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 microorganisms (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 seaweedes (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-Vivas 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 draw 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-phenthaldehdyde (OPA), ferrous sulphate, l-mercaptoethanol, hydrogen peroxide (H2O2), trifluoroacetic acid (TFA), ammonium carbonate, isoacetamide (IAA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ammonium bicarbonate (NaHCO3), and enzymes (pancreatin, trypsin, and pepsin) were provided by Sigma-Aldrich (Saint Louis, MO, USA). Sodium carbonate (Na2CO3) and bicarbonate (NaHCO3) were obtained from Panreac (Barcelona, Spain). Ethanol (EtOH) was provided by Thermo Fisher Scientific (Waltham, MA, USA). Bradford reagent (Coomasie Blue G-250), Mini-Protein 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 (Murcia, Spain).

2.2. Ultrasound-assisted extraction

UAE was carried out using a high intensity focused ultrasound (HFPU) 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:

\[
y = k_0 + \sum_{i=1}^{4} k_i x_i + \sum_{i=1}^{4} k_{ij} x_i x_j + \sum_{i=1}^{4} k_{ij} x_i x_j
\]

where \(x_i\) and \(x_j\) are the independent variables, \(k_i\) represents the constant term, and \(k_i, k_{ij}, k_{ij}\) 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 (R2) 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 R2.

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, Waltham, 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 μg 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 (silicon dioxide), wrapped in tin foil, and introduced in the equipment.

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. Electro spray conditions were: skimmer voltage, 60 V; drying gas flow, 12 L/min; gas temperature, 350 °C; fragmentor 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%, 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. Peptides with 10 IgP 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., Warrenton, 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). Experiments 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:

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 A² - 1.04249 AB + 0.0936948 AC + 0.0222346 AD - 1.29725 B² + 0.0299098 BC + 0.0415736 BE - 0.00704851 C² - 0.00076499 CE

This mathematical model predicted the 95.3% of proteins content.
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...
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-Garcia 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:

\[
\text{Proteins content (g protein/100 g MR)} = 0.48093 + 0.0044625 A + 0.0024575 B + 0.026325 C - 0.00012615 A^2 + 0.00022346 AB - 0.000097337 BC - 0.00084625 C^2
\]

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-Garcia 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 (Alvarez-Vinas 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 mechanism.
of hydroxyl radical formation).

could be assigned to proteins in the PLE extract. Tables 1 and 2 show the identification of proteins in the UAE extract while only 37 peptides were identified in the extract obtained using PLE. Eight of these 9 proteins identified in the extract obtained using 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, peroxima, 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), sucrose synthase 2 (P06473), 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 (Q04066), V-type proton ATPase catalytic subunit A (Q04002), 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, only protein P34824 was exclusively extracted with PLE. Eight of these 9 proteins were common to the UAE extract while only protein P34824 was identified in the UAE extract. Multiple 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 identified in the UAE extract obtained using UAE, only 9 were identified in the extract obtained using PLE (Table 1).

Table 1

| Accession number | Gene name | Protein name | Mass (Da) | Peptides in protein sequence |
|------------------|-----------|--------------|-----------|----------------------------|
| 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 |

3.4. 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, peroxima, 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), sucrose synthase 2 (P06473), 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 (Q04066), V-type proton ATPase catalytic subunit A (Q04002), 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, only protein P34824 was exclusively extracted with PLE. Eight of these 9 proteins were common to both extracts (SLVR, TLLR, LLER, ELLR, SLLR, LYVR, WLFR, LSLR, FELLR, LSFR, FLAR, VVVVR, LLSR, VGF, and KFTR).

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 ± 0.6 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) and in the case of the UAE extract, only protein P34824 was identified in the UAE extract obtained using UAE, only 9 were identified in the extract obtained using PLE. Eight of these 9 proteins were common to both extracts (SLVR, TLLR, LLER, ELLR, SLLR, LYVR, HLVR, LVL, WLFR, FELLR, LSFR, FLAR, VVVVR, LLSR, VGF, and KFTR).

![Antioxidant capacity](image-url) (PLE extract was diluted 4-times to avoid signal saturation in the evaluation of the inhibition of hydroxyl radical formation).

