (4:1, v/v); s_2, methanol–water (4:1, v/v); s_3, acetone–water (4:1, v/v). Other PLE settings were: cycle time 5 min (two cycles in static mode), solvent flush 60%, pressure extraction 20 MPa, extraction mixture ethanol–water (4:1, v/v), for all methods.

extract phenols from barley flour. Two grams of wholemeal barley flour were mixed with 4 g of Hydromatrix (Dionex, Germany) and placed in the extraction cell (33 mL). Two 5-min static cycles were applied at 20 MPa, using ethanol–water (4:1, v/v) as the extraction mixture. The extraction temperatures were set at 60, 90, and 120 °C (Table 1 shows the experimental plan and sample explanation). The solvent flush was 60% and purging time was 60 s. The fractions extracted were evaporated at 40 °C with a vacuum evaporator and reconstituted with 2 mL of water–formic acid (99.7:0.3, v/v).

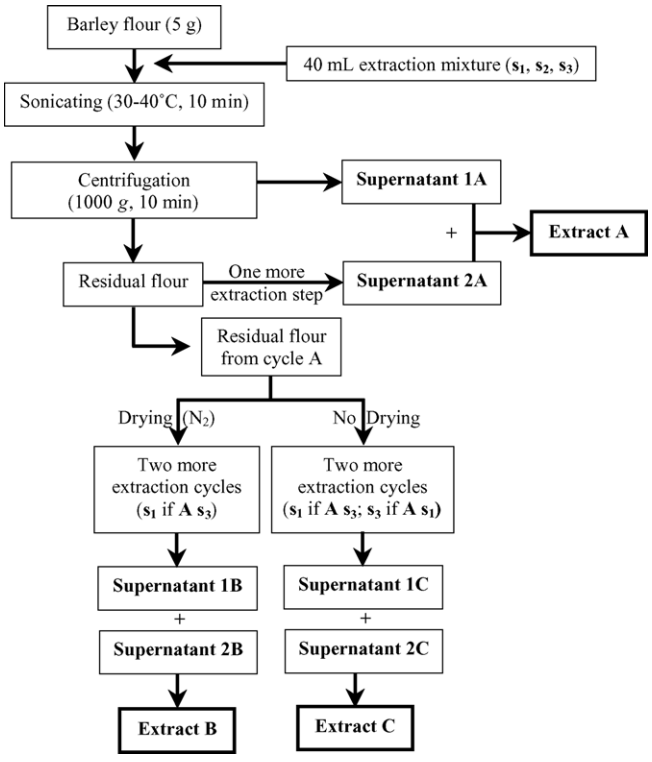

Fig. 1. Experimental extraction plan of phenolic compounds from barley flour. When extract A is an ethanol–water (4:1, v/v) extract (s_1) and extract C is an acetone–water (4:1, v/v) extract (s_3), and they were collected: the sample was called EtAc-N_2; extract A is an acetone–water (4:1, v/v) extract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

2.3. Extraction of bound phenolic compounds

2.3.1. Alkaline hydrolysis

One gram of wholemeal flour was digested with 100 mL of 2 M NaOH at room temperature and shaken under nitrogen gas for two different digestion periods (4 and 20 h). The mixture was then acidified at pH 2–3 in 10 M of hydrochloric acid in a cooling-ice bath and extracted with 500 mL of hexane to remove lipids by a separator funnel. The final solution was extracted five times with 100 mL of diethyl ether–ethyl acetate (1:1, v/v) by a separator funnel. The organic fractions were pooled and evaporated to dryness. The phenolic compounds were reconstituted with 5 mL of water–formic acid (99.7:0.3, v/v).

2.3.2. Soft-acid hydrolysis

One gram of wholemeal 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 diethyl ether–light petroleum (b.p. 40–60 °C) (1:1, v/v) were added to the digested samples. Using a separator funnel, the organic fraction was discarded and the residue was washed twice in 25 mL of diethyl ether–light petroleum (b.p. 40–60 °C) (1:1, v/v). Lastly, the aqueous fraction was washed five times in 100 mL of diethyl ether–ethyl acetate (1:1, v/v) to remove lipids by a separator funnel. The organic fractions were pooled and evaporated to dryness. Phenolic compounds were reconstituted with 5 mL of water–formic acid (99.7:0.3, v/v).

2.4. Spectrophotometric assays

The spectrophotometric analyses were performed using a UV-1601 spectrophotometer from Shimadzu (Duisburg, Ger-

many) and were replicated three times for each extract or calibration point ($n = 3$).

2.4.1. Determination of total phenolic compounds (TPC)

The total phenolic compounds of the extracts were determined by the Folin–Ciocalteu spectrophotometric method according to Singleton and Rossi [36]. A gallic acid calibration curve (range 1–1500 μg/mL) was plotted ($A = 1.0559c + 0.0178$, $r^2 = 0.999$) to assess the total content of phenolic compounds.

2.4.2. Phenols index (PI)

The phenols index was made according to Riberau-Gayon [37] and Maillard et al. [38], with slight modifications, as reported by Bonoli et al. [39]. A gallic acid calibration curve (range 1–2000 μg/mL) was plotted ($A = 0.4142c + 0.0017$, $r^2 = 0.999$) to assess the phenols index.

2.4.3. Flavonols (FI) and hydroxycinnamics indices (HI)

The flavonols and hydroxycinnamics indices were obtained by diluting 200 μL of each phenolic extract in 10 mL of methanol. The solution was shaken and absorbance was evaluated at 370 and 320 nm (at 25 °C), respectively, using quartz cuvettes. Quercetin (FI calibration range: 1–1000 μg/mL; FI equation: $A = 0.6346c + 0.0033$, $r^2 = 0.999$) and ferulic acid (HI calibration range: 1–1000 μg/mL; HI equation: $A = 0.8974c - 0.0119$, $r^2 = 0.996$) calibration curves were plotted.

2.4.4. o-Diphenols index (ODI)

The spectrometric determination of *o*-diphenols by Mateos et al. [40] was adopted although slightly modified as reported by Bonoli et al. [24]. A gallic acid calibration curve (range 1–2000 μg/mL) was plotted to assess the *o*-diphenols index. The equation of the gallic acid calibration curve was $A = 1.0673c + 0.0363$ and the correlation coefficient $r^2 = 0.999$.

