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Comparative study of factors affecting the recovery of proteins from malt rootlets using pressurized liquids and ultrasounds

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ABSTRACT

Malt rootlets (MR) are a waste from brewing with high protein content. This work proposes to study the impact of extracting parameters on the recovery of proteins and the characteristics of extracts from MR using ultrasound-assisted extraction (UAE) and pressurized liquid extraction (PLE). A Box-Behnken experimental design was employed to study the effect of extracting parameters on the protein yield, while characterization comprised the study of antioxidant properties, the identification of extracted proteins using high-resolution tandem mass spectrometry, and the evaluation of the co-extraction of phenolic compounds. Protein extraction was promoted at an ultrasounds amplitude of 68%, for 20 min at 52 °C in UAE, while adding 33% ethanol resulted in the highest yield in PLE. While UAE extracted 53 ± 5% of MR proteins, PLE reached a 73 ± 7%, using more sustainable conditions. Significant antioxidant activities were observed in the PLE extract, although undermined by gastrointestinal digestion. Proteomic analysis detected 68 proteins from *Hordeum vulgare* in the UAE extract and 9 in the PLE extract. Proteins in MR are very different to that from barley grains or brewer's spent grains. PLE also co-extracted phenolic compounds while this was not significant by UAE.

1. Introduction

Barley (*Hordeum vulgare* L.) is the fourth most cultivated cereal and the major grain used in brewing (Mahalingam, 2018). Brewing involves different steps being malting the first one. Malting is the process by which barley grains are germinated by soaking into water. It results in the growing of sprouts at the bottom of barley seeds and the release of starch from the seed endosperm that will turn into fermentable sugars in following brewing steps. Nevertheless, malt rootlets (MR) must be removed to avoid the bitter flavours they give to beer. Some 3–5 kg of MR are produced from every 100 kg of malt. While typically sold for cattle feed, MR has been also proposed as a growing media for micro-organisms (Cejas et al., 2017) and as a source of nutrients in formulations and functional foods (Waters et al., 2013).

MR contains around 10–35% of crude protein (Briggs, 1998), including an important quantity of essential amino acids, specially lysine (Briggs, 1998; Waters et al., 2013). Surprisingly, this waste material has scarcely been researched as a source of proteins unlike other brewing wastes such as the brewing spent grain. Only one work dealing with this target has been published. Chen et al. developed a method for the extraction of MR proteins using conventional solid-extraction under alkali conditions (pH 9.0 and 40 °C). Extraction took 60 min and the resulting extract showed a 33.7% of proteins in addition to phenolic compounds (Cheng et al., 2016). Further research focusing on the recovery of proteins from this complex matrix under more sustainable conditions and shorter times and reaching higher yields are urged.

Extraction of proteins can be promoted by favouring physical contact between proteins and the extracting medium using techniques such as pressurized liquid extraction (PLE) or ultrasound-assisted extraction (UAE) (Olivares-Galván et al., 2020). UAE is a well-known technique to make extractions faster and more efficient by providing mechanical energy through a phenomenon called *cavitation*. UAE has been extensively applied for the extraction of proteins, including the works performed by the own group (Olivares-Galván et al., 2020). Nevertheless, no work has explored the extraction and characterization of proteins from MR. PLE, on the other hand, allows solvents to achieve

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temperatures higher than their atmospheric boiling point without evaporation by the application of high pressures. These solvents show enhanced penetration into the sample and mass transfer. Thereby, higher yields are possible with less solvent and shorter times (Hernández-Corroto et al., 2020). Despite PLE was initially employed for the extraction of low-molecular weight compounds, its application for the extraction of proteins is quickly expanding. The extraction of proteins using PLE has been mainly focussed to the valorization of food byproducts (cereal bran, fish side streams, pomegranate peel, brewer spent grain, etc.) and seaweeds (*Spirulina*) due to the green characteristics of this technique. Many works addressing the extraction of proteins from food byproducts by PLE involved the use of water at high temperatures (160-270 °C), also called subcritical water extraction (SWE). These extractions result in protein degradation and suppose high-energy consumption (Álvarez-Viñas et al., 2021). Indeed, the solubility of proteins in water is low due to aggregation and association to cell walls. In other cases, PLE has been employed to obtain extracts with the highest content in bioactives, e.g., extracts with high antioxidant activity (de la Fuente et al., 2021; Zhou et al., 2021, 2022). These extracts contained many molecules (polyphenols, vitamins, saponins, chlorophyll, etc.), in addition to proteins but these works did not focus just on the extraction of proteins. Only few works have evaluated the effect of some parameters on the extractability of proteins in okara and rice bran (Wiboonsirikul et al., 2007, 2013), brewer's spent grain (Du et al., 2020) and flaxseeds (Ho et al., 2007). Moreover, our research group has discovered that the use of PLE with water-ethanol mixtures can be an interesting strategy for the extraction of proteins (González-García et al., 2021; Guzmán-Lorite et al., 2022; Hernández-Corroto et al., 2020). All these works demonstrate the potential of PLE for the extraction of proteins. Nevertheless, none of these works have targeted the extraction of proteins from MR neither have evaluated the characteristics of extracted proteins.

The aim of this work was to do a comparative study of the impact of extracting parameters on the recovery of proteins from MR by UAE and PLE. Chemometric tool Box-Behnken experimental design will be used to find out main factors affecting the extraction of proteins in every case. Extracts obtained by every technique will be characterized by the study of their antioxidant properties (in intact extracts and in gastrointestinal digested ones) and the identification of extracted proteins using high-resolution tandem mass spectrometry. Furthermore, the purity of extracts will be also evaluated by the study of the co-extraction of phenolic compounds. Comparison of results will enable to withdraw interesting conclusions about the conditions promoting extraction of MR proteins and their potential applications.

2. Materials and methods

2.1. Chemicals and samples

All reagents were of analytical grade and water was purified in a Milli-Q system from Millipore (Bedford, MA, USA). Folin-Ciocalteu reagent, sodium dodecyl sulphate (SDS), phosphate buffer (PB), hydrochloric acid (HCl), and tris (hydroxymethyl)aminomethane (TRIS) were obtained from Merck (Darmstadt, Germany). Ethylenediaminetetraacetate (EDTA) was from VELP Scientific (Usmate, Italy). Methanol (MeOH), urea, formic acid, acetic acid (AA), and acetonitrile (ACN) were obtained from Scharlau (Barcelona, Spain). Albumin from bovine serum (BSA), DL-dithiothreitol (DTT), 1,10-phenanthroline, L-glutathione (GSH), potassium persulphate, 2,2‟-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS), o-phthaldialdehyde (OPA), ferrous sulphate, β-mercaptoethanol, hydrogen peroxide (H_2O_2), trifluoroacetic acid (TFA), ammonium carbonate, iodoacetamide (IAA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ammonium bicarbonate (NH_4HCO_3), and enzymes (pancreatin, trypsin, and pepsin) were provided by Sigma-Aldrich (Saint Louis, MO, USA). Sodium carbonate (Na_2CO_3) and bicarbonate ($NaHCO_3$) were obtained from Panreac (Barcelona, Spain). Ethanol (EtOH) was provided by Thermo Fisher Scientific (Waltham, MA, USA). Bradford reagent (Coomassie Blue G-250), Mini-Protean precast gels, Tris/glycine/SDS running buffer, Laemmli buffer, Bio-Safe Coomassie G-250 stain, and Precision Plus Protein All Blue standards, were from Bio-Rad (Hercules, CA, USA). Dry and ground MR was provided by Estrella de Levante Fábrica de Cerveza SAU (Murcia, Spain).

2.2. Ultrasound-assisted extraction

UAE was carried out using a high intensity focused ultrasounds (HIFU) probe from Sonics Vibra-Cell (model VCX130, Hartford, CT, USA), with 130 W of maximum potential. MR was ground with a domestic miller. All extractions were performed by mixing 100 mg of ground MR and 5 mL of extracting buffer (100 mM Tris-HCl, pH 9.0). Extraction conditions (concentration of additives, ultrasounds amplitude, extraction time, and temperature) were optimized using a Box-Behnken incomplete factorial experimental design. Response variable was the protein content, expressed as g protein per 100 g MR. Experimental data were fitted to the following quadratic model:

$$y = k_0 + \sum_{i=1}^{4} kx_i + \sum_{i=1}^{4} k_{ii}x_i^2 + \sum_{i=1}^{4} k_{ij}x_i x_j$$

where x_i and x_j are the independent variables, k_0 represents the constant term, and k_i, k_{ii}, and k_{ij} are the terms for linear, second-order effects, and interactions between variables, respectively. The analysis of variance ANOVA ($\alpha = 0.05$) and the determination coefficient (R^2) were used to evaluate the fitting of the established mathematical model.

