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Fractionation of Antioxidants from Autohydrolysis of Barley Husks

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The liquid phase from nonisothermal autohydrolysis of barley husks was extracted with ethyl acetate and redissolved in ethanol to yield a crude extract (denoted BHEAE), which was subjected to further processing to enhance the antioxidant activity. A fractionation method, carried out for characterization purposes, consisted of the extraction of BHEAE with organic solvents of increasing polarity and further fractionation in Sephadex LH-20. Among the tested solvents, ethyl acetate allowed the highest yield, phenolic content, and antioxidant activity. Upon elution with methanol, products with high DPPH radical scavenging capacity (IC_{50} = 0.22 g/L) were obtained. The major compounds in the isolate were benzoic and cinnamic acids. Adsorption−desorption in commercial polymeric resins was carried out as an alternative strategy for BHEAE refining. This method is more suited for possible scale-up and provided a concentrate with a Trolox equivalent antioxidant capacity of 9 mM, which was obtained at a yield of 18 g/kg of barley husks.

KEYWORDS: Antioxidant compounds; autohydrolysis; barley husks; resins; Sephadex; solvent extraction

INTRODUCTION

Cleaning of whole barley grains before malting leads to a solid byproduct (mainly composed of husks), which shows drawbacks for further utilization: for example, its high ash content hinders fuel applications, and transportation is expensive owing to its low density.

Aqueous processing of barley husks (autohydrolysis or hydrothermal treatment) is an environmentally friendly technology leading to a liquid phase containing xylooligomers, monosaccharides, sugar decomposition products, acetic acid, and fractions derived from the extractives and acid-soluble lignin. Both extractives and compounds derived from acid-soluble lignin can be selectively extracted from autohydrolysis liquids with ethyl acetate, leading to extracts with activity comparable to that of synthetic antioxidants (*1*). However, the phenolic content of the said extracts is too low for some commercial applications (10−32 wt % of total dry solids).

Physicochemical processing of crude extracts can enhance both purity and activity. Solvent extraction can separate compounds of different polarities selectively. However, when food applications are envisaged, environmental and toxicity restrictions limit the number of suitable solvents. In the literature, extracts from several flowers, essential oils, and fermented food have been sequentially fractionated with a variety of solvents (hexane, dichloromethane, ethyl acetate, butanol, methanol, ethanol, and diethyl ether). Sephadex LH-20 gels have been used for processing extracts containing phenolic compounds, enabling both concentration and purification of active compounds (*2, 3*).

Alternatively, refining of extracts can be carried out with resins, which show a variety of favorable properties for industrial application, including easy regeneration, durability, and lack of toxicity. Acrylic, polystyrene-based, and formaldehyde−phenol resins have been used to recover anthocyanins, flavonoids, and hydroxycinnamates from waste streams of orange juice manufacture and peels (*4*), cauliflower byproducts (*5*), spinach (*6*), olive (*7*), mango peels (*8*), and orange pulp wash (*9, 10*) or for the selective removal of some terpenoids and flavonoids from juices (*11, 12*). Adsorbent resins have been used to recover and concentrate phenolics from aqueous plant extracts (*13, 14*) and from synthetic solutions (*15, 16*).

Under defined conditions, the adsorption of phenolic compounds can be favored with respect to other compounds in solution (such as carotenoids or sugars) (*11*). Polymeric resins are highly porous, and a variety of compounds can be adsorbed and desorbed, depending on the polarity of the solvent. Using polar solvents such as water, resins can adsorb organic nonpolar species, whereas in the presence of nonpolar solvents, some resins (especially those of acrylic and phenolic nature) exhibit hydrophilic or slightly polar behavior and can adsorb slightly polar species.

The aim of this work was to determine the antioxidant activity of refined concentrates obtained from autohydrolysis liquids of barley husks. In an initial approach carried out with characterization purposes, the barley husk ethyl acetate extract (BHEAE

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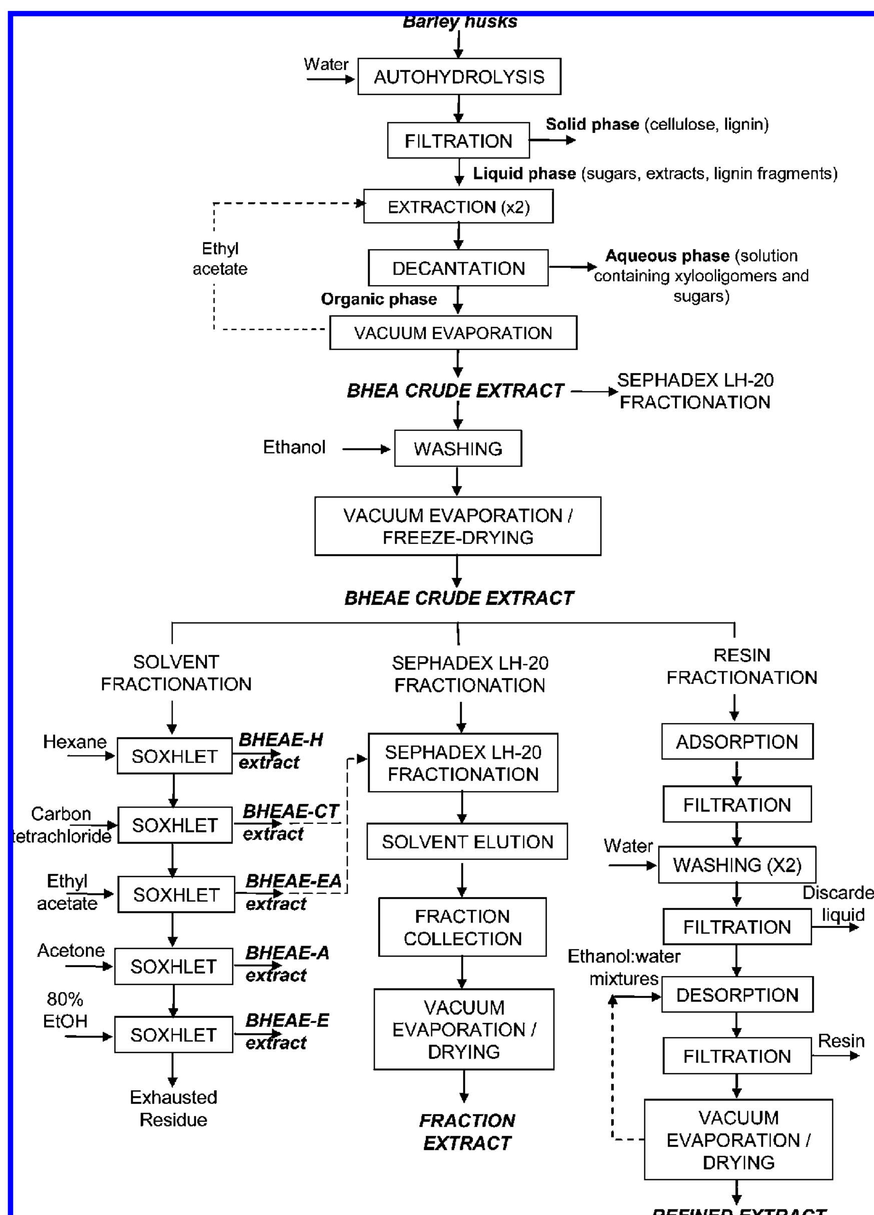