2.5. Evaluation of the free radical scavenging activity

In order to determine the free radical scavenging activity of the extracts, the 2,2-diphenyl-1-picrylhydrazyl assays was evaluated according to Parejo et al. [41] and Brand-Williams et al. [42], with some modifications. An aliquot of each extract (100 μL) was added to 2.9 mL of 100 μM DPPH (Sigma, St. Louis, MO, USA) solution in methanol–water (80:20, v/v). The decrease in absorbance was noticed at 517 nm in the 0–30 min range (at 25 °C). One hundred microliters of water–formic acid (99.7:0.3, v/v) added to 2.9 mL methanol–water (80:20, v/v) was used to zero the spectrometer. The exact initial DPPH concentration ($C_{DPPH} = 101.465$ μM) in the reaction medium was calculated from the DPPH calibration curve, having the equation: $A_{517\,nm} = 0.010C_{DPPH} + 0.055$ ($r^2 = 0.999$). A Trolox calibration curve ($A = 0.0270c + 0.0008$, $r^2 = 0.999$) was used to assess the free radical scavenging activity (FRSA). The results were expressed as μmol of Trolox equivalents/100 g of flour.

2.6. Capillary electrophoresis analysis

A Beckman capillary electrophoresis instrument P/ACE 5500 (Beckman Instruments, Fullerton, CA, USA) equipped with a diode array detector was used. Beckman P/ACE Station software was used for data acquisition and processing on a personal computer. The capillary cartridge contained uncoated fused silica tubing (50 μm i.d. × 375 μm o.d.) supplied by Beckman. Total capillary length was 47 cm, whereas the effective length was 40 cm. UV detection was carried out at 200 nm. Before use, the new capillary was conditioned by flushing 1 M sodium hydroxide solution (5 min), 0.1 M sodium hydroxide (5 min), HPCE-grade water (5 min) and, lastly, the running buffer (5 min). The capillary not in use was stored in water to prevent buffer crystallization.

A 20 mM sodium tetraborate, 5 mM KH_2PO_4, and 10 mM SDS buffer (pH 9.0) was used. The buffer was sonicated for 10 min before use. Samples were injected hydrodynamically at the anodic end in low-pressure mode (0.5 psi) for 3 s (1 psi = 6894.76 Pa). Electrophoretic separations were carried out using a positive power supply of 30 kV at 30 °C (current = 68–70 μA). Before each injection, the capillary was rinsed consecutively with 0.1 M NaOH (2 min), HPCE-grade water (2 min) in high-pressure mode (20 psi), and re-equilibrated with the running buffer (2 min). After each electrophoretic cycle, the capillary was rinsed with HPCE-grade water (2 min). All washing steps were performed at the same temperature as the run. The running buffer was changed after three runs. The capillary electrophoretic analysis was replicated three times for each extract or calibration point ($n = 3$).

2.7. 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, ANOVA) and Pearson's linear correlations, both at $p < 0.05$ level, were evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Optimization of the micellar electrokinetic chromatography method

When alcohol-based extraction mixtures are employed a higher recovery index should be allowed for all phenolic classes [8,9]. Therefore, the extract obtained by means of ethanol–water (4:1, v/v) was used to optimize the electrophoretic method. The method's optimization for separation of phenolic compounds extracted from vegetable foods involved the study of several parameters, as

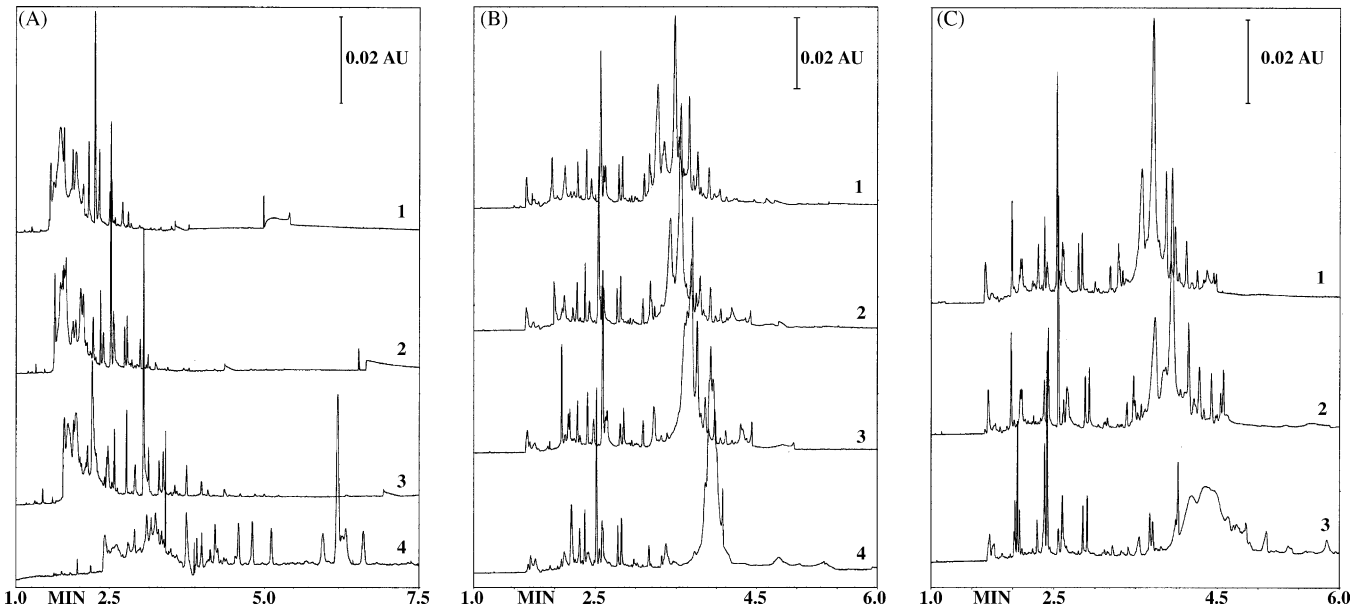

Fig. 2. Effect of tetraborate (A), SDS (B) and KH_2PO_4 (C) concentrations on the capillary electrophoresis separation of barley phenolic compounds. Running buffer in A: (1) 10 mM sodium tetraborate, (2) 20 mM sodium tetraborate, (3) 45 mM sodium tetraborate, (4) 100 mM sodium tetraborate. Running buffer in B: sodium tetraborate 20 mM containing (1) 5 mM SDS, (2) 10 mM SDS, (3) 20 mM SDS, (4) 45 mM SDS. Running buffer in C: (1) 20 mM sodium tetraborate, 10 mM SDS, 5 mM KH_2PO_4; (2) 20 mM sodium tetraborate, 10 mM SDS, 10 mM KH_2PO_4; (3) 20 mM sodium tetraborate, 20 mM SDS, 20 mM KH_2PO_4. Other conditions as in Section 2.