Extracted proteins were next precipitated overnight with cold acetone. After centrifugation, the pellet was stored at −20 °C until use. Before use, the pellet was solubilized in a 5 mM phosphate buffer (pH 8).

2.3. Pressurized liquid extraction

PLE was carried out using an accelerated solvent extractor system (ASE 150, Dionex, Sunnyvale, CA, USA). Sample was prepared by mixing 1.5 g of milled malt rootlets and 9 g of sand (used as dispersive agent) which was displayed in a stainless-steel extraction cell (10 mL). A cellulose filter (2.5 cm diameter, Whatman) was fixed at the cell bottom to avoid particles filtration. Initial equipment conditioning involved preheating at 1500 psi for 6 min followed by 100 s of nitrogen purge. Solvents were previously degassed in an ultrasonic bath for 30 min. Optimization of extraction conditions (extraction time, percentage of EtOH, and extraction temperature) was performed by a Box-Behnken incomplete factorial experimental design using the protein content as response variable and fitting the data to a quadratic equation similar to the previously shown for the optimization of UAE variables. As previously, the fitting of the regression model was assessed by ANOVA and the determination of R^2.

All extracts were evaporated in a centrifugal concentrator (Eppendorf AG, Hamburg, Germany) and stored at −20 °C until use. Before use, solid samples were solubilized in a 5 mM phosphate buffer (pH 8).

2.4. Proteins content determination

Three different methods were employed for determining the proteins content in extracts: Bradford method, Bicinchoninic Acid Protein (BCA) assay, and Dumas assay. Bradford and BCA assays were used for the determination of the proteins content during optimization of extracting parameters since they are based on spectrometric measurements that are fast and easy to obtain. Dumas assay was used to determine the proteins content in the final extract obtained under optimal conditions due to its higher accuracy. In all cases, all measurements were made by triplicate.

Bradford assay required the dilution of Bradford reagent in water (1:4 (v/v)). Then, 1 mL of this solution was mixed with 12.3 μL of sample

(or standard) and, after standing at room temperature for 5 min, the absorbance at 595 nm was measured in a spectrophotometer UV/Vis Lambda 35 (PerkinElmer, Walthman, MA, USA). A calibration curve using BSA (from 0 to 0.3 mg/mL) was prepared.

As SDS is an important interfering agent in Bradford assay, protein quantification in extracts obtained by UAE was performed by the BCA assay using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For that purpose, 25 μL of sample (or standard) were mixed with 200 μL of working solution by agitation for 30 min at 37 °C. Afterwards, the absorbance at 562 nm of the resulting solution was measured. A calibration curve using BSA (from 0 to 0.5 mg/mL) was employed.

DUMAS assay was carried out in a NDA 702 Dumas Nitrogen Analyzer (VELP scientifica, Usmate, Italy). For that purpose, 100 mg of liquid extract was adsorbed on 50 mg of a Super-Absorbent Powder™ (silicon dioxide), wrapped in tin foil, and introduced in the equipment. When analysing solid samples, the sample (100 mg) was wrapped directly in tin foil and introduced in the equipment. High purity EDTA was used as standard to obtain the calibration curve (0–50 mg).

2.5. Characterization of extracts

Separation of proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Mini-Protean system from Bio Rad following the procedure described in González-García et al. (2021).

In vitro gastrointestinal digestion of extracts was carried out using pepsin (pH 2.0, 1:35 enzyme:subtrate ratio, 1 h, 37 °C) and pancreatine (pH 7.5, 1:25 enzyme:subtrate ratio, 2h, 37 °C), successively. Incubations were performed in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany). The solution was kept at 100 °C for 10 min to stop the process. After centrifugation (10 min, 4000×*g*), the supernatants were collected. The performance of the reaction was assured by evaluating the peptides content before and after the hydrolysis by the OPA method, following the procedure described by Hernandez-Corroto et al. (2018).

Antioxidant capacity of extracts and hydrolysates was evaluated using three *in vitro* antioxidant assays to determine their ability to scavenge free radicals (ABTS and DPPH) and to inhibit the formation of hydroxyl radicals. All methods were described in González-García et al. (2014). All measurements were made by triplicate.

2.6. Proteomic analysis

Proteins in extracts were hydrolysed using trypsin enzyme. Briefly, proteins were diluted up to 10 mg/mL with 50 mM NH_4HCO_3 and 8 M urea, reduced by adding DTT (10.6 μL of 100 mM DTT to 200 μL aliquot), incubated for 1 h at 37 °C, alkylated with 50 μL of 75 mM iodoacetamide, and incubated for 30 min in darkness and at room temperature. Afterwards, the sample was diluted 4 times in 50 mM NH_4HCO_3 and trypsin enzyme (10 μL of 10 g/L) was added. The resulting mixture was submitted to overnight incubation at 37 °C. The enzymatic digestion was quenched by addition of 100 μL of 50% TFA. After digestion, samples were desalted through a weak cation exchange STRATA® column (30 mg of solid phase) from Phenomenex® (Torrance, CA, USA). Desalted samples were dried in a Speedvac system (Eppendorf™, Hamburg, Germany) and redissolved in the initial mobile phase before mass spectrometer analysis.

A hybrid Quadrupole-Orbitrap Q Exactive mass spectrometer (MS) coupled to an Ultra High Performance Liquid Chromatography (UHPLC) from Thermo Fisher Scientific (Waltham, MA, USA) was employed to perform the identification. Separation of peptides was achieved by reversed-phase chromatography in an Ascentis Express Peptide ES-C18 column (100 mm × 2.1 mm, 2.7 μm particle size) using a guard column (5 mm × 2.1 mm, 2.7 μm particle size), both from Supelco (Bellefonte, PA, USA). Chromatographic conditions were: flow rate, 0.3 mL/min; column temperature, 25 °C; mobile phase A, 0.3% (v/v) AA in water; mobile phase B, 0.3% (v/v) AA in ACN; and injection volume, 10 μL. The optimized elution gradient was: 5% B for 10 min, 5–40% B in 20 min, 40–95% B in 10 min. The separation gradient was followed by restoring initial eluting conditions in 5 min.

MS analysis was carried out in the positive ionization mode using full-scan from 100 to 1700 m/z. Electrospray conditions were: skimmer voltage, 60 V; drying gas flow, 12 L/min; gas temperature, 350 °C; fragmentator voltage, 200 V; nozzle voltage, 0 V; nebulizer pressure, 50 psig; capillary voltage, 3500 V. The Jet Stream sheath gas flow was 12 L/min and temperature was 400 °C. Auto mode was selected for tandem mass spectrometry (MS/MS) with the following conditions: a collision energy of 4 V per 100 Da, 3 precursors per cycle, and active exclusion after two spectra (released after 1 min). An internal standard, warfarin, yielding an ion at 309.1121 m/z, was used throughout the analysis. All samples were injected by triplicate.

PEAKS Studio Version 7 software (Bioinformatics Solutions Inc. Waterloo, Canada) was employed to analyse MS/MS spectra using the *de novo* tool or the database search tool (PEAKS DB). Peptide sequences were accepted by *de novo* if the expected percentage of correct amino acids (average local confidence, ALC) was equal or higher to 90%. I and L amino acids could not be differentiated using the *de novo* tool, so only isoforms with L were displayed. However, isoforms with I are equally possible.

Hordeum vulgare protein sequences extracted from UNIPROT database was used to analyse peptide sequences. Carbamidomethylation and oxidation of methionine residues were set as fixed and variable modifications, respectively. Peptides mass tolerance was less than 10 ppm and fragment mass tolerance less than 0.5 Da. Peptides should have a False Discovery Rate (FDR) lower than 1% for acceptance. Proteins with −10 lgP lower than 20 were discarded. Only peptides and proteins that were identified in, at least, three different injections distributed between two or more different replicates, were considered. Functionality of proteins was obtained from QuickGo (www.ebi.ac.uk/QuickGo).

2.7. Total phenolics content and identification of phenolic compounds by UHPLC-Q-Orbitrap-MS/MS

The co-extraction of phenolic compounds was initially estimated by the determination of the total phenolics content (TPC) following the Folin–Ciocalteu assay. Results were expressed as mg gallic acid equivalent (GAE) per g of sample (mg GAE/g BMR) and measured by triplicate.

A further evaluation of the co-extraction of phenolic compounds was carried out by their identification in the extracts using MS/MS analysis. Identification of phenolic compounds was carried out in the negative ionization mode under the same chromatographic and spectrometric conditions previously detailed for the identification of peptides. A negative ion from warfarin (307.0965 m/z) was employed as internal standard throughout the analysis.