Figure 1. Flow diagram of the fractionation protocol followed for the ethyl acetate extracts from the liquid phase generated during autohydrolysis of barley husks.

fraction) was subjected to sequential extractions with solvents of increasing polarity followed by fractionation in Sephadex LH-20 gels. A more practical technology, based on the adsorption into polymeric resins, was optimized to select the optimal operational conditions leading to higher phenolic content and antioxidant activity.

MATERIALS AND METHODS

Materials. Barley husks were collected in a local malting factory (San Martín, San Cibrao das Viñas, Ourense, Spain), air-dried, milled, screened to pass 1 mm sieve, homogenized in a single lot, and stored in a dry dark place until use.

Preparation of Barley Husk Extracts. Barley husks (BH) were contacted with water in a batch reactor (Parr Instrument Co., Moline, IL) using a liquid/solid ratio of 8 (g/g), operating at nonisothermal conditions to achieve 226 °C (15 min). According to reported data (*1*), these operational conditions lead to the maximum yield in phenolics (9 g of ethyl acetate soluble/100 g; 25–29 g of ethyl acetate soluble phenolics/100 g). After cooling, the liquid phase was recovered by filtration and extracted twice with ethyl acetate at a liquor/ethyl acetate volume ratio of 1:3 (v/v) for 15 min. The organic phase was separated by decantation, and the solvent was removed by vacuum evaporation. The resulting crude extract (denoted BHEA) was further dissolved in 60% ethanol, subjected to vacuum evaporation, and freeze-dried to yield the crude extract (denoted BHEAE) employed for further fractionation (**Figure 1**).

Solvent Extraction. BHEAE was successively extracted with hexane, carbon tetrachloride, ethyl acetate, acetone, and 80% ethanol (Soxhlet operation, duration 9 h per solvent). The corresponding extracts were obtained by vacuum evaporation (40 °C) of the considered solvent and are denoted BHEAE-solvent. The extraction yield was determined gravimetrically. Selected extracts were fractionated with solvent mixtures in Sephadex LH-20 gel.

Sephadex Fractionation. Before applying the sample, the column was equilibrated with the eluent to be used in the separation until the baseline became stable (at least 2 bed volumes). Five milligrams of BHEAE was dissolved in 5 mL of ethanol and loaded onto a 40 cm × 3.5 cm column containing Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), which was eluted with either methanol or methanol/butanol and methanol/acetone mixtures at a flow rate of 0.5 mL/min. Absorbances (280 nm) were recorded online using an Agilent 8453 spectrophotometer, and fractions were collected using a Gilson FC 203B fraction collector with 3 mL test tubes.

Table 1. Solvent Extraction Yield, Total Phenolic Content, DPPH and ABTS Radical-Scavenging Capacity, FRAP, and Reducing Power Values of the Different Fractions and Crude Extract from Barley Husks (BHEAE)

	extraction yield (g of extract/100 g of BHEAE)	phenolic content (GAE/g of extract)	DPPH IC_{50} (g of extract/L)	ABTS IC_{50} (g of extract/L)	FRAP[a] (mM ascorbic acid)	reducing power[a] (mM ascorbic acid)
BHEAE		0.324	0.493	0.364	0.248	0.244
BHEAE−hexane	1.65	0.142	3.00	1.70	0.045	0.049
BHEAE−carbon tetrachloride	9.46	0.465	0.496	0.358	0.185	0.201
BHEAE−ethyl acetate	75.9	0.495	0.717	0.362	0.197	0.216
BHEAE−acetone	7.32	0.150	0.841	0.463	0.077	0.131
BHEAE−80% ethanol	5.30	0.093	2.37	1.53	0.039	0.050

[a] Values determined with an extract concentration of 100 ppM.

Resin Adsorption. BHEAE was dissolved in distilled water and contacted with commercial food grade resins. An acrylic polymer (Amberlite XAD 7HP) and two resins (Amberlite XAD 761 and Amberlite XAD 1180) with formaldehyde−phenol polycondensed matrices were supplied by Sigma Chemical Corp. PS−DVB copolymers with different hydrophobicities (Sepabeads SP700, Sepabeads SP207, and Diaion HP20) were kindly supplied by Resindion S.R.L. (Mitsubishi Chemical Corp.). For activation purposes, resins were mixed with methanol, shaken for 1 min, and stirred (175 rpm, 25 °C) for 15 min. Before use, resins were rinsed with deionized water at a liquid to solid ratio of 5. The moisture content of resins was determined by oven-drying at 100 °C. In adsorption experiments, the phases were contacted in an orbital shaker (25 °C, 175 rpm) for 20 min. Previous experiments (data not shown) confirmed that equilibrium was achieved under these conditions.

Washing. Distilled water was used to remove unadsorbed compounds capable of reducing the purity of the extracts desorbed in further stages. Washing was performed in two stages by contacting distilled water in an orbital shaker (25 °C, 175 rpm, 3 g of water/g of resin) for 20 min.

Desorption. Ethanol/water mixtures were selected for desorption owing to their suitability for food uses and reported cleanup capability (*9*). Desorption was assessed by means of a Box−Behnken factorial design. The independent variables and their considered ranges were pH (2−6), temperature (25−45 °C), and ethanol content (40−96 v/v). In desorption experiments, saturated resins were contacted with ethanol/water solutions at a mass ratio of 1 g of moist resin/3 mL of ethanol−aqueous solutions (resin moisture, about 65%).

Analytical Methods. The total phenolic content was determined according to the Folin−Ciocalteu colorimetric method and expressed as gallic acid equivalents (GAE). The content of anthrone reactive substances (ARS) was determined by using the anthrone method (*17*) and expressed as hydroxymethylfurfural equivalents (HMFE).

The antioxidant activities of both crude extract and solvent extracts were evaluated using the tests listed below.