reported by Bonoli et al. [24–26]. In order to obtain the best separation of the electrophoretic peaks the type of buffer, its concentration and pH, the running voltage and applied temperature were varied. The effect of type of buffer and its concentration, at constant voltage and temperature (30 kV and 30 °C, respectively), are shown in Fig. 2: when the tetraborate concentration was increased from 10 to 100 mM the migration time of peaks increased, owing to the increased ionic strength of the running buffer, which determined a lower electroosmotic flow [43]. Using a 100 mM sodium tetraborate buffer (Fig. 2A), peak resolution increased even when separation efficiency decreased and the analysis took more than 10 min. When the SDS was added to the buffer (Fig. 2B), the first electrophoretic portion shifted to the right of the electropherogram, probably due to this fraction's affinity with the micellar phase. Increasing the SDS concentration to over 20 mM, led to a loss in peak resolution. When KH_2PO_4 was added to the buffer at a concentration of 5 mM, an improvement in peak resolution was detected, whereas adding over 20 mM produced a loss in peak resolution (Fig. 2C). Thus, the best background electrolyte, in terms of peak resolution and overall analysis time, was found to be 20 mM tetraborate, 10 mM SDS and a 5 mM KH_2PO_4 buffer.

In order to improve peak resolution, the pH of the optimized buffer (20 mM tetraborate, 10 mM SDS and 5 mM KH_2PO_4, at pH 9.0) was adjusted, adding appropriate amounts of 0.1 M HCl or 0.1 M NaOH to the following values: 8.0, 8.5, 8.8, 9.2, 9.4 and 9.8. When the buffer pH was increased, the peak migration times increased due to the higher ionization state of the phenols and increased ionic strength, which caused a lower electroosmotic flow [24,43,44]. When the pH of the buffer was lowered, the peak migration times and resolution decreased; thus, and the best peak resolution was found for pH 9.0 buffer (data not shown).

Voltage and temperature were changed from 20 to 30 kV and from 25 to 35 °C, respectively. When the voltage and temperature applied were raised, peak migration times decreased due to the positive effect of these two parameters on electrophoretic mobility. However, no significant effects on peak resolution were reported when 30 kV and 30 °C were applied, which were kept as the best separation conditions.

3.2. Validation of the MECK method

Repeatability was assessed for the ethanol–water (4:1, v/v) original extract and for the extract diluted 10-fold. Both extracts were injected 12 times on the same day (intraday precision, $n = 12$) and on three consecutive days (interday precision, $n = 36$). The relative standard deviations (R.S.D.s) of the peak areas and migration times were determined for each electrophoretic peak detected.

Intraday repeatability (expressed as R.S.D.) of the migration times was 0.20–0.57% for the undiluted extract and 0.33–0.62% for the extract diluted 10-fold, whereas interday repeatability was 0.51–0.65 and 0.60–0.80% for the undiluted extract and the extract diluted 10-fold, respectively.

Intraday repeatability (expressed as R.S.D.) of the total peak area was 5.43 and 7.35% for the undiluted extract and the extract diluted 10-fold, respectively, whereas interday repeatability was 6.16 and 8.12% for the undiluted extract and the extract diluted 10-fold, respectively. As expected, intra-

day precision was greater than interday precision and the method demonstrated good overall repeatability.

The method's sensitivity was assessed on four phenolic compounds found in barley samples identified by UV-DAD spectral analysis: gallic acid, (+)-catechin, ferulic acid and tannic acid, at a wavelength of 200 nm and solutions of 0.5, 0.1, 0.5 and 3.5 μg/mL, respectively, which gave a signal-to-noise ratio of approximately 3 (S/N ≈ 3), corresponding to the limit of detection (LOD) of the method.

The quantification procedure was performed in the range 1–2000, 1–1000, 1–2000, and 10–5000 μg/mL for the four above-mentioned phenolic compounds, respectively, using the peak area versus analyte concentration to make the calibration curves. The linearity range was assessed for each analyte using 11, 8, 10, and 8 concentration levels, respectively, which were injected three times ($n = 3$). Linear regression results were as follows ($A = mc \pm q$, where A is the peak area, c is the analyte concentration expressed as μg/mL, q is the y-intercept and r^2 is the correlation coefficient): $A = 322.45c + 872.82$ ($r^2 = 0.999$), $A = 393.95c + 3103.6$ ($r^2 = 0.997$), $A = 162.06c + 867.4$ ($r^2 = 0.999$), $A = 242.26c + 11202$ ($r^2 = 0.994$), for gallic acid, (+)-catechin, ferulic acid and tannic acid, respectively.