Data sets were processed using Compound Discoverer 2.1 (Thermo Fisher Scientific, San Jose, CA, USA) software, which performed peak extraction, peak alignment, peak matching, and metabolite identification. An extensive database of phenolic compounds, including molecular weights and formulas, was implemented for a more reliable and faster manual validation of MS/MS spectra. Extracted masses were filtered to discard compounds without fragmentation, background compounds identified in the blank sample, and compounds whose m/z ratios could not derive from masses present in databases. Finally, the fragmentation spectra of filtered compounds were manually matched to spectra previously reported in literature for validation. When data were lacking, the identification of phenolic compounds was tentatively performed by matching the spectra with predicted ones generated by online software Competitive Fragmentation Modelling for Metabolite Identification (CFM-ID 3.0). Only polyphenols identified, at least, in two different injections, were considered.

2.8. Statistical analysis

Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warranton, VA, USA) was used for the statistical analysis. Values are displayed as mean ± standard deviation (SD) of, at least, three independent experiments.

3. Results and discussion

Two different techniques, never used before for the extraction of proteins from MR, were employed and compared to recover proteins from this sample. UAE is widely employed for the extraction of proteins and it usually requires a final step for the purification of proteins. It will be used as control. PLE is being considered as an alternative to UAE and this work will evaluate its performance in the extraction of MR proteins. Since different parameters can affect the extraction process in both techniques, the optimization of experimental conditions was firstly addressed.

3.1. Optimization of the extraction of proteins from MR using ultrasound-assisted extraction

The first optimized variable was the extracting buffer. Fifteen buffers covering from pH 5.5 to pH 13.0 were employed. This range was selected taking into account previous authors experience working with plant proteins (Olivares-Galván et al., 2020; Ma et al., 2022). In some cases, different buffers with identical pH were used. Extraction conditions employed in these experiments were: 30% of ultrasounds amplitude, 5 min extraction time, and room temperature. After extraction and centrifugation, proteins in supernatants were precipitated with cold acetone at 4 °C for 2 h. Relative proteins contents (expressed as % related to the higher yield) are shown in Fig. 1. Tris-HCl buffer (pH 9.0 and 8.5) yielded the highest protein recovery, while buffers at higher pHs showed lower yields. This result has been observed in other plant proteins such as pea, chickpea, and other legumes (Ma et al., 2022). Extraction yields also depended on the kind of buffer. Indeed, significant differences (p-value < 0.05) were observed when comparing results obtained with ammonium bicarbonate and phosphate buffers at pH 6.5, phosphate and Tris-HCl buffers at pH 7.5, and sodium hydroxide and sodium bicarbonate buffer at pH 11.0. As expected, extractability was promoted with organic buffers. Therefore, a Tris-HCl buffer (pH 9.0) was selected henceforth.

After extraction, purification of proteins was carried out to get rid of other compounds that usually are co-extracted with proteins. Four different protocols were compared: precipitation with acetone or HCl for 2 h or overnight. No precipitation was observed when using HCl at 2 h or overnight probably due to the solubility of proteins at very high pHs. Regarding acetone, increasing the precipitation time from 2 h to overnight allowed to double the amount of recovered proteins. Therefore, overnight precipitation with acetone was the chosen procedure.

After selecting the extracting buffer and the precipitation protocol, a three factor Box-Behnken experimental design was applied to optimize remaining experimental conditions: presence of additives (SDS (variable A) and DTT (variable B) at concentrations from 0 to 1% (w/v)), extraction time (variable C, 1–20 min), ultrasound amplitude (variable D, 30–70%), and temperature (variable E, 25 to 60 °C). A total of 50 experiments were established: 40 points corresponding to the own factorial design and 10 central points that reflected the experimental error. Proteins content was used as response variable (Y). Table S1 includes all experiments and proteins contents determined by BCA assay (due to the presence of SDS in some of them). Extractions used as central points (experiments 1, 2, 4, 5, 15, 17, 20, 32, 42, and 45) were highly reproducible. The addition of DTT and, especially, SDS in the extracting buffer clearly promoted the extraction of proteins. In fact, the lowest extraction of proteins was determined in experiment 27 where no SDS or DTT was added while the addition of 0.5–1% of both (experiments 1 and 11) led to a huge increase in protein extractability. The second-order polynomial model best fitting variables to predict the proteins content was:

$$\text{Proteins content (g protein/100 g MR)} = 0.580815 + 8.1924\,A + 0.917987\,B + 0.263416\,C + 0.0250572\,D + 0.00432436\,E - 5.78422\,A^2 - 1.04249\,AB + 0.0936948\,AC + 0.0222346\,AD - 1.29725\,B^2 + 0.0299098\,BC + 0.0415736\,BE - 0.00704851\,C^2 - 0.00076499\,CE$$

This mathematical model predicted the 95.3% of proteins content

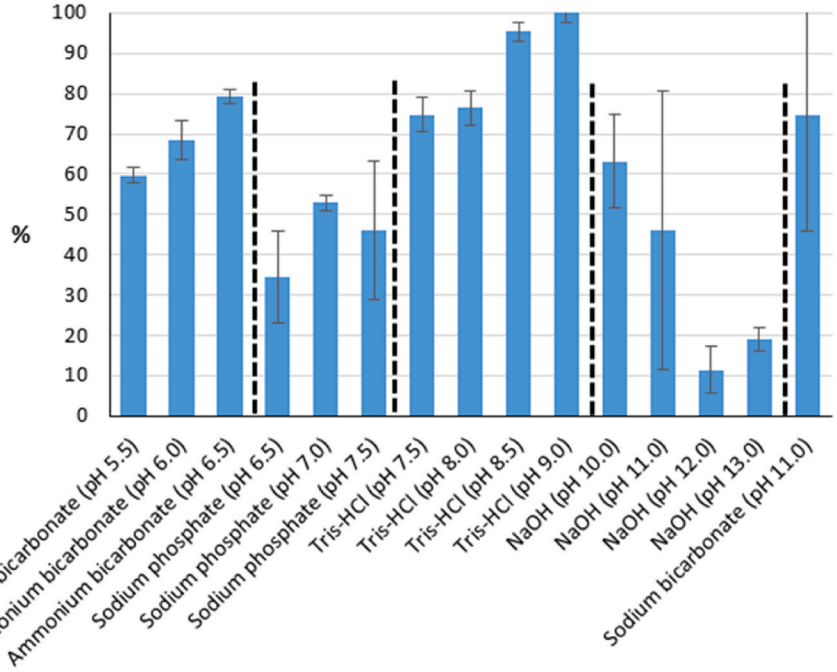

Fig. 1. Relative recovery of proteins obtained at different pHs and buffers using UAE. Other experimental conditions were: 30% of ultrasounds amplitude, 5 min extraction time, and room temperature.

variability and ANOVA confirmed its suitability (p-value for the lack-of-fit > 0.05). Moreover, ANOVA also predicted that the SDS concentration, the extraction time, the probe amplitude, and the DTT concentration were the variables with more significant effects on the proteins yield (p-value < 0.05).

The effect of the temperature, extraction time, and amplitude, at constant SDS and DTT concentrations, on the protein yield is shown in Fig. 2A as 3-D contour plot. Proteins extraction was promoted at higher ultrasounds amplitudes, times, and temperatures. Accordingly, optimal extraction conditions were: extraction buffer, Tris-HCl (100 mM, pH 9.0) containing 0.92% (w/v) SDS and 1% (w/v) DTT; temperature, 52 °C; HIFU amplitude, 68% (88 W); and extraction time, 20 min. Under these conditions and after overnight precipitation with acetone and centrifugation, the proteins yield was 16 ± 1 mg of proteins/100 mg of MR (determined by Dumas). Taking into account that the proteins content in MR is 29.7 ± 0.7 mg/100 mg MR, the extraction yield was 54%. Comparing with results obtained by Cheng et al. (2016) using a conventional extraction, also at pH = 9, the application of UAE enabled