α,α-Diphenyl-β-picrylhydrazyl (DPPH) Radical Scavenging. Two milliliters of a 6×10^{-5} M methanolic solution of DPPH was added to 50 μL of a methanolic solution of the antioxidant, and the decrease in absorbance at 515 nm after 16 min was recorded in an Agilent 8453E UV−visible spectrophotometer. The inhibition percentage (IP) of the DPPH radical was calculated as the percent of absorbance reduction after 16 min with respect to the initial value. IC_{50} was calculated as the concentration of phenolic compounds required to quench 50% of the initial DPPH radical.

Trolox Equivalent Antioxidant Capacity (TEAC). This assay is based on the scavenging of ABTS radical [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)]. ABTS radical cation ($ABTS^{\bullet +}$) was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration). The mixture was allowed to stand in the dark at room temperature for 12−16 h before use and then diluted with phosphate buffer saline (PBS) (pH 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 30 °C. After the addition of 1.0 mL of diluted $ABTS^{\bullet +}$ solution to 10 mL of antioxidant compounds or Trolox standards in ethanol or PBS, the absorbance readings were taken at 1 min intervals up to 6 min. Appropriate solvent blanks were run in each assay. The percentage inhibition of absorbance at 734 nm was calculated as a function of the concentration of extracts and Trolox. Decrease by 50% of the initial ABTS concentration was defined as IC_{50}.

Ferric Reducing Antioxidant Power (FRAP). The method is based on the ability of a compound to reduce Fe^{3+}, which is relevant due to the role of this cation as an initiator of oxidation processes in organisms. The reagent was made by mixing 25 mL of 300 mmol/L acetate buffer (pH 3.6) and 2.5 mL of a 10 mmol/L 2,4,6-tripyridyl-*s*-triazine (TPTZ) solution in 40 mmol/L HCl and 20 mmol/L $FeCl_3 \cdot 6\ H_2O$ in distilled water. The reagent was always freshly prepared and used as a blank. Aqueous solutions of ascorbic acid were used for calibration. Samples (100 mL) were mixed with 3 mL of the reagent, and the absorbance was monitored at 593 nm.

Reducing Power. One milliliter of extract (dissolved in methanol) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1.0% potassium ferricyanide, and the mixture was incubated at 50 °C for 30 min. After the addition of 2.5 mL of 10% trichloroacetic acid, the mixture was centrifuged, and the supernatant (2.5 mL) was mixed with water (2.5 mL) and 0.5 mL of 0.1% ferric chloride before the absorbance at 700 nm was read. Results are expressed as ascorbic acid equivalents.

HPLC. Analyses were carried out using an Agilent HPLC 1100 instrument equipped with a Supelcosil LC18 column and a DAD detector, operating with a flow rate of 1 mL/min. The injection volume was 20 μL. A nonlinear gradient of solvent A ($MeOH/H_2O/CH_3COOH$, 10:89:1, v/v/v) and solvent B ($MeOH/H_2O/CH_3COOH$, 90:9:1, v/v/v) was used. Elution gradients were used as follows: 0 min, 100% A, 0% B; 30 min, 60% A, 40% B; 40 min, 60% A, 40% B; 42 min, 100% A, 0% B. Samples from initial liquors were analyzed directly, after saponification and after acid hydrolysis. Saponification of the phenolic compounds present in liquors was carried out with 0.75 M NaOH at 25 °C for 40 min under nitrogen atmosphere. Acid hydrolysis was carried out by adding sulfuric acid to the liquids to reach 4% (weight basis), and the solutions were kept for 30 min at 121 °C in stirred reactors.

RESULTS AND DISCUSSION

To assess the fractionation of BHEAE on the basis of polarity and molecular size, extractions with a sequence of solvents of increasing polarity were performed, and selected extracts were subjected to further fractionation in Sephadex LH-20. Additionally, a more practical approach (with regard to the gels and eluting solvents) was tried, and adsorption into polymeric resins and further desorption with ethanol were explored as a refining method.

Solvent Extraction of BHEAE. The extraction yields and the phenolic contents of the extracts obtained with the various solvents are listed in **Table 1**. Even though the BHEAE fraction was prepared by ethyl acetate extraction of the liquid phase from autohydrolysis, it can be observed that about 12.6% of BHEAE was not solubilized by the sequence hexane−carbon tetrachloride−ethyl acetate, a fact ascribed to compositional variations caused during vacuum concentration, washing, freeze-drying, and/or storage. Considering the extracts from individual extractions, ethyl acetate resulted in the highest recovery yield, whereas carbon tetrachloride, acetone, and 80% ethanol showed yields of lower than 10%. Hexane extraction recovered less than 2% of the initial weight.

Table 2. Determination of Phenolic Acid Content (Means of Samples in Milligrams per Gram) by HPLC Analysis with Diode Array Detector[a]

sample[b]	GA	DHB	VA	SA	V	p-CA
BHEAE	1.03	6.99	2.61	1.53	3.97	1.25
BHEAE-ah	1.06	6.84	0.83	1.09	3.79	nd
BHEAE-s	0.73	5.22	1.41	1.06	2.33	1.31
BHEAE-CT	nd	9.28	nd	nd	17.3	0.88
BHEAE-CT-s	nd	7.91	nd	nd	18.1	1.49
BHEAE-EA	nd	3.07	nd	nd	2.23	1.36
BHEAE-EA-s	nd	3.08	nd	nd	1.92	1.13
expt 2	nd	15.6	nd	nd	4.59	0.81
expt 3	nd	3.76	nd	nd	1.69	0.41
expt 9	nd	9.49	0.65	nd	3.45	1.50
expt 14	nd	3.67	0.58	0.61	1.76	0.46

[a] All values are dry basis. Values are means of three determinations. GA, gallic acid; DHB, 3,4-dihydroxybenzaldyde; VA, vanillic acid; SA, syringic acid; V, vanillin; p-CA, *p*-coumaric acid. [b] ah, after acid hydrolysis; s, after saponification.

The fractions soluble in ethyl acetate and carbon tetrachloride showed higher phenolic content than the initial crude extracts, whereas the extracts in hexane, acetone, and 80% ethanol contained around 15% or less of gallic acid equivalents (GAE). The results suggest that these solvents are suitable for the selective removal of nonphenolic compounds, leading to enriched products in solid phase.

Carbon tetrachloride and ethyl acetate provided the most active extracts on the DPPH and ABTS radical-scavenging assays, with IC_{50} values in the range of those found for BHEAE, whereas the compounds soluble in hexane or in ethanol exhibited low activity. The experimental results showed that the phenolic content varied according to the antioxidant activity. The extracts obtained with ethyl acetate and carbon tetrachloride were the most reducing ones, both of them being slightly less potent than BHEAE.