3.3. MEKC analysis of free phenolic compounds in barley samples

The electropherogram of an ethanol-based extract is given in Fig. 3. When aqueous ethanol was used as the only extraction solvent (s_1, EtH_2O sample) or as the first extraction solvent, without drying the residual flour with N_2 between extraction cycles A and C (s_1 followed by s_3, EtAc-N_2 sample), the electropherogram presented two separate zones (as Fig. 3A shows): in the former, several sharp and baseline separated peaks were detected between 1.9 and 3.3 min, whereas in the latter, a large group of unresolved peaks were found

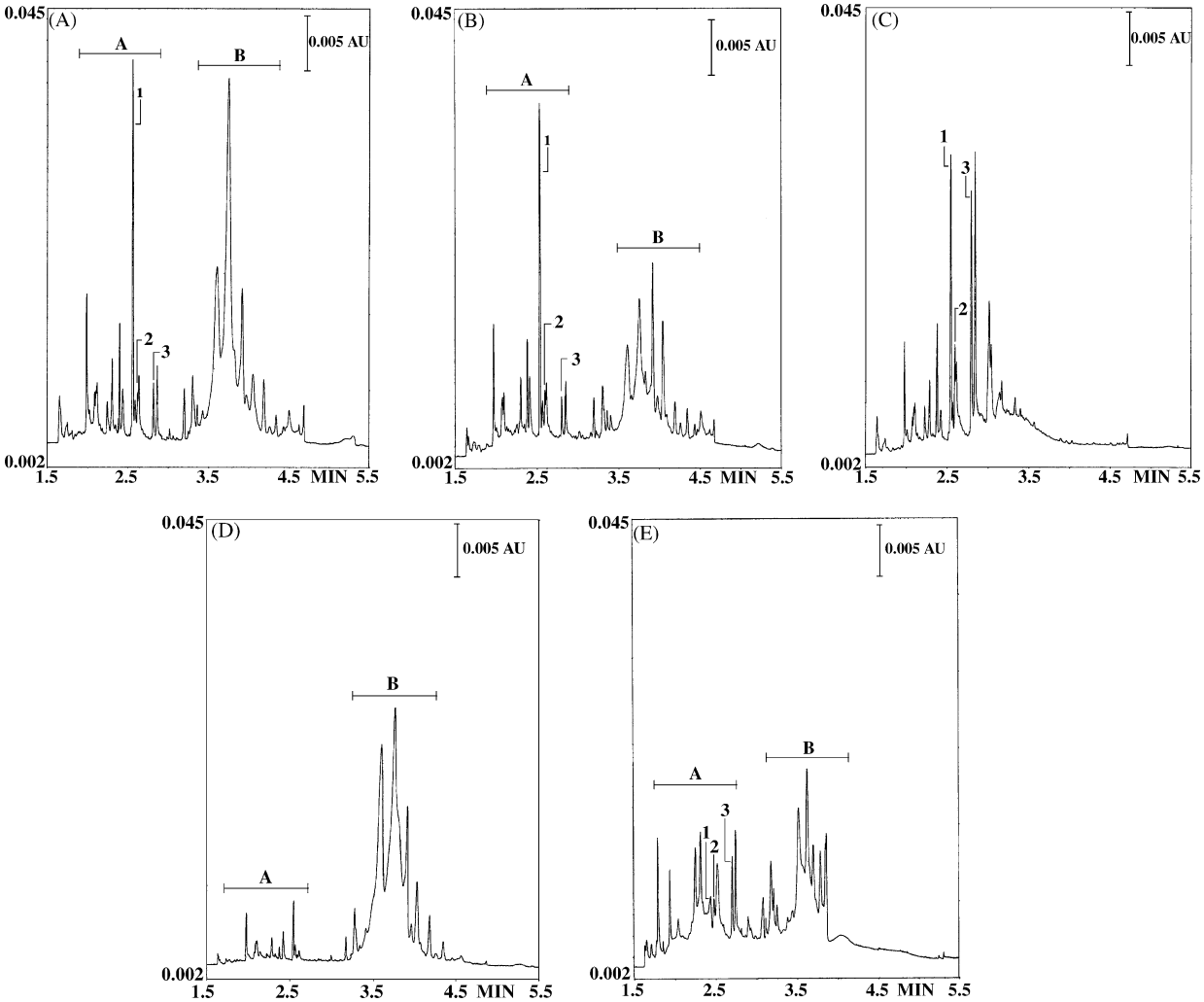

Fig. 3. Electropherograms of the ethanol-based (A), MetH_2O (B), Fract Ac (C), Fract Et (D) and PLE120-2/4 (E) extracts. In zone A are mainly catechins and proanthocyanidins, while in zone B are hydrolysable tannins (well matching with tannic acid). Peak identification: 1, (−)-epicatechin; 2, (+)-catechin; 3, prodelphinidin B3. Conditions as in Section 2.

between 3.4 and 4.7 min, which had greater affinity with the SDS than the first peaks, as reported in the paragraph on the optimization method. Spiking attempts with flavan-3-ols (usually called catechins) and their oligomers (also known as proanthocyanidins) showed that these compounds were in the first electrophoretic zone, while the second zone was well matched with the tannic acid standard. This behavior was confirmed by capillary electrophoresis UV-DAD spectral analysis, where most of the compounds in zone A had typical spectra of flavan-3-ols. Therefore, as reported in Fig. 3A, (−)-epicatechin, (+)-catechin, prodelphinidin B3 and tannic acid were identified in barley samples. To simplify the discussion, the zone A, which mainly consists of catechins and proanthocyanidins, was called zone of simple phenols, while zone B was called zone of hydrolysable tannins (well matching with tannic acid).

The electrophoretic profile of the simple phenols (zone A) of the aqueous methanol extract (s_2, $MetH_2O$ sample) was similar to that of the aqueous ethanol extract, whereas the extraction yield of hydrolysable tannins was slightly lower (see Fig. 3B).

When aqueous acetone was used as the only extraction solvent (s_3, AcH_2O sample and Fract Ac sample, where the supernatant collected from the extraction cycle A was kept separate from the supernatant of cycle B, as reported in Table 1 and Fig. 1), the electropherogram only showed the simple phenols zone (Fig. 3C). Therefore, the aqueous acetone selectively enhanced the catechins and proanthocyanidins extraction yield. In particular, the prodelphinidin B3 peak was markedly recovered, while hydrolysable tannins were not extracted.

Since aqueous ethanol extracted both flavan-3-ols and hydrolysable tannins when used as the first blend, in order to verify the selective recovery of the phenolic classes, the residual flour from cycle A, extracted with acetone–water (4:1, v/v) (s_3, Fract Ac sample), was extracted two more times with ethanol–water (4:1, v/v) (s_1, Fract Et sample), after being dried in an N_2 flow, and the fractions were kept separate. Fig. 3D shows that hydrolysable tannins were selectively recovered by aqueous ethanol when acetone was used as the first extraction mixture and the residual flour was dried in an N_2 flow. Fig. 3D also shows that the two extraction steps (cycle A) with aqueous acetone almost fully extracted the simple phenols.