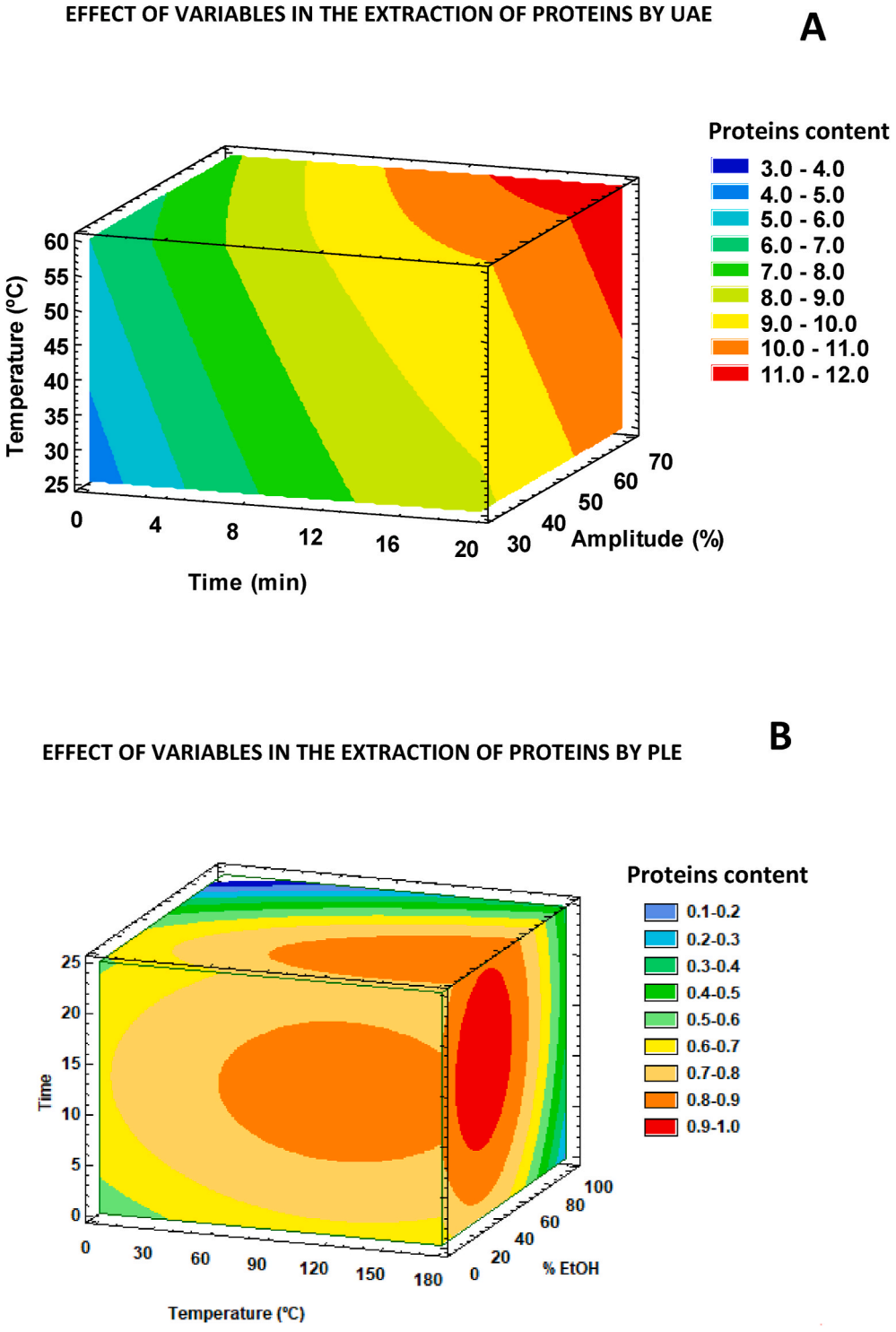

Fig. 2. Response surface 3-D contour plots showing the effect of the temperature, time, and ultrasounds amplitude (at 0.92% (w/v) SDS and 1% (DTT)) in the amount of proteins recovered by UAE (A) and the effect of the percentage of ethanol, temperature, and time in the amount of proteins recovered by PLE (B).

to increase the protein recovery and reduce the required extraction time (from 60 to 20 min). Moreover, the method of Cheng et al. (2016) co-extracted phenolic compounds. Comparing with methods using UAE for the extraction of proteins from other matrices different from MR, such as the brewer's spent grain, the optimized method enabled a better extraction of proteins (González-García et al., 2021).

3.2. Optimization of the extraction of proteins from MR using pressurized liquid extraction

An incomplete factorial Box-Behnken experimental design was utilized to optimize the variables affecting the extraction in PLE: percentage of EtOH (variable A, 0–100% (v/v)), temperature (variable B, 40-170 °C), and time (variable C, 5–25 min). These conditions were chosen according to previous authors experience and instrumentation limitations. Seventeen experiments were fixed: 12 points corresponding to the own factorial design and 5 central points to evaluate the experimental error. Table S2 includes all experiments and proteins contents determined by Bradford assay. Extractions used as central points (experiments 5, 6, 8, 13, and 15) showed high reproducibility. The lowest proteins recoveries were observed at high EtOH percentages while temperature seems to be the variable most enhancing proteins extraction. Darker extracts were observed when using temperatures of 170 °C (see extracts 9, 11, 16, and 17, Fig. S1). This could be due to the extraction of coloured phenolic compounds or the development of Maillard reactions that are promoted at high temperatures (Martins et al., 2000).

The second-order polynomial model best fitting variables to predict proteins content was:

$$\text{Proteins content (g protein/100 g MR)} = 0.48093 + 0.0046265\ A + 0.0024575\ B + 0.026325\ C - 0.00012615\ A^2 + 0.000022462\ AB - 0.0000097337\ B^2 - 0.00084625\ C^2$$

This mathematical model predicted the 99.4% of the variability in the proteins content. Moreover, ANOVA confirmed the suitability of the model (p-value for the lack-of-fit > 0.05). Most significant variable was the EtOH concentration (p-value < 0.05). Fig. 2B shows as 3-D contour plot the effect of the three variables on the proteins yield. Extraction of proteins by PLE was promoted at low-medium percentages of EtOH, medium-high temperatures, and high times. The positive effect of high temperatures and times on the protein yield was also observed in the case of *Spirulina* (Zhou et al., 2021) while the addition of low-medium percentages of EtOH and high temperatures enabled to increase the extraction of proteins from brewer's spent grain (González-García et al., 2021) and pomegranate seeds (Guzmán-Lorite et al., 2022). Nevertheless, the extraction of proteins from pomegranate peels (Hernández--Corroto et al., 2020) required the use of high EtOH percentages and temperatures. Therefore, the evaluation of the effect of extraction parameters on the proteins yield depends on the matrix and requires a suitable optimization.

In comparison with other works using PLE with subcritical water (Álvarez-Viñas et al., 2021), the addition of low percentages of EtOH seems to favour the extraction of proteins avoiding the use of high temperatures or longer times than can result in proteins degradation and development of undesirable Maillard reactions. Thus, optimal extraction conditions were: EtOH, 33% (v/v); temperature, 164°C; time, 15 min. Moreover, the number of statics cycles also affected the extraction of proteins (see Table S3) observing the highest value when using 5 cycles (15 min every one). Under these conditions, it was possible to extract 22 ± 2 mg of proteins/100 mg of MR (determined by Dumas assay), which means a 73 ± 7% of extraction yield. This content is higher than the previously obtained by UAE (53 ± 5%) confirming the capacity of PLE, usually employed for the extraction of small molecules, as a sustainable and efficient technique for the extraction of proteins. It is not possible a comparison with other works dealing with the extraction of proteins from MR but we can compare it with the extraction of proteins from other matrices. Wiboonsirikul et al. (2007) used subcritical water to extract proteins from rice bran and observed the highest recovery (20% of proteins) at 200 °C and 5 min. Zhou et al. (2022) extracted proteins from *Spirulina* using dimethylsulfoxide at 40 °C and 15 min obtaining a 4.5% yield, de la Fuente et al. (2021) used water for extracting proteins from fish side streams observing extraction yields ranging from 28 to 48%, depending on the byproduct, and Ho et al. (2007) recovered 22.5% of proteins from flaxseeds. This recoveries are lower than the obtained in the extraction of proteins from MR under optimized conditions.

3.3. Characterization of extracts obtained using UAE and PLE

Characterization of extracts was carried out by the separation of proteins by SDS-PAGE and the evaluation of antioxidant properties in the intact extracts and in the extracts after submission to a simulated gastrointestinal digestion.

Separation of proteins by SDS-PAGE (see Fig. 3) showed numerous bands ranging from 5 to 100 kDa in both extracts being difficult to observe significant differences on their profile.

Antioxidant capacity is an important characteristic of proteins, which justifies its study. The antioxidant capacity of extracts was evaluated by measuring their capability to inhibit the formation of hydroxyl radicals and to scavenge free radicals (ABTS and DPPH radicals). Results were graphically summarized in Fig. 4. Although extracts showed capacity to scavenge free radicals, the inhibition of hydroxyl radicals formation was the main antioxidant mechanism in both extracts. It was remarkable the capacity shown by the PLE extract in this assay since it had to be diluted 4-times to avoid signal saturation. The higher antioxidant capacity observed for the PLE extract in two of the antioxidant assays could be attributed to the own extracted proteins, although phenolic compounds could also be co-extracted and, then, could be contributing to this activity. Moreover, antioxidant activity of extracts was also measured after a simulated gastrointestinal digestion to evaluate its effect. Gastrointestinal digestion severely reduced the capacity to inhibit the formation of hydroxyl radicals and the DPPH radical scavenging capacity. This fact supports that the antioxidant activity observed by these two mechanisms could be associated to intact proteins and also to intact phenolics compounds, in case they were co-extracted. However, the ABTS radical scavenging capacity kept or even increased after *in vitro* digestion. In this case, released peptides may be contributing to this antioxidant antioxidant mechanism.