HPLC-DAD analysis of the most active extracts showed that major phenolics were (in decreasing order), 3,4-dihydroxybenzaldehyde, vanillin, vanillic acid, syringic acid, *p*-coumaric acid, and gallic acid (**Table 2**). These compounds have been found in the ethyl acetate extracts of hydrolysates from autohydrolysis of lignocellulosics (*1*). The benzoic and cinnamic acid derivatives are typical products of lignin depolymerization during hydrothermal treatments of barley husks.

Sephadex Fractionation. Several solvents (methanol, methanol/butanol, and methanol/acetone) were selected as eluents to fractionate BHEAE, the carbon tetrachloride extract of BHEAE (denoted BHEAE-CT), and the ethyl acetate extract of BHEAE (denoted BHEAE-EA), after dissolution in methanol (concentration = 1 g/L).

BHEAE Fractionation. **Figure 2a** shows the Sephadex LH-20 elution profile obtained for this fraction (operational conditions: concentration, 1 g/L; eluent, methanol). Three fractions (F1, F2, and F3) with light color and manageable texture were obtained. The corresponding recovery yields and properties (phenolic content, antioxidant activity) are listed in **Table 3**. The eluates before F1, between F1 and F2, between F2 and F3, and after F3, which showed low phenolic contents, were discarded. F1, F2, and F3 contained jointly about 81% of the initial material. Again, the experimental data showed proportionality between phenolic content and scavenging capacity: a correlation coefficient of 0.958 was determined for the linear fitting of data from ABTS radical-scavenging capacity versus phenolic content of extracts. Among the isolated fractions, only F3 showed higher potency than BHT (IC_{50} = 2.79 g/L). For comparative purposes, a fresh extract obtained by ethyl acetate extraction from autohydrolysis liquor (fraction BHEA) was eluted with methanol (see **Figure 2b**). In this case two major fractions (see **Table 3**) were obtained for yield, phenolic content, and antioxidant activity. Both fractions showed phenolic contents slightly higher than the one of the initial extract, but the radical scavenging capacity was similar to the value determined for the initial sample. Additionally, BHEAE was eluted with methanol/butanol mixtures (first with a linear gradient from 70 to 90% butanol to yield fraction F1, followed by isocratic elution with 95% butanol to obtain F2, see **Figure 2a**). The fractions obtained with this operational mode did not improve the phenolic content or activity with respect to the initial extract. Similarly, elution with a linear gradient of methanol/acetone (100 to 30%) yielded two fractions accounting for 52% of the injected material, but no purification effect was observed.

BHEAE-EA Fractionation. When BHEAE-EA was eluted with methanol (see **Figure 2c**), three fractions of fair color were obtained, which showed phenolic contents comparable to or higher than that of the starting extract (see **Table 3**). F3 was the most active fraction, suggesting that the elution of high molecular weight compounds of nonphenolic nature took place in the initial stages of fractionation. Further elution resulted in fractions with increased phenolic content: for example, F3 exhibited a remarkable content of GAE (89%) and a high antioxidant activity (IC_{50} = 0.24, in the range of BHA). The recovery yield was less than 10% of the initial weight. In a related work, Cruz et al. (*3*) obtained a fraction more active than BHA by eluting a crude extract from hydrolytic treatment of distilled red grape pomace with methanol.

BHEAE-CT Fractionation. Elution with methanol resulted in two fractions (see **Figure 2d**). The results in **Table 3** show that the second fraction presented the highest recovery yield, as well as an improved ABTS radical scavenging capacity in comparison with the initial extract.

Adsorption−Desorption in Commercial Polymeric Resins. The above results confirm that fractionation of both BHEAE and BHEA with organic solvents is feasible, but limited improvements in phenolic content and radical-scavenging capacity were achieved (except for fraction F3 from BHEAE-EA elution in Sephadex LH-20 gel). However, in this latter case, the recovery yield accounted for just 8 wt % of barley husks.

As a practical alternative for refining purposes, resin adsorption (which avoids the utilization of undesirable solvents for food manufacture) was assayed. This operational mode is suitable for the recovery of hydroxycinnamic and benzoic compounds, which are the active components of the extracts employed in this work.

Preliminary experiments were carried out to select the best resin and to assess the effects of the most influential operational variables (liquid to solid ratio and initial phenolics concentration). Because the feed is not viscous and low temperatures enhance the phenolic adsorption capacity (*18*), adsorption experiments were performed at room temperature. This operational mode has been followed in the literature frequently (*12, 14, 15, 22, 23*).

Resin to Liquid Ratio. BHEAE was dissolved in distilled water at concentrations of 0.16 or 1.63 g of GAE/L, a value in the range reported in related studies. **Table 4** shows data concerning both adsorption capacity (variable *q*, defined as grams of phenolics adsorbed per gram of adsorbent) and adsorption percentages obtained with the tested resins (SP700, SP207, XAD7HP, XAD761, XAD1180, and HP20) at the considered liquid to resin ratios operating at selected concentra-