In an attempt to find a satisfactory compromise between ethanolic and acetonic extractions, ethanol was mixed with acetone to make an ethanol–acetone–water (7:7:6, v/v/v) mixture (data not reported). However, it was found that ethanol had greater extraction power than acetone since the degree of extraction of simple phenols and hydrolysable tannins was similar to that obtained with the aqueous ethanol mixture (Fig. 3A).

PLE was carried out with ASE 200 instrumentation. Since the alcoholic mixture allowed the recovery of all the phenolic classes (simple phenols and hydrolysable tannins) the ethanol–water (4:1, v/v) was selected as the extraction blend. In order to achieve the maximum extraction area-to-solvent ratio, related to the capacity of the extraction cells used (33 mL), 2 g of wholemeal barley were mixed with 4 g of Hydromatrix, to avoid any packing effects. A 5:3 barley flour/Hydromatrix ratio had also been tested in a previous work [39], but the results were unsatisfactory (data not reported). Two 5-min static cycles at 20 MPa were used and 60, 90, and 120 °C were set as the extraction temperatures. Fig. 3E shows a typical electropherogram obtained by injecting a PLE sample, that of the PLE120-2/4 extract. The PLE samples had a worse peak resolution than the conventional solid–liquid extraction samples and, although the hydrolysable tannins were efficiently recovered, most of the peaks of the simple phenols zone were not extracted.

3.4. Quantification of free phenolic compounds in the barley samples by the MEKC method and statistical correlations with spectrophotometric results

In order to make a direct comparison between the extraction yield of phenolic compounds by conventional solid–liquid extractions and the 2/4 PLE sample series (barley flour weight/Hydromatrix weight), electrophoretic peaks were quantified. Therefore, all the peaks in the catechins and proanthocyanidins zone were quantified using the (+)-catechin calibration curve, while the peaks in the hydrolysable tannins zone were quantified using the tannic acid calibration curve. The peaks that migrated between both zones, having a typical phenolic UV spectrum, were quantified using the gallic acid calibration curve and added to the simple phenols. Tables 2 and 3 report the quantification list of the samples obtained by capillary electrophoresis. As can be seen, the highest value of simple phenols was detected by the AcH_2O extract (0.51 ± 0.04 mg phenols/g flour) that was not significantly different ($p < 0.05$) from Fract Ac. Thus, the amount of simple phenols extracted by aqueous acetone was almost twice that of other samples. However, the alcohol-based extractions (EtH_2O, $MetH_2O$, and EtAc-N_2) enabled the recovery of considerable amounts of catechins and proanthocyanidins (from 0.26 ± 0.00 to 0.31 ± 0.02 mg catechin and gallic acid/g flour, for EtH_2O and $MetH_2O$, respectively) as well as of hydrolysable tannins (from 1.26 ± 0.03 to 2.05 ± 0.37 mg tannic acid/g flour, for $MetH_2O$ and Fract Et, respectively).

Generally, the 2/4 PLE series extracted less simple phenols and hydrolysable tannins compared to the conventional extraction methods. Moreover, increasing the extraction temperature decreased the amount of simple phenols detected, probably due to their degradation at higher PLE temperatures.

Thus, the highest amount of total phenolic compounds recovered was obtained when ethanol was used as the extraction mixture, mainly because it extracted more hydrolysable tannins (2.14 ± 0.06 and 2.13 ± 0.36 mg phenols/g flour for EtH_2O and Fract Et, respectively).

Table 2
Spectrophotometric indices of barley extracts, expressed as average ± standard deviation ($n = 3$, unless otherwise stated)

Experiment name	TPC[a]	PI[a]	ODI[a]	FI[b]	HI[c]	FRSA[d]
AcH_2O	0.68 ± 0.09	0.30 ± 0.09	0.66 ± 0.03	0.02 ± 0.00	0.06 ± 0.00	421.07 ± 9.76
EtH_2O	0.38 ± 0.02	0.34 ± 0.01	0.56 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	122.11 ± 4.50
$MetH_2O$	0.29 ± 0.04	0.29 ± 0.04	1.08 ± 0.04	0.01 ± 0.01	0.03 ± 0.02	103.87 ± 11.56
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 ± 6.29
Fract Ac[e]	0.65 ± 0.21	0.39 ± 0.09	0.72 ± 0.28	0.02 ± 0.01	0.05 ± 0.01	383.24 ± 61.70
Fract Et[e]	0.13 ± 0.04	0.17 ± 0.02	0.64 ± 0.22	0.01 ± 0.00	0.02 ± 0.00	25.01 ± 7.22
PLE60-2/4	0.32 ± 0.03	0.39 ± 0.04	0.82 ± 0.18	0.03 ± 0.01	0.01 ± 0.01	97.96 ± 5.25
PLE90-2/4	0.30 ± 0.06	0.37 ± 0.04	0.71 ± 0.04	0.03 ± 0.01	0.02 ± 0.00	90.07 ± 34.97
PLE120-2/4	0.22 ± 0.07	0.37 ± 0.11	0.69 ± 0.24	0.06 ± 0.01	0.07 ± 0.02	43.24 ± 27.23
Acid hydrolysis[e]	0.49 ± 0.14	6.85 ± 1.65	1.19 ± 0.33	0.04 ± 0.03	0.24 ± 0.07	426.74 ± 124.65[f]
Alkaline hydrolysis for 4 h[e]	0.27 ± 0.15	0.56 ± 0.14	0.59 ± 0.19	0.19 ± 0.11	0.26 ± 0.15	20.08 ± 24.04
Alkaline hydrolysis for 20 h	0.24 ± 0.21	1.10 ± 0.05	0.43 ± 0.15	0.42 ± 0.06	1.43 ± 0.05	133.70 ± 5.35

Abbreviations: TPC, total phenolic compounds; PI, phenols index; ODI, *o*-diphenols index; FI, flavonols index; HI, hydroxycinnamics index; FRSA, free radical scavenging activity.