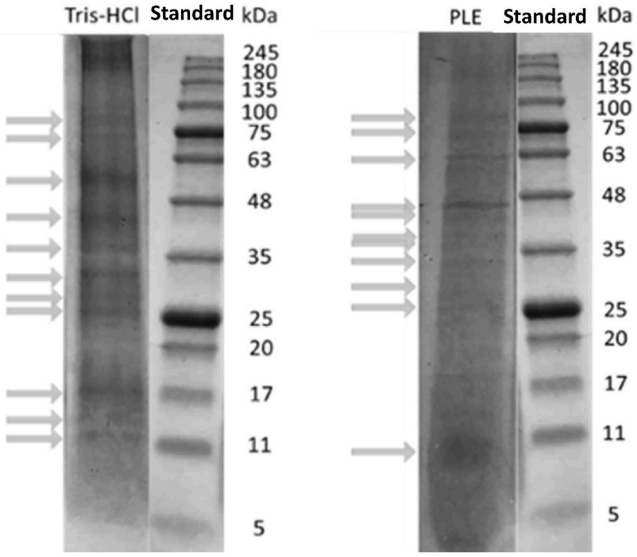

Fig. 3. SDS-PAGE separation of proteins extracted from MR by UAE and PLE.

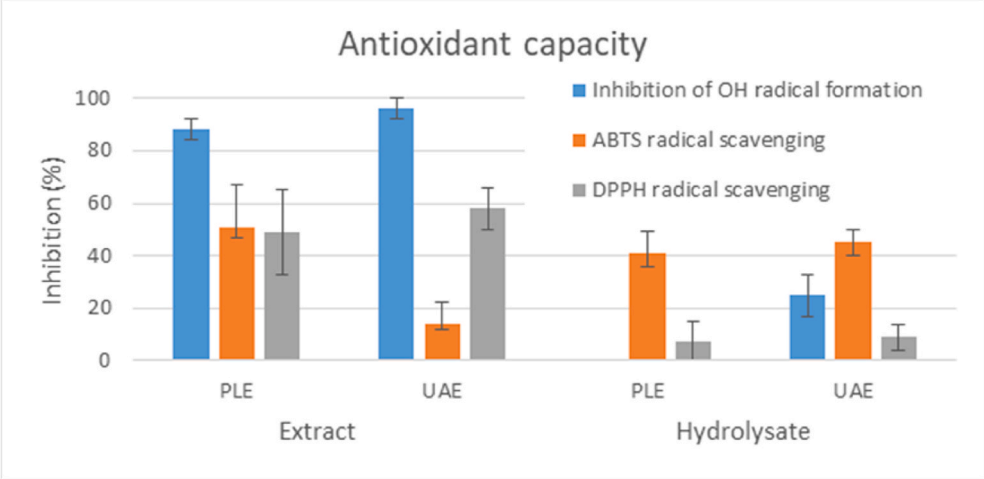

Fig. 4. Antioxidant capacity of the extracts obtained by PLE and UAE. (* PLE extract was diluted 4-times to avoid signal saturation in the evaluation of the inhibition of hydroxyl radical formation).

3.4. Proteomic analysis of extracts

A direct in-solution digestion of proteins with trypsin was performed for the identification of proteins in extracts followed by RP-UHPLC-Q-Orbitrap-MS/MS analysis. Peptides were searched against Uniprot_*Hordium vulgare* database. A total of 275 peptides enabled the identification of proteins in the UAE extract while only 37 peptides could be assigned to proteins in the PLE extract. Tables 1 and 2 show the proteins identified from these peptides. While 68 proteins from *Hordeum vulgare* were identified in the extract obtained using UAE, only 9 were identified in the extract obtained using PLE. Eight of these 9 proteins were common to the UAE extract while only protein P34824 was exclusively extracted with PLE. Table 3 shows the location, the activity, and the biological processes in which identified proteins are involved. Most proteins were located in the cytoplasm, the extracellular region, and the nucleus although some proteins from the ribosome, perixoma, vacuole, and mitochondrion were also identified. Regarding their functionality, many proteins showed metal and nucleotide binding, catalytic, and inhibitory capacities. Just three proteins with nutrient reservoir activity were identified (B3-hordein (P06471), serpin-Z4 (P06293), and B1-hordein (P06470)) while most identified proteins were involved in carbohydrate metabolism. This fact is in agreement with Mahalingam (2020) who suggested that the production of sugars was a key process in rootlets. Additionally, other identified proteins were involved in defence and stress responses, which was also in accordance with Mahalingam (2018). They attributed this fact due to the stressful environment occurring during germination. This is very different to the observed in the brewer's spent grain (BSG) where many identified proteins were subunits of hordeins (González-García et al., 2021). In addition to the previously mentioned storage proteins, other proteins in common with BSG were: beta-amylase (P16098 and P82993), serpin Z4 (P06293), sucrose synthase 2 (P31923), signal recognition particle 54 kDa protein 1 and 2 (P49968 and P49969), alpha-amylase trypsin inhibitor CMb (P32936), phytepsin (P42210), pyrophosphate-energized vacuolar membrane proton pump (Q06572), S-adenosylmethionine synthase 4 (Q4LB21), serpin ZX (Q40066), V-type proton ATPase catalytic subunit A (Q40002), and elongation factor 1-alpha (Q40034). Furthermore, an additional peptide (YEEIVK) appearing in both extracts was assigned to different virus proteins (capsid proteins) and, in the case of the UAE extract, some peptides were assigned to proteins from other plants (*Sorghum bicolor* and *Agrostis stolonifera*) or virus (barley yellow mosaic and dwarf viruses).

Additionally, the *de novo* sequencing enabled to observe 70 peptides in the extract obtained by PLE and 66 in the case of the UAE extract. Peptides sequences are shown in Tables S4 and S5 and Figs. S2 and S3 show the MS/MS spectra corresponding to some of these peptides. Peptides from the PLE extract ranged from 4 to 11 amino acids, while in the case of the UAE extract peptides ranged from 4 to 7. Sixteen peptides were common to both extracts (SLVR, TLLR, LLER, ELLR, SLLR, LYVR, HLVLR, LVLR, WLFR, FELLR, LSLR, FLAR, VVVVR, LLSR, VGFR, and KFTR).

Table 1
Proteins identified in the extract obtained by the optimized PLE method.

Accession number	Gene name	Protein name	Mass (Da)	Peptides in protein sequence	−10 logP
P06471		B3-hordein (Fragment)	30195	3	137.19
P14928	HVA1	ABA-inducible protein PHV A1	21820	1	105.8
P33044		Antifungal protein R (Fragment)	4453	2	104.94
P93176	TUBB	Tubulin beta chain	50194	1	108.17
P26517	GAPC	Glyceraldehyde-3-phosphate dehydrogenase 1 cytosolic	36514	4	149.68
P12948	DHN3	Dehydrin DHN3	16162	1	87.32
P34824		Elongation factor 1-alpha	49178	8	180.46
Q40034		Elongation factor 1-alpha	49142	8	180.46
P08477	GAPC	Glyceraldehyde-3-phosphate dehydrogenase 2 cytosolic	33236	9	151.86

3.5. Evaluation of the co-extraction of phenolic compounds

Phenolic compounds are frequently co-extracted with proteins. In order to evaluate this fact, TPC was firstly evaluated. TPC value was much higher in the case of the PLE extract than in the UAE extract: 3.1 ± 0.3 mg GAE/g of MR in the UAE extract and 14.8 ± 0.6 mg GAE/g of MR in the PLE extract. TPC obtained using the UAE method was in agreement with those given by Budaraju et al. (2018) (3.76 mg GAE/g of MR) while TPC in PLE extract was similar to the obtained from a BSG extract by PLE under similar conditions (35% EtOH, 155 °C, 17 min, 5 cycles): 17.2 ± 0.07 mg GAE/g of BSG (González-García et al., 2021). Nevertheless, since the assay used (Folin-Ciocalteu method) is based on the measurement of the capacity to reduce a probe molecule, it was not

Table 2
Proteins identified in the extract obtained by the optimized UAE method.