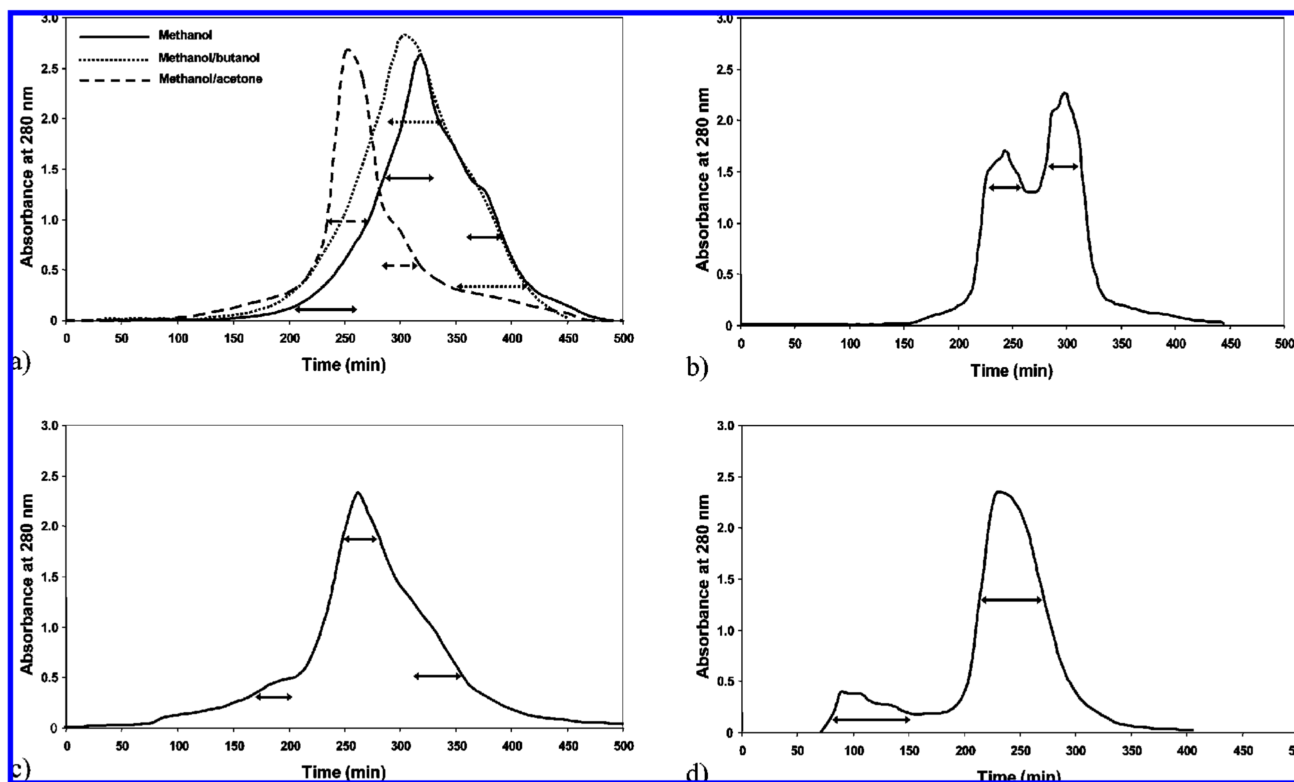

Figure 2. Chromatographic profile of the ethyl acetate extracts in Sephadex LH-20 for (**a**) BHEAE, (**b**) BHEA, (**c**) BHEAE-EA, and (**d**) BHEAE-CT.

Table 3. Recovery Yield, Total Phenolic Content, and DPPH Radical-Scavenging Capacity of the Ethyl Acetate Extract from Barley Husks Fractionated with Sephadex LH-20

	elution time (min)	recovery yield (g/100 g of extract)	phenolic content (g of GAE/g)	DPPH IC_{50} (g/L)	ABTS IC_{50} (g/L)
BHEAE			0.324	0.493	0.364
Crude Extract in Ethyl Acetate (BHEAE) Eluted with Methanol					
fraction 1 (F1)	204−258	31.2	0.212	>1	0.869
fraction 2 (F2)	288−330	33.6	0.257	>1	0.570
fraction 3 (F3)	360−390	16.0	0.299	0.742	0.440
Direct Extraction of Hydrolysate with EA (BHEA) and Eluted with Methanol					
fraction 1 (F1)	232−256	19.6	0.328	>1	0.496
fraction 2 (F2)	286−304	19.4	0.452	0.467	0.264
Crude Extract in Ethyl Acetate (BHEAE) Eluted with Methanol/Butanol					
fraction 1 (F1)	290−340	20.0	0.38	0.68	0.385
fraction 2 (F2)	350−415	24.0	0.36	>1	0.541
Crude Extract in Ethyl Acetate (BHEAE) Eluted with Methanol/Acetone					
fraction 1 (F1)	233−267	30.0	0.220	>1	0.742
fraction 2 (F2)	283−316	22.0	0.046	>1	>1
EA Fraction (BHEAE-EA) Eluted with Methanol					
fraction 1 (F1)	172−202	4.00	0.431	>1	
fraction 2 (F2)	250−280	20.0	0.583	0.613	
fraction 3 (F3)	316−352	8.00	0.894	0.224	
other fractions		54.0	0.370	0.692	
Carbon Tetrachloride Fraction (BHEAE-CT) Eluted with Methanol					
fraction 1 (F1)	82−148	20.0	0.198	>1	>1
fraction 2 (F2)	214−268	40.0	0.500	0.554	0.275

tions of phenolics. The maximum values of q were obtained with the resins SP700, XAD761, and XAD1180 and were higher than the ones reported for phenolics from alfalfa (*21*), but lower than those reported for the adsorption of synthetic (*15, 22, 23*) or single compounds (*24*).

The adsorption yields determined in this study lie in the range 96.8−76.9% (see **Table 4**). Yields in the range 60−80% have been reported for the adsorption of orange pulp pigments into styrene−divinylbenzene (SDVB) copolymers (*19*), whereas 80−90% phenolics from citrus peel juices were adsorbed into polystyrene−divinylbenzene and cross-linked polyacrylate resins. Scordino et al. (*15*) reported an almost linear increase in the adsorption percentage of a hesperidin solution (from 60.5 to 95.5%) when the resin/liquid ratio was increased from 0.5 to 1.5 g/L. Increasing the resin/liquid ratio from 1 to 10% (w/v) improved the adsorption of alfalfa polyphenols onto polystyrene-based resins from 35 to 86%, but the influence was not significant at higher ratios (*21*). A typical pattern of decrease

Table 4. Effect of the Solid to Liquid on the Adsorption of Phenolic Compounds from BHEAE in the Selected Commercial Resins

resin/liquid ratio (g/mL)	phenolic concn (g GAE/L)	SP700	SP207	XAD7HP	XAD761	XAD1180	HP20
		Phenolics Adsorption (%)					
2:5	0.916	92.6 ± 0.07	92.5 ± 0.05	76.9 ± 1.21	88.9 ± 0.05	87.7 ± 0.02	84.0 ± 0.29
3:5	0.916	95.6 ± 0.00	95.5 ± 0.09	80.2 ± 0.22	92.7 ± 0.03	91.1 ± 0.12	87.5 ± 0.16
	1.63	95.2 ± 0.05	94.6 ± 0.17	80.7 ± 0.11	92.4 ± 0.03	89.0 ± 0.54	87.1 ± 0.24
4:5	0.916	96.8 ± 0.01	96.7 ± 0.04	82.6 ± 0.12	94.4 ± 0.02	93.3 ± 0.10	91.5 ± 0.01
		Adsorption Capacity *q* (Milligrams of GAE per Gram of Dry Resin)					
2:5	0.916	6.06 ± 0.00	4.17 ± 0.00	5.34 ± 0.08	5.99 ± 0.00	5.91 ± 0.00	4.81 ± 0.02
3:5	0.916	4.17 ± 0.11	2.87 ± 0.00	3.71 ± 0.01	4.16 ± 0.00	4.09 ± 0.01	3.34 ± 0.01
	1.63	7.40 ± 0.00	5.06 ± 0.01	6.65 ± 0.01	7.39 ± 0.00	7.12 ± 0.04	5.92 ± 0.02
4:5	0.916	3.17 ± 0.00	2.18 ± 0.00	2.87 ± 0.00	3.18 ± 0.00	3.14 ± 0.00	2.62 ± 0.00