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).
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           | Protein disulfide-isomerase | 56463 | 10 | 184.64 |
| P52572           | PER1-1-Cys peroxidixin | 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 |
| Q9ZR8S           | Tubulin alpha-3 chain | 49729 | 6 | 211.38 |
| Q96460           | Tubulin alpha-2 chain | 49701 | 6 | 211.38 |
| P34937           | Triosephosphate isomerase cytosolic isoenzyme | 26737 | 6 | 170.76 |
| Q9M5G3           | TCTP | 18884 | 5 | 143.5 |
| P14928           | HVA1 | 21820 | 4 | 156.89 |
| Q43473           | Tubulin alpha-1 chain | 49597 | 4 | 152.43 |
| P96471           | B3-hordein | 30195 | 4 | 142.75 |
| Q40078           | V-type proton ATPase subunit B 1 | 54027 | 3 | 155.13 |
| P62162           | CAM | 16832 | 3 | 134.11 |
| P29114           | LOX1-1 | 96393 | 7 | 191.29 |
| P06293           | PAZ1 | 43276 | 6 | 187.61 |
| Q9M5G3           | TCTP | 79533 | 5 | 165.78 |
| Q4LB21           | SAM4 | 43221 | 2 | 110.74 |
| P93176           | TUBB | 50194 | 3 | 157.29 |
| P33044           | Antifungal protein R | 4453 | 2 | 103.31 |
| Q43472           | bHLH01 | 15926 | 3 | 126.05 |
| P42210           | Pyrithrin | 54226 | 1 | 123.81 |
| Q49410           | RPS7 | 22078 | 4 | 132.2 |
| Q01548           | Peroxidase 2 (Fragment) | 18882 | 4 | 147.98 |
| P28814           | Barvin | 13737 | 1 | 94.24 |
| P12948           | DHN3 | 16162 | 2 | 71.33 |
| P55238           | Glucose-1-phosphate adenylyltransferase small subunit chloroplastic/amylolytic | 56049 | 1 | 64.98 |
| P32936           | IAT2 | 16526 | 2 | 77.32 |
| P12952           | Dehdyrin DHN2 | 14442 | 1 | 65.52 |
| Q40079           | V-type proton ATPase subunit B 2 | 53726 | 3 | 155.13 |
| A1E98           | atpA | 55295 | 4 | 114.95 |
| Q96562           | CHS2 | 43189 | 1 | 84.88 |
| P48560           | Oxalate oxidase | 21203 | 1 | 75.7 |
| P20026           | Myb-related protein | 29740 | 1 | 71.96 |
| P27968           | NAR-7 | 98630 | 1 | 59.67 |
| Q4LB22           | SAM3 | 42766 | 4 | 147.68 |

Table 2 (continued)