Accession number	Gene name	Protein name	Mass (Da)	Peptides in protein sequence	−10 logP
P29305		14-3-3-like protein A	29352	14	226.03
Q43470		14-3-3-like protein B	29691	8	182.48
P80284	PDI	Protein disulfide-isomerase	56463	10	184.64
P52572	PER1	1-Cys peroxiredoxin PER1	23963	7	178.14
Q43772		UTP–glucose-1-phosphate uridylyltransferase	51644	14	196.9
Q40002		V-type proton ATPase catalytic subunit A (Fragment)	64098	8	168.57
Q9ZRR5	TUBA3	Tubulin alpha-3 chain	49729	6	211.38
Q96460	TUBA2	Tubulin alpha-2 chain	49701	6	211.38
P34937		Triosephosphate isomerase cytosolic	26737	6	170.76
Q9M5G3	TCTP	Translationally-controlled tumor protein homolog	18884	5	143.5
P14928	HVA1	ABA-inducible protein PHV A1	21820	4	156.89
Q43473	TUBA1	Tubulin alpha-1 chain	49597	4	152.43
P31922	SS1	Sucrose synthase	92211	9	177.29
P06471		B3-hordein (Fragment)	30195	4	142.75
Q40078		V-type proton ATPase subunit B 1	54027	3	155.13
P62162	CAM	Calmodulin OS=Hordeum vulgare	16832	3	134.11
P29114	LOX1.1	Linoleate 9S-lipoxygenase 1	96393	7	191.29
P06293	PAZ1	Serpin-Z4 OS=Hordeum vulgare	43276	6	187.61
Q06572		Pyrophosphate-energized vacuolar membrane proton pump	79533	5	165.78
Q4LB21	SAM4	S-adenosylmethionine synthase 4	43211	2	110.74
P93176	TUBB	Tubulin beta chain OS=Hordeum vulgare	50194	3	157.29
P33044		Antifungal protein R (Fragment)	4453	2	103.31
Q43472	blt801	Glycine-rich RNA-binding protein blt801	15926	3	126.05
P42210		Phytepsin	54226	1	123.81
Q949H0	RPS7	40S ribosomal protein S7	22078	4	132.2
Q01548		Peroxidase 2 (Fragment)	18882	4	147.98
P28814		Barwin	13737	1	94.24
P12948	DHN3	Dehydrin DHN3	16162	2	71.33
P55238		Glucose-1-phosphate adenylyltransferase small subunit chloroplastic/amyloplastic	56049	1	64.98
P32936	IAT2	Alpha-amylase/trypsin inhibitor CMb	16526	2	77.32
P12952	DHN2	Dehydrin DHN2	14442	1	65.52
Q40079		V-type proton ATPase subunit B 2	53726	3	155.13
A1E9I8	atpA	ATP synthase subunit alpha chloroplastic	55295	4	114.95
Q96562	CHS2	Chalcone synthase 2	43189	1	84.88
P45850		Oxalate oxidase 1	21203	1	75.7
P20026	MYB1	Myb-related protein Hv1	29740	1	71.96
P27968	NAR-7	Nitrate reductase [NAD (P)H]	98630	1	59.67
Q4LB22	SAM3	S-adenosylmethionine synthase 3	42766	4	147.68
P12951	DHN1	Dehydrin DHN1	14236	1	65.52
Q9XHS0	RPS12	40S ribosomal protein S12	15295	2	70.56
P35266		60S ribosomal protein L17-1	19504	4	133.8
Q40024		Betaine aldehyde dehydrogenase	54290	3	114.5
P06470		B1-hordein	33422	4	137.25
Q4LB23	SAM2	S-adenosylmethionine synthase 2	42828	4	133.05
Q06378		Glutamine synthetase	39128	4	93.11
Q40066	PAZX	Serpin-ZX	42947	1	102.88
P82993	BMY1	Beta-amylase	59639	2	93.81
P16098	BMY1	Beta-amylase	59647	2	93.81
P50888	RPL24	60S ribosomal protein L24	18400	2	90.53
Q9ZRI8		Formate dehydrogenase mitochondrial	41546	1	81.67
P00828	atpB	ATP synthase subunit beta chloroplastic	53875	5	143.39
P13691	IAD1	Alpha-amylase inhibitor BDAI-1	16429	2	68.5
Q00531		60 kDa jasmonate-induced protein	60362	2	102.12
Q43492	PAZ7	Serpin-Z7	42821	1	88.09
P00693	AMY1.1	Alpha-amylase type A isozyme	47796	1	49.3
P12949	DHN4	Dehydrin DHN4	22573	1	64.14
P49969	SRP54-2	Signal recognition particle 54 kDa protein 2	54508	1	60.18
P49968	SRP54-1	Signal recognition particle 54 kDa protein 1	54512	1	60.18
P31923	SS2	Sucrose synthase 2	92575	1	107.51
P35267		60S ribosomal protein L17-2	19705	3	107.64
P45851		Oxalate oxidase 2	23479	1	72.25
P92392	rpoA	DNA-directed RNA polymerase subunit alpha	38905	1	58.67
P08477	GAPC	Glyceraldehyde-3-phosphate dehydrogenase 2 cytosolic	33236	8	215.81
P0CG86	MUB1	Ubiquitin-40S ribosomal protein S27a	17655	6	179.32
P0CG87	MUB2	Ubiquitin-40S ribosomal protein S27a	17671	6	179.32
P06353		Histone H3.3	9233	6	125.35
P26517	GAPC	Glyceraldehyde-3-phosphate dehydrogenase 1 cytosolic	36514	12	203.02
Q40034	BLT63	Elongation factor 1-alpha	49142	17	212.14

possible to assure that the higher TPC value in the PLE extract was due to the presence of phenolic compounds. Indeed, any molecule with antioxidant capacity, including extracted proteins, could reduce this probe molecule. In order to have a more solid knowledge on the presence of phenolic compounds in the PLE and UAE extracts, they were analysed by RP-UHPLC-Q-Orbitrap-MS/MS. Thirteen phenolic compounds were identified in the PLE extract while no one was identified in the UAE extract. This is in agreement with previous TPC results. Table S6 groups the phenolic compounds identified in the PLE extract and Fig. S4 shows their mass spectra. Compounds 4, 8, 9, 10, 11, and 12 were validated by matching their fragmentation spectra with others reported in literature.

Table 3
Location, activity, and biological process in which identified proteins are involved.

Accession number	Cell location	Molecular function	Biological process	Extract
P29305	–	Protein kinase C inhibitor	Negative regulation of catalytic activity	UAE
Q43470	Nucleus	DNA binding	Regulation of transcription	UAE
Q40002				
P80284	Endoplasmatic reticulum	Isomesare	Catalysis of disulfide bonds	UAE
P52572	Cytoplasm and nucleus	Antioxidant	Cellular detoxification	UAE
Q43772	Cytoplasm	Transferase	Glycolysis	UAE
Q9ZRR5	Cytoplasm (microtubule)	Nucleotide binding; cytoskeleton constituent	Cytoskeleton organization	UAE/
Q96460				PLE
P93176				
P34937	Cytoplasm	Isomerase	Glycolysis	UAE
Q9M5G3	Cytoplasm	–	–	UAE
P14928	–	–	Environmental stress[1]	UAE/
				PLE
Q43473	Cytoplasm (microtubule)	Nucleotide binding; cytoskeleton constituent	Glycolysis	UAE
P31922	–	Transferase	Sucrose metabolism	UAE
P31923				
P06471	–	Nutrient revervoir	–	UAE/
P06470				PLE
Q40078	–	proton transmembrane transportation	ATP metabolism	UAE
P62162	–	Ca^{2+} binding	–	UAE
P29114	–	Oxidoreductasa Metal binding	Lipid metabolism	UAE
P06293	Extracellular region	Nutrient reservoir Peptidase inhibitor	Negative regulation of peptidase activity Defence response	UAE
Q06572	Vacuole membrane	Metal binding	Proton transmembrane transport	UAE
Q4LB21	Cytoplasm	Nucleotide binding	Biosynthesis of S-adenosylmethionine	UAE
Q4LB22		Metal binding		
Q4LB23		Transferase		
P33044	–	–	Defence response	UAE/
				PLE
Q43472	–	DNA/RNA binding	Response to cold tolerance	UAE
P42210	Vacuole	Endopeptidase activity	Proteolysis	UAE
Q949H0	Ribosome	Translation	Structural constituent of ribosome	UAE
Q9XHS0				
P35266				
Q01548	–	Oxidoreductase activity and metal binding	Response to oxidative stress	UAE
P28814		Ribonuclease Carbohydrate binding	Defence response	UAE
P12948[2]	–	–	Response to water	UAE/
P12951				PLE[2]
P12952				
P12949				
P55238	Chloroplast/amyloplast	Transferasa Nucleotide binding	Starch biosynthesis	UAE
P32936	Extracellular region	Peptidase inhibitor	Negative regulation of peptidase activity	UAE
Q40066				
P13691				
Q43492				
Q40079	Part of a proton-transporting V-type ATPase complex in vacuolar membrane	Catalyzes of ATP hydrolysis	ATP metabolism	UAE
A1E9I8	Part of a proton-transporting ATP synthase complex	Nucleotide binding	ATP metabolism	UAE
P00828	Chloroplast			
Q96562		Transferase	Flavonoid biosynthetic process	UAE
P45850	Extracellular region	Metal binding Oxidoreductase	Membrane organization Removal of superoxide radicals	UAE
P20026	Nucleus	DNA binding	–	UAE
P27968	–	Nitrate reductase Metal binding	Nitrate assimilation	UAE
Q40024	Perixoma	Oxido-reductase	Response to anoxia and osmotic stress	UAE
Q06378	Cytoplasm	Nucleotide binding Catalytic	Glutamine biosynthesis	UAE
P82993	–	Hydrolase	Carbohydrate metabolism	UAE
P16098				
P50888	Ribosome	–	–	UAE
Q9ZRI8	Mitochondrion	Catalysis of oxidation of formate to carbon dioxide	Formate catabolic process	UAE
Q00531	–	Toxin; rRNA glycosylase activity	Negative regulation of translation Defence respond	UAE
P00693	Extracellular region	Metal binding α-amylase activity	Carbohydrate metabolism	UAE
P49969	Cytoplasm	Nucleotide and RNA binding	Targeting of proteins to membrane	UAE
P49968				
P45851	Extracellular region	Oxidoreductase activity; metal binding	–	UAE