[a] Expressed as mg gallic acid/g flour.
[b] Expressed as mg quercetin/g flour.
[c] Expressed as mg ferulic acid/g flour.
[d] Expressed as μmol Trolox equivalents/100 g flour.
[e] Average value from six repetitions.
[f] Average value from three repetitions.

The values of the spectrophotometric indices and the capillary electrophoresis quantifications are given in Tables 2 and 3, and Fig. 4 illustrates the closest relationships that were found between TPC, FRSA, and spCEQ.

Since interfering compounds might be extracted together with the phenolic compounds during the extraction steps, which may affect the spectrophotometric measurements, the FRSA spectrophotometric assay could provide accurate information on the extracted compounds having authentic antioxidant power (as free radical scavengers). It is interesting to note Pearson's positive correlations between FRSA and TPC ($r^2 = 0.876$, $p < 0.0001$), FRSA and spCEQ ($r^2 = 0.887$, $p < 0.0001$), and, evidently, between TPC and spCEQ ($r^2 = 0.845$, $p < 0.0001$).

Table 3
Capillary electrophoresis quantifications of simple phenols (spCEQ), bound phenols (bpCEQ, for hydrolysis), hydrolysable tannins (htCEQ) and total phenols (tpCEQ) capillary electrophoresis quantification of barley extracts, expressed as average ± standard deviation ($n = 3$, unless otherwise stated)

Experiment name	spCEQ[a] or bpCEQ[b]	htCEQ[c]	tpCEQ[d]
AcH_2O	0.51 ± 0.04	–	0.51 ± 0.04
EtH_2O	0.26 ± 0.00	1.89 ± 0.06	2.14 ± 0.06
$MetH_2O$	0.31 ± 0.02	1.26 ± 0.03	1.57 ± 0.05
EtAc-N_2	0.28 ± 0.02	1.27 ± 0.41	1.54 ± 0.41
Fract Ac[e]	0.40 ± 0.11	–	0.40 ± 0.11
Fract Et[e]	0.08 ± 0.02	2.05 ± 0.37	2.13 ± 0.36
PLE60-2/4	0.23 ± 0.04	1.15 ± 0.03	1.38 ± 0.06
PLE90-2/4	0.19 ± 0.05	0.56 ± 0.26	0.75 ± 0.30
PLE120-2/4	0.16 ± 0.09	1.58 ± 0.13	1.79 ± 0.14
Acid hydrolysis[e]	0.91 ± 0.16[f]		
Alkaline hydrolysis for 4 h[e]	0.14 ± 0.08		
Alkaline hydrolysis for 20 h	0.59 ± 0.08		

[a] Expressed as mg (+)-catechin and gallic acid/g flour.
[b] Expressed as mg ferulic acid/g flour.
[c] Expressed as mg tannic acid/g flour.
[d] Expressed as mg phenols/g flour.
[e] Average value from six repetitions.
[f] Average value from three repetitions.

No significant correlations between ODI, FI, HI and FRSA were found (nor between the first three spectrophotometric indices and the TPC, PI and spCEQ) denoting that it was the free phenolic compounds (detected using the Folin–Ciocalteu method, by absorption at 280 nm and capillary electrophoresis) as a whole, that gave the highest significant contribution to the free radical scavenging activity, rather than a specific group of compounds. Moreover, the spCEQ values were close to those obtained with the Folin–Ciocalteu method, therefore it should be noted that the compounds selected represent the profile of the total phenolic compounds in barley.

No correlations were found between the capillary electrophoresis quantification of the hydrolysable tannins and most of the spectrophotometric indices. Therefore, this class of phenolic compounds did not contribute to the antioxidant capacity of the extracts (expressed as radical scavenging activity evaluated by the DPPH assay), while the simple phenols (catechins and proanthocyanidins) produced the greatest antioxidant power in the extract. For example, even though the Fract Et sample had one of the highest amounts of hydrolysable tannins (2.05 ± 0.37 mg tannic acid/g flour), it had the lowest significant values ($p < 0.05$) for spCEQ (0.08 ± 0.02 g (+)-catechin and gallic acid/g flour), FRSA (25.01 ± 7.22 μmol Trolox equivalents/100 g flour) and TPC (0.13 ± 0.04 mg gallic acid/g flour) compared to other samples. In the same way, the AcH_2O and Fract Ac samples had the highest TPC and spCEQ values (see Tables 2 and 3), due to the high amount of catechins and proanthocyanidins recovered, which matched the highest significant FRSA values (421.07 ± 9.76, 383.24 ± 61.70, 227.60 ± 7.55 μmol Trolox