Table 5. Effect of pH in Water Solubility of the BHEAE and on the Adsorption onto Sepabeads SP700

	before adsorption		after adsorption				after washing	
	phenolics (g of GAE/g of extract)	ARS (g of HMFE/g of extract)	phenolics adsorption (%)	*q* (mg of GAE/g of dry resin)	ARS adsorption (%)	*q* (mg of HMFE/g of dry resin)	phenolics removal (%)	ARS removal (%)
BHEAE	0.324 ± 0.00	0.110 ± 0.00	95.2 ± 0.01	7.40 ± 0.00	93.5 ± 0.12	3.45 ± 0.13	4.68 ± 1.16	5.60 ± 1.05
pH 6	0.315 ± 0.01	0.106 ± 0.01	96.0 ± 0.20	7.37 ± 0.17	95.7 ± 0.11	3.56 ± 0.20	5.44 ± 0.09	2.03 ± 1.71
pH 4	0.322 ± 0.00	0.098 ± 0.00	99.4 ± 0.01	7.48 ± 0.05	97.0 ± 0.29	3.37 ± 0.11	0.871 ± 0.03	0.00 ± 0.00
pH 2	0.304 ± 0.00	0.092 ± 0.00	99.7 ± 0.04	6.99 ± 0.09	98.1 ± 0.19	3.16 ± 0.12	0.555 ± 0.02	0.00 ± 0.00

in adsorption capacity for increasing loadings of resin was observed in this study, a finding similar to the ones reported for adsorption of oleuropein and rutin on silk fibroin (for solid/liquid ratios in the range of 0.025−0.1 g of resin/g of olive leaf extract) (*25*) and for the adsorption of flavonoids (naringin and limonin) onto XAD resins at 1−2% w/v (*18*).

A comparative evaluation of data listed in **Table 4** shows that SP resins performed significantly better than the rest and that the best results corresponded to SP700. On the basis of the results achieved with this selected resin, operation at a resin/liquid ratio of 3:5 g/mL was selected for further experiments, owing to its favorable balance between phenolic adsorption and adsorption capacity. It can be noted that increases in the concentration of phenolics from 0.16 to 1.63 g of GAE/L did not result in significantly different phenolic adsorption.

Adsorption of Phenolics onto SP700 Resin. BHEAE dissolved in distilled water at a concentration of 1.63 g of GAE/L had a pH of 6.4. Because the adsorption of phenolics could be promoted under acidic conditions (due to the affinity of nonpolar resins for the protonated form of low molecular weight compounds), the pH was adjusted to 2, 4, or 6 by adding 1 M HCl. Acidification resulted in the formation of precipitates, which were removed by centrifugation. The contents of phenolics and anthrone reactive substances (ARS) were unaffected at pH 4, but slightly reduced at pH 2 (see **Table 5**). However, TEAC values were not significantly altered. Adsorption of phenolics from BHEAE was hardly affected by pH, in agreement with results reported for the adsorption of hesperidin (*15*) or cyanidin 3-glucoside (*23*) in resins with the same structure (PS-DBV).

Water-soluble impurities were easily removed by water washing of the resin, whereas the bound phenolic compounds accounted for less than 5% of the total adsorbed. **Table 5** lists data concerning the removal of phenolics and ARS during washing. ARS removal was highest at the original pH of BHEAE and decreased by acidic pH. Operation in the column at water/resin volume ratios in the range of 2−5 allowed the removal of sugars and other solubles from orange pulp (*19*) and from aqueous spinach extract (*6*).

Desorption Assessment. The structure of the Box−Behnken experimental design employed to assess desorption is shown in **Table 6**. The contact time was fixed in 20 min, because previous experiments (data not shown) indicated that this time was long enough to ensure equilibrium conditions. This period was quite short in comparison to other values reported in the literature: for example, 4 h has been reported for phenolics from apple juice (*12*), and more prolonged times (20 h−4 days) have been employed in the desorption of antioxidants from olive leaves (*25*). The independent variables and the respective variation ranges were pH, 2−6; temperature, 25−45 °C; and ethanol weight percent, 40−96%. The coded, dimensionless values of the independent variables [with variation ranges (−1, 1)] were named *p*, *t*, and *C*. The set of conditions employed in the various experiments is also shown in **Table 6**.

The dependent variables selected in this study were as follows: phenolics desorption yield (g of GAE desorbed/100 g of GAE adsorbed; denoted y_1); phenolic content of the desorbed product (g of GAE/g of desorbed product; denoted y_2); ARS desorption yield (g of HMFE in desorbed product/100 g of adsorbed HMFE; denoted y_3); ARS content of the desorbed product (g of HMFE/g of product; denoted y_4); total solids desorption yield (g of desorbed product/100 g of adsorbed product; denoted y_5); and antioxidant activity (AA) (mM Trolox; denoted y_6).

The experimental results for the dependent variables are also shown in **Table 6**. Empirical modeling was carried out by fitting the data to second-order equations containing interaction terms, according to the generalized equation

$$y_i = a_{0i} + a_{pi}p + a_{ti}t + a_{Ci}C + a_{pti}pt + a_{pCi}pC + a_{tCi}tC + a_{ppi}p^2 + a_{tti}t + a_{CCi}C \quad (1)$$

where all of the coefficients (a_{pi}, a_{ti}, a_{Ci}, a_{pti}, a_{pCi}, a_{tCi}, a_{ppi}, a_{tti}, a_{CCi}) were calculated by minimization of the sum of deviation squares. **Table 7** lists the values determined for the regression parameters, their significance, and the values of the statistical parameters R^2 and *F*.

The experimental data determined for y_1 and y_2 (see **Table 6**) showed that the maximum yield of phenolics desorption