(*continued on next page*)

Table 3 (*continued*)

Accession number	Cell location	Molecular function	Biological process	Extract
P92392	Chloroplast	DNA binding Transferase activity	Transcription	UAE
P26517	Cytoplasm	Oxidoreductase activity	Glucose metabolism	UAE/ PLE
P34824[2] Q40034	Cytoplasm	Nucleotide binding	Translational elongation	UAE/ PLE[2]
P08477	Cytoplasm	Oxidoreductase activity	Glucose metabolism	UAE/ PLE
P0CG86 P0CG87	Ribosome, nucleus, and cytoplasm	Metal and nucleotide binding, structural constituent of ribosome	Translation	UAE
P06353	Nucleus	Nucleotide binding	Constituent of chromatin	UAE

[1] From Hong et al. (1992).
[2] Only identified in the PLE extract.

For the rest of compounds, no spectra were found in the literature and validation was tentatively performed by matching the spectra with predicted ones.

Eight of these polyphenols were hydroxycinnamic acids: vanillactic acid (2), ethyl caffeate (7), sinapic acid (8), ferulic acid (10), and other derived compounds (1, 3, 5, and 11). Some of these phenolic compounds were previously observed in beer such as caffeic acid (Jandera et al., 2005), ferulic acid (Jandera et al., 2005), sinapic acid (Jandera et al., 2005). Others have been observed in foods such as vinegars (Alonso et al., 2004) (ethyl caffeate and isoferulic acid). Many of these compounds have shown relevant biological activities. Ethyl caffeate yielded anticancer (Lee et al., 2014), antifibrotic (Boselli et al., 2009), antidiabetic (Williams et al., 2012), and anti-inflammatory activities (Chiang et al., 2005) and sinapic acid has been studied for his antioxidant, anti-inflammatory, antimicrobial, and anticancer activities (Balagangadharan et al., 2019). Isoferulic acid has shown inhibitory effects against *Staphylococcus aureus* (Qiao and Chen, 1991), while hydroferulic acid demonstrated antioxidant activities by radical scavenging (Deters et al., 2008).

Another phenolic compound, also observed in beer (Jandera et al., 2005), is syringic acid (4) which has been associated to antioxidant, antimicrobial, anti-inflammatory, anticancer, and antidiabetic activities (Srinivasulu et al., 2018). Homovanillic acid (9), by the other hand, is a metabolite synthesized from dopamine, and it was also reported in beer (Floridi et al., 2003). Furthermore, hesperetin (12) is a flavonoid with capacity of lowering cholesterol (Wilcox et al., 2001) that has also been observed in wine (Jandera et al., 2005). Finally, phenolic compound 6 is a phenolic glycoside consisting of a phenolic structure attached to a glycosyl moiety and phenolic compound 13 is an O-methylated isoflavone.

4. Conclusions

Recovery of malt rootlets proteins has been possible by PLE and UAE. Proteins extraction was promoted at high ultrasounds amplitudes, time, and temperature in UAE while the addition of low amounts of EtOH clearly increased the protein extraction yield in PLE. PLE extracted 73% of the proteins in malt rootlets, which is 38% more proteins than the extracted by UAE, using a shorter time and in a more sustainable way. PLE extract also showed higher antioxidant capacity than the UAE extract although simulated gastrointestinal digestion led to a decreasing activity in both extracts. Proteomic analysis enabled to identify many proteins, especially in the UAE extract in addition to other peptides that could not be assigned to any protein. Unlike barley grains and brewer's spent grain, with high amounts of hordeins, only three storage proteins were detected in malt rootlets supporting that malt rootlets protein profile is very different to that of barley grains or brewer's spent grains. Most identified proteins in malt rootlets were located in the cytoplasm and were involved in carbohydrate metabolic processes and in defence and stress responses. The determination of the total phenolic compounds value and, especially, the analysis by MS/MS enabled to confirm the co-extraction of some phenolic compounds by PLE while no one was observed in the UAE extract. The presence of these phenolic compounds, in addition to its higher proteins content, could justify the higher antioxidant activity observed in the PLE extract related to the UAE extract. The high recovery of proteins from malt rootlets along with the properties of extracts make them deserve the attention of those looking for the revalorization of malt rootless.

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CRediT authorship contribution statement

Saúl Olivares Galván: Investigation, Validation, Formal analysis, Writing – original draft. **Estefanía González-García:** Investigation, Validation, Formal analysis. **María Luisa Marina:** Supervision, Resources, Funding acquisition, Project administration, Writing – review & editing. **María Concepción García:** Conceptualization, Investigation, Supervision, Resources, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: M Concepcion Garcia reports financial support was provided by Spain Ministry of Science and Innovation. M Luisa Marina reports financial support was provided by Community of Madrid. M Concepcion Garcia reports a relationship with University of Alcala that includes: board membership. No relationships or activities to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2022.10.009.

References

Alonso, Á.M., Castro, R., Rodriguez, M.C., Guillén, D.A., Barroso, C.G., 2004. Study of the antioxidant power of brandies and vinegars derived from Sherry wines and correlation with their content in polyphenols. Food Res. Int. 37, 715–721. https://doi.org/10.1016/j.foodres.2004.03.007.

Álvarez-Viñas, M., Rodríguez-Seoane, P., Flórez-Fernández, N., Torres, M.D., Díaz-Reinoso, B., Moure, A., Domínguez, H., 2021. Subcritical water for the extraction and hydrolysis of protein and other fractions in biorefineries from agro-food wastes and algae: a review. Food Bioprocess Technol. 14, 373–387. https://doi.org/10.1007/s11947-020-02536-4.

Balagangadharan, K., Trivedi, R., Vairamani, M., Selvamurugan, N., 2019. Sinapic acid-loaded chitosan nanoparticles in polycaprolactone electrospun fibers for bone regeneration in vitro and in vivo. Carbohydr. Polym. 216, 1–16. https://doi.org/10.1016/j.carbpol.2019.04.002.

Boselli, E., Bendia, E., Di Lecce, G., Benedetti, A., Frega, N.G., 2009. Ethyl caffeate from Verdicchio wine: chromatographic purification and in vivo evaluation of its antifibrotic activity. J. Separ. Sci. 32, 3585–3590. https://doi.org/10.1002/jssc.200900304.

Briggs, D.E., 1998. Malts and Malting, first ed. Blackie Academic & Professional, London, UK.

Budaraju, S., Mallikarjunan, K., Annor, G., Schoenfuss, T., Raun, R., 2018. Effect of pre-treatments on the antioxidant potential of phenolic extracts from barley malt rootlets. Food Chem. 266, 31–37. https://doi.org/10.1016/j.foodchem.2018.05.110.

Cejas, L., Romano, N., Moretti, A., Mobili, P., Golowczyc, M., Gómez-Zavaglia, A., 2017. Malt sprout, an underused beer by-product with promising potential for the growth and dehydration of lactobacilli strains. J. Food Sci. Technol. 54, 1–9. https://doi.org/10.1007/s13197-017-2927-7.