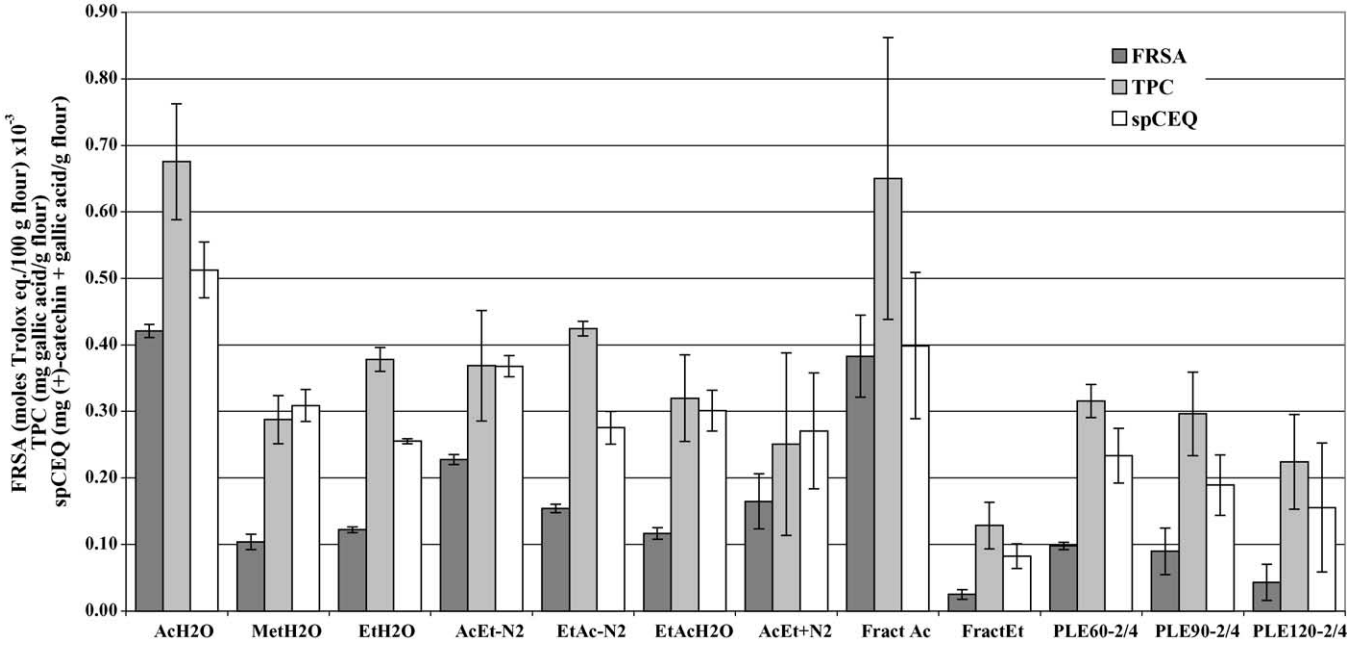

Fig. 4. Total phenolic compounds (TPC, expressed as mg gallic acid/g flour), free radical scavenging activity (FRSA, expressed as μmol Trolox equivalents/100 g flour, $\times 10^{-3}$) and simple phenols capillary electrophoresis quantification (spCEQ, expressed as mg (+)-catechin + gallic acid/g flour) of free phenolic extracts.

equivalents/100 g flour, respectively) ($p < 0.05$), which were two to four times greater than other samples.

Generally, the PLE samples had lower FRSA and TPC values than those of traditional solid–liquid extractions. Moreover, by increasing the extraction temperature, the FRSA and TPC values decreased, probably because of the degradation of the phenolic compounds due to the high temperature reached. This result did not always correspond to the other spectrophotometric indices (PI, ODI, HI and FI), which, in most cases, gave similar or higher results for the PLE extracts than for the other samples. Such behavior might be explained by the extraction principle of this 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 both for the analytes and interfering compounds, highly correlated to the sample matrix. Therefore, the non-phenolic compounds extracted with the PLE 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 radical scavenging activity and capillary electrophoresis quantification, since the former assay is based on a specific phenolic-structure reaction, while the latter is based on a specific detection wavelength.

3.5. MEKC analysis of bound phenolic compounds in barley samples

Fig. 5 shows the electropherogram obtained by injecting the barley flour extracts after alkaline (4 and 20 h as digestion

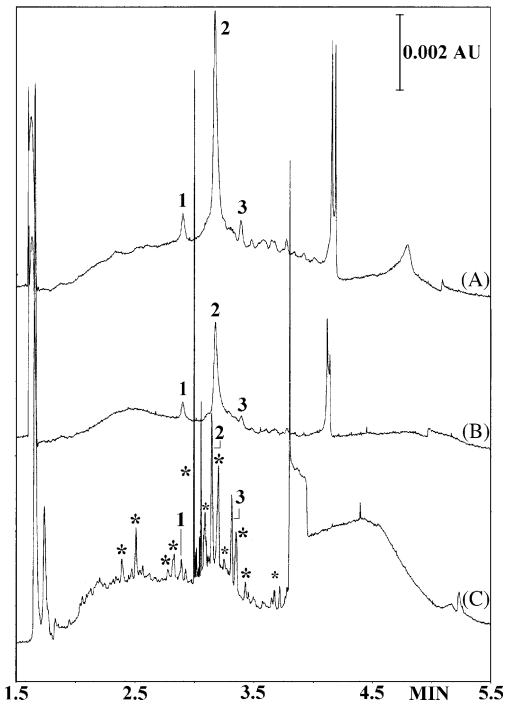

Fig. 5. Electropherograms of the alkaline and acid hydrolysis extracts. (A) Alkaline hydrolysis with 20 h as digestion time; (B) alkaline hydrolysis with 4 h as digestion time; (C) soft-acid hydrolysis. Peaks marked with asterisk might be derivatives of hydroxycinnamic acids or other phenolic compounds extracted by acid hydrolysis. Peak identification: 1, cinnamic acid; 2, *trans*-ferulic acid; 3, *trans*-*p*-coumaric acid. Conditions as in Section 2.

time) and acid hydrolyses, under the conditions described in the optimization paragraph. In the case of alkaline hydrolysis, the main peaks detected were *trans*-ferulic, cinnamic, and *trans*-*p*-coumaric acids, identified by spiking with commercial standards and spectral analysis by UV-DAD. In particular, *trans*-ferulic acid was the most abundant phenolic compound extracted by alkaline hydrolysis, as reported in literature [11–15]. The recovery of these compounds was clearly enhanced by increasing digestion time from 4 to 20 h (see Fig. 5).

The electropherogram of the acid hydrolysis of the barley flour detected the above-mentioned hydroxycinnamic acids, even though their abundance were relatively diminished. In fact, a lower amount of *trans*-ferulic and a higher number of *trans*-*p*-coumaric acids were recovered, compared to those obtained by alkaline hydrolysis. Moreover, several sharp peaks were observed (marked with asterisks in Fig. 5), which show a characteristic phenolic UV spectrum. Probably, these compounds could be derivatives of hydroxycinnamic acids, particularly *trans*-ferulic acid, or other phenolic compounds that have a similar structure, especially those extracted under acid conditions, such as benzoic acids and their derivatives [12,16].