| Accession number | Gene name | Protein name | Mass (Da) | Peptides in protein sequence | –10 logP |
|------------------|-----------|--------------|-----------|-----------------------------|-----------|
| P12951           | DHN1 | Dehdyrin DHN1 | 14236 | 1 | 65.52 |
| Q9XH50           | RPS12 | 40S ribosomal protein S12 | 15295 | 2 | 70.56 |
| P32566           | 60S ribosomal protein L17-1 | 19504 | 4 | 133.8 |
| Q40024           | Betaine aldehyde dehydrogenase | 54290 | 3 | 114.5 |
| P6470            | B1-hordein | 33422 | 4 | 137.25 |
| Q4LRK3           | SAM2 | S-adenosylmethionine synthase 2 | 42828 | 4 | 133.05 |
| Q96378           | Glutamine synthetase | 39128 | 4 | 93.11 |
| Q40066           | PAZX | Serpin-ZX | 42947 | 1 | 102.88 |
| P82993           | Beta-amylose | 59639 | 2 | 93.81 |
| P16498           | Beta-amylose | 59647 | 2 | 93.81 |
| P50888           | RPL24 | 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-amylose 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 |
| P00963           | AMY1.1 | Alpha-amylose type A isozyme | 47796 | 1 | 49.3 |
| P12949           | DHN4 | Dehdyrin DHN4 | 22573 | 1 | 64.14 |
| P49969           | SRP54-1 | 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 | 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           | GACP | Glyceraldehyde-3-phosphate dehydrogenase 2 | 33236 | 8 | 215.81 |
| P0CG86           | MUB1 | 40S ribosomal protein S27a | 17655 | 6 | 179.32 |
| P0CG87           | MUB2 | Ubiquitin-40S ribosomal protein S27a | 17671 | 6 | 179.32 |
| P60353           | HI24 | Histone H2.4 | 9233 | 6 | 125.35 |
| P26517           | GACP | Glyceraldehyde-3-phosphate dehydrogenase 1 | 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 anti-oxidant 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           | –                              | Endoplasmic reticulum            | –                                                          |         |
| P90284           | Cytoplasm and nucleus          | Isomerase                        | Catalysis of disulfide bonds                               | UAE     |
| P2272            | Cytoplasm                      | Antioxidant                      | Cellular detoxification                                    | UAE     |
| Q43772           | Cytoplasm                      | Transferase                      | Glycolysis                                                 | UAE     |
| Q92RR5           | Cytoplasm (microtubule)        | Nucleotide binding; cytoskeleton constituent | Cytoskeleton organization | UAE/PLE |
| Q96460           | –                              | –                                | –                                                          |         |
| P93176           | –                              | –                                | –                                                          | UAE/PLE |
| P34937           | Cytoplasm                      | Isomerase                        | Glycolysis                                                 | UAE     |
| Q9M5G3           | –                              | –                                | –                                                          |         |
| P14928           | –                              | –                                | Environmental stress                                      | UAE/PLE |
| P94373           | Cytoplasm (microtubule)        | Nucleotide binding               | Glycolysis                                                 | UAE     |
| Q31922           | –                              | Transferase                      | Sucrose metabolism                                         | UAE     |
| P31923           | –                              | Nutrient reservoir               | –                                                          | UAE/PLE |
| Q40078           | –                              | –                                | ATP metabolism                                             | UAE     |
| P32162           | –                              | Ca²⁺ binding                     | –                                                          | UAE     |
| P29114           | –                              | Oxidoreductase                   | Lipid metabolism                                           | UAE     |
| P6293            | Extracellular region           | Nutrient reservoir               | Negative regulation of peptidase activity                  | UAE     |
| Q60572           | Vacuole membrane               | Metal binding                    | Defence response                                           | UAE     |
| Q4LB22           | Cytoplasm                      | Metal binding                    | Biogenesis of S-adenosylmethionine                         | UAE     |
| P31923           | –                              | –                                | –                                                          |         |
| Q10548           | –                              | Oxidoreductase activity and metal binding | Defeance response                              | UAE     |
| P28814           | –                              | Ribonuclease                     | Response to oxidative stress                               | UAE     |
| P12948²          | –                              | Carbohydrate binding             | Response to oxidative stress                               | UAE     |
| P12951           | –                              | –                                | –                                                          |         |
| P12952           | –                              | –                                | –                                                          |         |
| P12949           | –                              | –                                | –                                                          |         |
| P55238           | Chloroplast/amyloplast         | Transferase                      | Starch biosynthesis                                        | UAE     |
| P32936           | Extracellular region           | Peptidase inhibitor              | Negative regulation of peptidase activity                  | UAE     |
| Q40066           | –                              | –                                | –                                                          |         |
| P13681           | –                              | –                                | –                                                          |         |
| Q40079           | Part of a proton-transporting V-type ATPase complex in vacuolar membrane | Catalyzes of ATP hydrolysis | ATP metabolism                                             | UAE     |
| A1E918           | Part of a proton-transporting ATP synthase complex | Nucleotide binding              | ATP metabolism                                             | UAE     |
| P00828           | Chloroplast                    | –                                | –                                                          |         |
| Q96562           | Extracellular region           | Transferase                      | Flavonoid biosynthetic process                             | UAE     |
| P45850           | Extracellular region           | Metal binding                    | Membrane organization                                      | UAE     |
| P20026           | Nucleus                        | DNA binding                      | Removal of superoxide radicals                             | UAE     |
| P27968           | –                              | Nitrate reductase                | Nitrate assimilation                                       | UAE     |
| Q40024           | Perixoma                       | Oxido-reductase                  | Response to anoxia and osmotic stress                      | UAE     |
| Q60378           | Cytoplasm                      | Nucleotide binding               | Glutamine biosynthesis                                     | UAE     |
| P82993           | –                              | Hydrolase                        | Carbohydrate metabolism                                    | UAE     |
| P16998           | –                              | –                                | –                                                          |         |
| P50888           | Ribosome                       | –                                | –                                                          |         |
| Q92RR8           | Mitochondrion                  | Catalysis of oxidation of formate to carbon dioxide | Formate catabolic process | UAE     |
| Q00531           | –                              | Toxin; RNA glycosylase activity  | Negative regulation of translation                         | UAE     |
| P00693           | Extracellular region           | Metal binding                    | Defence respond                                            | 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)
Homovanillic acid (9), by the other hand, is a antimicrobial, anti-inflammatory, anticancer, and antidiabetic activities metabolite synthesized from dopamine, and it was also reported in beer (Jandera et al., 2005). Syringic acid (4) which has been associated to antioxidant, anti-inflammatory, antimicrobial, and anticancer activities (Bala et al., 2003). Furthermore, hesperetin (12) is a flavonoid with capacity of lowering cholesterol (Wilcox et al., 2001) that has also been 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., 2004) and sinapic acid has been studied for its 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 (Flordi 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.

### Table 3 (continued)

| Accession number | Cell location | Molecular function | Biological process | Extract |
|------------------|--------------|--------------------|--------------------|---------|
| P92392           | Chloroplast  | DNA binding        | Transcription      | UAE     |
| P26517           | Cytoplasm    | Transferase activity| Glucose metabolism | UAE/PLE |
| F34824           | Cytoplasm    | Oxidoreductase activity| Glucose metabolism | UAE/PLE |
| Q40034           | Cytoplasm    | Nucleotide binding | Translational elongation | UAE/PLE |
| P08477           | Cytoplasm    | Oxidoreductase activity| Glucose metabolism | UAE/PLE |
| P0CG86           | Ribosome, nucleus, and cytoplasm | Metal and nucleotide binding, structural constituent of ribosome | Translation | UAE |
| P0CG87           | 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 hydroxycinnameric acids: vanillic 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). 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., 2004) and sinapic acid has been studied for its 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 (Flordi 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 ultrasound 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

Saul Olivares Galván: Investigation, Validation, Formal analysis, Writing – original draft. Estefanía González-Garcia: 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.crf.s.2022.10.009.
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