Cheng, H.-J., Zhang, L.-M., Duan, H.-X., Li, J., Dai, Y.-J., 2016. Optimization of extraction technology of the alkali-soluble components of barley malt roots and its composition analysis. In: Takahashi, M. (Ed.), Medicine and Biopharmaceutical (Proceedings of the 2015 International Conference). World Scientific Publishing, Singapur, pp. 1497–1505. https://doi.org/10.1142/9778.

Chiang, Y.-M., Lo, C.-P., Chen, Y.-P., Wang, S.-Y., Yang, N.-S., Kuo, Y.-H., Shyur, L.-F., 2005. Ethyl caffeate suppresses NF-kappaB activation and its downstream inflammatory mediators, iNOS, COX-2, and PGE2 in vitro or in mouse skin. Br. J. Pharmacol. 146, 352–363. https://doi.org/10.1038/sj.bjp.0706343.

de la Fuente, B., Pallarés, N., Barba, F.J., Berrada, H., 2021. An integrated approach for the valorization of sea bass (Dicentrarchus labrax) side streams: evaluation of contaminants and development of antioxidant protein extracts by pressurized liquid extraction. Foods 10, 546. https://doi.org/10.3390/foods10030546.

Deters, M., Knochenwefel, H., Lindhorst, D., Koal, T., Meyer, H.H., Hänsel, W., Resch, K., Kaever, V., 2008. Different curcuminoids inhibit T-lymphocyte proliferation independently of their radical scavenging activities. Pharm. Res. (N. Y.) 25, 1822–1827. https://doi.org/10.1007/s11095-008-9579-2.

Du, L., Arauzo, P.J., Meza Zavala, M.F., Cao, Z., Olszewski, M.P., Kruse, A., 2020. Towards the properties of different biomass-derived proteins via various extraction methods. Molecules 25, 488. https://doi.org/10.3390/molecules25030488.

Floridi, S., Montanari, L., Marconi, O., Fantozzi, P., 2003. Determination of free phenolic acids in wort and beer by coulometric array detection. J. Agric. Food Chem. 51, 1548–1554. https://doi.org/10.1021/jf0260040.

González-García, E., Marina, M.L., García, M.C., 2021. Impact of the use of pressurized liquids on the extraction and functionality of proteins and bioactives from brewer's spent grain. Food Chem. 359, 129874 https://doi.org/10.1016/j.foodchem.2021.129874.

González-García, E., Marina, M.L., García, M.C., 2014. Plum (Prunus Domestica L.) by-product as a new and cheap source of bioactive peptides: extraction method and peptides characterization. J. Funct.Foods 11, 428–437. https://doi.org/10.1016/j.jff.2014.10.020.

Guzmán-Lorite, M., Marina, M.L., García, M.C., 2022. Pressurized liquids vs. high intensity focused ultrasounds for the extraction of proteins from a pomegranate seed waste. Innovat. Food Sci. Emerg. Technol. 77, 102958 https://doi.org/10.1016/j.ifset.2022.102958.

Hernandez-Corroto, E., Marina, M.L., Garcia, M.C., 2018. Multiple protective effect of peptides released from Olea europaea and Prunus persica seeds against oxidative damage and cancer cell proliferation. Food Res. Int. 106, 458–467. https://doi.org/10.1016/j.foodres.2018.01.015.

Hernández-Corroto, E., Plaza, M., Marina, M.L., García, M.C., 2020. Sustainable extraction of proteins and bioactive substances from pomegranate peel (Punica granatum L.) using pressurized liquids and deep eutectic solvents. Innovat. Food Sci. Emerg. Technol. 60, 102314 https://doi.org/10.1016/j.ifset.2020.102314.

Ho, C.H.L., Cacace, J.E., Mazza, G., 2007. Extraction of lignans, proteins and carbohydrates from flaxseed meal with pressurized low polarity water. LWT–Food Sci. Technol. 40, 1637–1647. https://doi.org/10.1016/j.lwt.2006.12.003.

Hong, B., Barg, R., Ho, T.H., 1992. Developmental and organ-specific expression of an ABA- and stress-induced protein in barley. Plant Mol. Biol. 18, 663–674. https://doi.org/10.1007/BF00020009.

Jandera, P., Skeifíková, V., Rehová, L., Hájek, T., Baldriánová, L., Skopová, G., Kellner, V., Horna, A., 2005. RP-HPLC analysis of phenolic compounds and flavonoids in beverages and plant extracts using a CoulArray detector. J. Separ. Sci. 28, 1005–1022. https://doi.org/10.1002/jssc.200500003.

Lee, H.N., Kim, J.-K., Kim, J.H., Lee, S.-J., Ahn, E.-K., Oh, J.S., Seo, D.-W., 2014. A mechanistic study on the anti-cancer activity of ethyl caffeate in human ovarian cancer SKOV-3 cells. Chem. Biol. Interact. 219, 151–158. https://doi.org/10.1016/j.cbi.2014.05.017.

Ma, K.K., Greis, M., Lu, J., Nolden, A.A., McClements, D.J., Kinchla, A.J., 2022. Functional performance of plant proteins. Foods 11, 594–616. https://doi.org/10.3390/foods11040594.

Mahalingam, R., 2020. Analysis of the barley malt rootlet proteome. Int. J. Mol. Sci. 21, 179. https://doi.org/10.3390/ijms21010179.

Mahalingam, R., 2018. Temporal analyses of barley malting stages using shotgun proteomics. Proteomics 18, e1800025. https://doi.org/10.1002/pmic.201800025.

Martins, S.I.F.S., Jongen, W.M.F., van Boekel, M.A.J.S., 2000. A review of Maillard reaction in food and implications to kinetic modelling. Trends Food Sci. Technol. 11, 364–373. https://doi.org/10.1016/S0924-2244 (01)00022-X.

Olivares-Galván, S., Marina, M.L., García, M.C., 2020. Extraction and characterization of antioxidant peptides from fruit residues. Foods 9, 1018. https://doi.org/10.3390/foods9081018.

Qiao, Z., Chen, R., 1991. Isolation and identification of antibiotic constituents of propolis from Henan. Zhongguo Zhongyao Zazhi 16, 481–482.

Srinivasulu, C., Ramgopal, M., Ramanjaneyulu, G., Anuradha, C.M., Suresh Kumar, C., 2018. Syringic acid (SA) – A review of its occurrence, biosynthesis, pharmacological and industrial importance. Biomed. Pharmacother. 108, 547–557. https://doi.org/10.1016/j.biopha.2018.09.069.

Waters, D.M., Kingston, W., Jacob, F., Titze, J., Arendt, E.K., Zannini, E., 2013. Wheat bread biofortification with rootlets, a malting by-product. J. Sci. Food Agric. 93, 2372–2383. https://doi.org/10.1002/jsfa.6059.

Wiboonsirikul, J., Hata, S., Tsuno, T., Kimura, Y., Adachi, S., 2007. Production of functional substances from black rice bran by its treatment in subcritical water. LWT–Food Sci. Technol. 40, 1732–1740. https://doi.org/10.1016/j.lwt.2007.01.003.

Wiboonsirikul, J., Mori, M., Khuwijitjaru, P., Adachi, S., 2013. Properties of extract from okara by its subcritical water treatment. Int. J. Food Prop. 16, 974–982. https://doi.org/10.1080/10942912.2011.573119.

Wilcox, L.J., Borradaile, N.M., de Dreu, L.E., Huff, M.W., 2001. Secretion of hepatocyte apoB is inhibited by the flavonoids, naringenin and hesperetin, via reduced activity and expression of ACAT2 and MTP. J. Lipid Res. 42, 725–734.

Williams, L.K., Li, C., Withers, S.G., Brayer, G.D., 2012. Order and disorder: differential structural impacts of myricetin and ethyl caffeate on human amylase, an antidiabetic target. J. Med. Chem. 55, 10177–10186. https://doi.org/10.1021/jm301273u.

Zhou, J., Wang, M., Berrada, H., Zhu, Z., Grimi, N., Barba, F.J., 2022. Pulsed electric fields (PEF), pressurized liquid extraction (PLE) and combined PEF + PLE process evaluation: effects on Spirulina microstructure, biomolecules recovery and Triple TOF-LC-MS-MS polyphenol composition. Innovat. Food Sci. Emerg. Technol. 77, 102989 https://doi.org/10.1016/j.ifset.2022.102989.

Zhou, J., Wang, M., Carrillo, C., Zhu, Z., Brncic, M., Berrada, H., Barba, F.J., 2021. Impact of pressurized liquid extraction and pH on protein yield, changes in molecular size distribution and antioxidant compounds recovery from spirulina. Foods 10, 2153. https://doi.org/10.3390/foods10092153.