3.6. Quantification of bound phenolic compounds in the barley samples by the MEKC method and statistical correlations with the spectrophotometric results

The MEKC quantification of bound phenolic extracts was carried out using the ferulic acid calibration curve since most of the compounds found in the hydrolyzed samples were hydroxycinnamics acids. In order to make a direct comparison between the extraction recovery of both types of hydrolysis, unidentified peaks of the acid hydrolysis extract (presenting typical UV spectra of phenolic compounds) were also quantified using the ferulic acid calibration curve. The amounts of bound phenolics in the hydrolyzed extracts are given in Table 2. With regard to basic hydrolysis, when digestion time was increased from 4 to 20 h, the amount of bound phenols extracted (bpCEQ) significantly increased ($p < 0.05$). However, the highest significant recovery yield of bound phenols (bpCEQ) was produced by acid hydrolysis ($p < 0.05$).

Table 2 and Fig. 6A show the effects of digestion time on the recovery of bound phenol compounds in alkaline hydrolysis. The 20-h alkaline hydrolysis showed significantly higher HI, FI, PI, FRSA and bpCEQ values ($p < 0.05$) than the 4-h alkaline hydrolysis, as previously reported. No significant differences were detected between both alkaline hydrolyses with regard to TPC and ODI. As can be seen, increasing the alkaline hydrolysis digestion time from 4 to 20 h clearly increased the HI and FRSA. Therefore, longer digestion times led to higher phenol compound extraction yields [7,23–25], and the bound phenols were found to be mainly hydroxycinnamic acids [11–15].

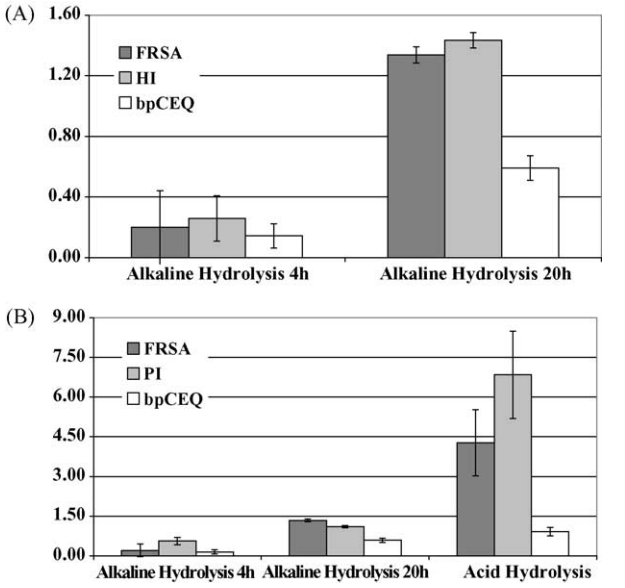

Fig. 6. Free radical scavenging activity (FRSA, expressed as μmol Trolox equivalents/100 g flour, $\times 10^{-2}$), hydroxycinnamics index (HI, expressed as mg ferulic acid/g flour), phenols index (PI, expressed as mg gallic acid/g flour), and bound phenols capillary electrophoresis quantification (bpCEQ, expressed as mg ferulic acid/g flour) of alkaline hydrolysis (A) and soft-acid hydrolysis (B) extracts.

Acid hydrolysis had higher FRSA, TPC, PI, ODI and bpCEQ values, and lower HI and FI values than 4- and 20-h alkaline hydrolyses ($p < 0.05$), as Table 2 reports (Fig. 6B illustrates the level of FRSA, PI and bpCEQ for the alkaline and acid hydrolysis extracts).

Therefore, higher amounts of hydroxycinnamic acids and flavonols were extracted when 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, since acid hydrolysis produced higher TPC, ODI and PI indices than 20-h alkaline hydrolysis (two, six, and three times higher, respectively) and, similarly, a FRSA value about three times higher, this could suggest that the phenolic compounds extracted by soft-acid hydrolysis had greater radical scavenging 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.

Interestingly, correlations between capillary electrophoresis quantifications, spectrophotometric determinations and free radical scavenging activity results of the free and bound phenolic compounds were not noted, probably due to different electrophoretic and spectrophotometric response factors and differences in the antioxidant power of the main compounds recovered by each extraction method (catechins and proanthocyanidins as free phenols, and hydroxycinnamic acids as bound phenols) [9].

4. Conclusions

As reported in this study, free phenolic compounds can be selectively extracted from barley flour by simple solid–liquid extraction using different extraction solvent mixtures, while bound phenolic compounds can only be recovered by either acid or alkaline hydrolytic digestion. However, collecting both free and bound phenols in one extraction procedure does not seem to be feasible, due to the amount of free phenols lost in the defatting steps required to remove lipids during hydrolysis. Therefore, in order to thoroughly investigate the entire phenolic antioxidant activity of barley, both free and bound phenols must be extracted. In order to verify the efficiency, reliability and suitability of extracting free and bound phenolic compounds from barley, several extraction trials were tested in this study, using different solvent mixtures and methods. Moreover, the selectivity of each extraction solvent and the recovery methodology used for a number of phenol classes was evaluated. In order to reach these objectives, a rapid capillary electrophoresis method was optimized and the results were correlated to several spectrophotometric assays.

The use of an acetone-based solid–liquid extraction led to higher extraction yields of flavan-3-ols and proanthocyanidins (almost twice that of other samples), while the use of alcohol-based methods (aqueous ethanol or methanol) produced a higher recovery index for all the phenolic classes (catechins and hydrolysable tannins). Pressurized liquid extractions did not produce a satisfactory recovery of the free phenolic compounds in barley. Prolonging alkaline hydrolysis appeared to be a reliable method to collect hydroxycinnamic acids, while acid hydrolysis allowed a higher recovery of generic phenols, that showed interesting radical scavenging activity. Therefore, complementary information on the bound phenolic pattern of barley can be obtained by both hydrolysis methods.

Since positive correlations between FRSA, total phenolic compounds (by the Folin–Ciocalteu method), hydroxycinnamics index and capillary electrophoresis quantification of free (simple) and bound phenol compounds in barley have been reported in this work, capillary electrophoresis, coupled with these spectrophotometric indices, could be used as a rapid screening tool to evaluate phenol content in barley. In fact, the use of the micellar electrokinetic chromatography method optimized herein enables a rapid evaluation (about 10 min, rinsing steps included) of free and bound phenolic compounds, providing suitable selectivity (using UV-DAD spectral information) and satisfactory precision.

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