Table 6. Structure of the Box−Behnken Factorial Design: Independent Variables, Coded (Dimensionless) Independent Variables, Operation Applied to Desorption of Phenolic Compounds from Crude Extracts from Barley Husks (BHEAE) Adsorbed onto Sepabeads SP700

	independent variables			coded variables			dependent variables[a]					
expt	pH	T	EtOH%	p	t	C	y_{1EXP}	y_{2EXP}	y_{3EXP}	y_{4EXP}	y_{5EXP}	y_{6EXP}
1	2	25	68	−1	−1	0	59.5	0.294	74.3	0.134	64.9	6.39
2	2	35	40	−1	0	−1	20.8	0.225	44.9	0.177	29.6	2.68
3	2	35	96	−1	0	1	76.5	0.342	86.2	0.140	71.8	8.15
4	2	45	68	−1	1	0	65.0	0.304	79.8	0.136	68.7	7.54
5	4	25	68	0	−1	0	50.4	0.383	60.4	0.138	87.1	7.39
6	4	25	96	0	−1	1	62.6	0.456	67.3	0.147	91.0	8.32
7	4	35	68	0	0	0	52.4	0.427	59.9	0.146	81.4	7.98
8	4	35	68	0	0	0	52.7	0.416	59.3	0.141	83.8	7.74
9	4	35	68	0	0	0	53.5	0.396	59.6	0.133	89.5	7.70
10	4	45	40	0	1	−1	29.1	0.344	45.7	0.162	56.2	3.77
11	4	45	96	0	1	1	70.1	0.441	72.4	0.137	100	8.88
12	6	25	40	1	−1	−1	34.7	0.397	45.4	0.172	53.8	4.39
13	6	35	68	1	0	0	62.8	0.391	65.2	0.135	99.0	7.23
14	6	35	96	1	0	1	68.4	0.477	69.3	0.160	88.4	9.11
15	6	45	68	1	1	0	62.4	0.422	68.3	0.153	91.2	7.60
16	4	45	68	0	1	0	57.9	0.464	60.9	0.147	82.5	8.08

[a] y_1, phenolics desorption yield (g GAE desorbed/100 g GAE adsorbed); y_2, phenolic content (g of GAE/g of product); y_3, anthrone-RS desorption yield (g of HMFE desorbed/100 g of HMFE adsorbed); y_4, anthrone-RS content (g of HMFE/g of product); y_5, solids desorption yield (%); y_6, antioxidant activity (mM Trolox).

Table 7. Regression Coefficients from ANOVA for the Studied Objective Functions during Desorption of Barley Husks Phenolics from Sepabeads SP700*

	y_1	y_2	y_3	y_4	y_5	y_6
a_0	53.0[b]	0.408[b]	59.4[b]	0.139[b]	86.1[b]	7.70[b]
a_P	0.852[NS]	0.065[b]	−4.46[b]	0.004[NS]	12.2[b]	0.429[b]
a_T	3.17[b]	0.004[NS]	2.49[b]	0.000[NS]	1.88[NS]	0.198[NS]
a_C	21.5[b]	0.051[b]	15.3[b]	−0.012[b]	19.9[b]	2.54[b]
a_{PT}	−0.853[NS]	−0.009[NS]	−0.420[NS]	0.001[NS]	0.414[NS]	−0.420[a]
a_{PC}	−5.77[b]	−0.006[NS]	−4.58[b]	0.006[NS]	−2.18[NS]	−0.097[NS]
a_{TC}	−0.436[NS]	0.003[NS]	−1.39[NS]	0.000[NS]	0.310[NS]	0.147[NS]
a_{PP}	7.28[b]	−0.051[b]	9.77[b]	0.003[NS]	−8.44[b]	−0.359[a]
a_{TT}	1.65[NS]	0.006[NS]	2.44[a]	0.000[NS]	0.853[NS]	−0.002[NS]
a_{CC}	−9.54[b]	−0.008[NS]	−7.00[b]	0.018[b]	−14.5[b]	−1.45[b]
R^2	0.992	0.918	0.986	0.826	0.956	0.987
F	80.2	7.50	45.5	3.16	14.4	50.1

* y_1, phenolics desorption yield (g of GAE desorbed/100 g of GAE adsorbed); y_2, phenolic content (g of GAE/g of product); y_3, anthrone-RS desorption yield (g of HMFE desorbed/100 g of HMFE adsorbed); y_4, anthrone-RS content (g of HMFE/g of product); y_5, solids desorption yield (%); y_6, antioxidant activity (mM Trolox). [a], statistically significant at $p < 0.1$; [b], statistically significant at $p < 0.05$; [NS], statistically nonsignificant.

(76.5%) was achieved under the conditions of experiment 3 (pH 2, 35 °C, 96% ethanol), whereas the desorbed product with highest phenolic content (0.477 g of GAE/g of desorbed product) was obtained in experiment 14. Similar phenolic content was obtained in experiments 6, 11, and 16. Among them, only experiment 11 reached 70% desorption of phenolic compounds. Operation with 96% ethanol favored both the desorption of phenolics and the purity of the products, as it happened in experiments 14 (pH 6, 35 °C, 96% ethanol), 11 (pH 4, 45 °C, 96% ethanol), and 6 (pH 4, 25 °C, 96% ethanol). The lowest values of y_1 and some of y_2 were obtained in experiments 2, 10, and 12, all of them performed with 40% ethanol. The maximal values of y_1 and y_2 did not correspond to the same operational conditions, revealing that the enhancement of desorption was not selective for phenolic compounds.

The values of the regression parameters listed in **Table 7** showed that the most important effects on the phenolics desorption yield (variable y_1) were caused by the ethanol concentration, which contributed significantly through linear interaction and quadratic terms. Oppositely, temperature was scarcely influential (the linear term was the only one with statistical significance, and its absolute value was low in comparison with other coefficients involved in the model). The predicted dependence of y_1 on pH and ethanol concentration is shown **Figure 3a**. Enhanced desorption yields (above 75%) were predicted for operational conditions defined by reduced pH and high ethanol concentration. Desorption yields in the same range have been reported for rutin and oleuropein recovery from silk fibroin with 70% ethanol (*25*), from anthocyanins from citrus pulp wash with methanol (*9*), and from apple phenolics with aqueous ethanol or aqueous methanol from ethylene glycol cross-linked poly(methyl methacrylate) resin (*12*). Higher yields were reported for 96% ethanol desorption of anthocyanins from different resins (*22, 23*) or for patuletin and spinacetin 3-*O*-glycosides with 75% ethanol from XAD7HP and XAD16HP (*6*).

Temperature caused limited nonsignificant effects on the phenolic content (variable y_2). The model predicted high phenolic content (in the vicinity of 0.46 g of GAE/g of product) for operation at pH and ethanol concentrations near the upper limits considered in this study. Decreased ethanol concentrations resulted in reduced phenolic contents, particularly during operation at low pH. The concentration factor achieved under optimal desorption conditions was 1.5:1. Concentration factors reported in related studies are significantly higher: up to 25 for hesperidin desorbed with ethanol/NaOH solutions (*4*); in the range of 3−6 for phenolic compounds from cauliflower (*5*), lettuce, chicory (*26*), or spinach (*6*); and 7.5 for hydroxycinnamates from citrus byproducts (*9*). However, the phenolic content of the desorbed products obtained in this study compares well with the above data, whereas higher values have been obtained for antioxidants from citrus fruits (*27*).

Variable y_3 (ARS desorption yield) was favored by high ethanol concentrations, with limited influence of both pH and temperature. Under the conditions of experiment 3 (pH 2, 35 °C, 96% ethanol), 86% ARS was desorbed (see **Table 6**). Oppositely, minimal values of y_3 (about 45%) were obtained in experiments 2, 10, and 12, all of them performed with 40% ethanol. The behavior predicted by the empirical model (see **Figure 3c**) confirms these general patterns.

Variable y_4 (ARS content) varied in a narrow range, making a detailed discussion on the influence of the operational conditions difficult. The predicted behavior (see **Figure 3d**) was mainly affected by the second-order contribution of the ethanol

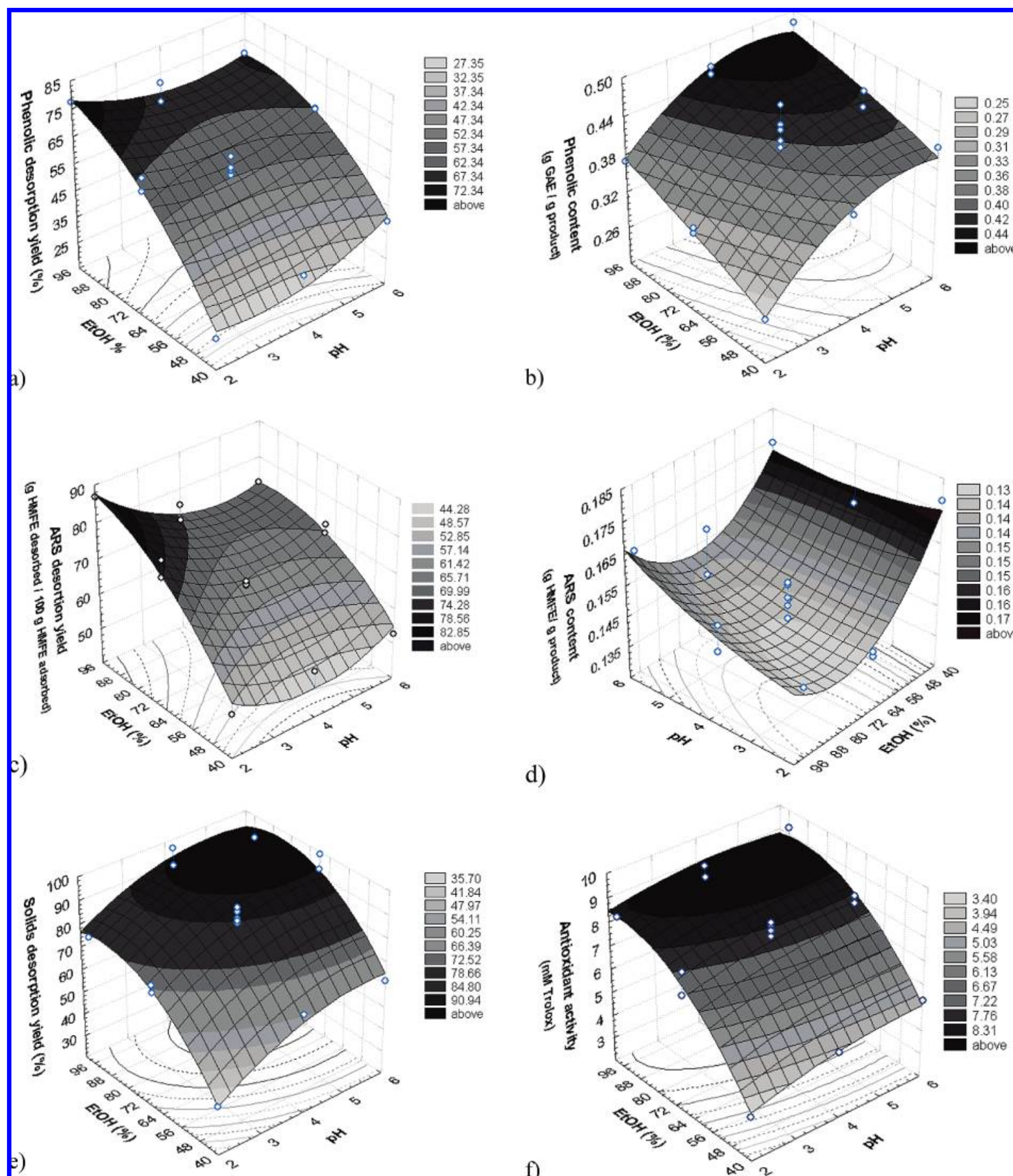

Figure 3. Response surface for the (**a**) phenolics desorption yield, (**b**) phenolic content in the desorbed product, (**c**) anthrone-RS desorption yield, (**d**) anthrone-RS content in the extract, (**e**) solids desorption yield, and (**f**) antioxidant activity as Trolox equivalents as a function of the pH and ethanol concentration during desorption of BHEAE from Sepabeads SP700.

concentration, leading to ARS contents of higher than 0.17 g/g in operation with diluted ethanol solutions.

Variable y_5 (desorption yield of total solids) varied strongly with both pH and ethanol concentration, which presented significant terms of first and second order, predicting a defined maximum (>91%, see **Figure 3e**) operating at pH between 4 and 6 and ethanol concentration higher than 4 and 68%, respectively.

Variable y_6 (antioxidant activity of the desorbed extracts, measured as Trolox equivalents) showed values in the range from 2.68 to 9.11 mM Trolox. The statistical parameters in **Table 7** confirmed significant contributions of the linear and second-order terms of the ethanol concentration, as well as for the linear contribution of pH. The response surface (see **Figure 3f**) presents a variation pattern governed by the ethanol concentration, with higher ethanol concentrations leading to products of increased antioxidant activity. The extract desorbed under conditions of experiment 14 showed higher purity and content of phenolic and antioxidant activity (**Tables 2** and **6**).

In the present study the autohydrolysis processing of barley husks at 226 °C, followed by ethyl acetate extraction of the liquid phase, ethanol resuspension to yield a crude extract (BHEAE), solvent extraction, and further fractionation in Sephadex LH-20, was studied. Only one fraction from the ethyl

acetate extract, eluted with methanol/acetone, showed both high purity and antioxidant potency. However, the low recovery yield (less than 8%) and the type of solvents employed limit the potential of this process for food-related applications. A more practical process employing more competitive resins and biorenewable eluting solvents was optimized. The active compounds present in extracts (benzoic and hydroxycinnamic acids) can be selectively recovered by means of adsorption into polymeric resins and further desorption. On the basis of the above data, it can be concluded that adsorption (SP700 at pH 4), washing with distilled water, and desorption at pH 4−6 with more than 80% ethanol resulted in a product (obtained at a yield of 18 g of GAE/kg of barley husks) with 48% phenolic content and an antioxidant activity equivalent to 1.64 g of Trolox.

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