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RESEARCH

Ultrasound and Microwave-assisted Extraction of Proteins from Coffee Green Beans: Effects of Process Variables on the Protein Integrity

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Abstract
The demand for proteins is constantly increasing and green extraction methodologies are needed to achieve environmental sustainability goals. The recovery of the by-products of the agri-food chain has also become a priority from a circular economy perspective. Some by-products are still little exploited for the extraction of proteins, such as coffee by-products. In this work, various innovative extraction technologies were applied to recover the protein fraction from the non-compliant coffee green beans (CGB), using a methodological approach that allowed to correlate the process parameters with the final quality of the extracted proteins. The ultrasound-assisted extraction (UAE) technique has been shown to have a minor impact on the quality of the proteins, thanks to the possibility of refrigerating the system, while the microwave-assisted extraction (MAE) shows a certain degree of degradation due to the high temperatures reached. The results indicate that strict temperature control is required during alkaline extraction to preserve the quality of the protein fraction.

Keywords Ultrasound · Microwave · Racemization · Temperature · Proteins

Introduction

The alkaline aqueous solutions have been widely applied to solubilize proteins from agri-food by-products since a better breakdown of cell wall components, fat saponification, and a higher protein solubility have been observed (Contreras et al., 2019; De Schouwer et al., 2019). Once the proteins have been solubilized in the extracting solution: sodium hydroxide or phosphate-buffered saline (PBS) are added to adjust the pH of the mixture to 3.0–3.5, which for this study was selected as the isoelectric precipitation point — where the net charge of the protein becomes zero — (Ciborowski & Silberring, 2016). Alkaline extraction exhibits a low extraction rate, and it can be enhanced by applying non-conventional techniques like power ultrasound or microwave (Wen et al., 2021).

Ultrasound-assisted extraction (UAE) is an innovative technique that leverages ultrasonic waves to enhance the extraction of different target compounds (e.g., bioactive compounds, proteins, lipids, and others) (Bhargava et al., 2021). The extraction exploits the diffusion of the target compounds in a liquid medium and has been used to extract molecules of interest from food and plant materials. High-intensity sonication (10–1000 W/cm^2) is used for extraction and processing applications. The main driving force for sonication extraction is acoustic cavitation (Tiwari, 2015). Cavitation produces transient bubbles, and their subsequent collapse generates microcracks, microchannel and pores on the surface of the matrix leading to better permeability (Peralta-Jiménez et al., 2013). Solvent selection plays an important role during a solid–liquid extraction such as the UAE. Its choice must ensure the delivery of a protein extract with high bioactivity, good nutritional properties, free of nucleic acids and with almost no odor and taste (Gong et al., 2016). The greater accessibility of the solvent to the internal structure of the cells facilitates the release of target compounds and their diffusion from the matrix to the solvent (Panda & Manickam, 2017). The main UAE advantages

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are the reduction of extraction processing time, quantity of energy and solvents used, unit operations and CO_2 emissions (Chemat et al., 2017). UAE has also drawn particular attention to protein extraction, as the demand for proteins is continuously increasing and green extraction methodologies are needed to meet environmental sustainability goals. UAE has been applied since years for protein extraction from different matrices (Moreno-Gonzalez et al., 2021): algae (Hildebrand et al., 2020), rice bran (Bedin et al., 2020), peanut (Ochoa-Rivas et al., 2017; Sun et al., 2020), and recently the valorization of agri-food byproducts has been covered by applying ultrasound in the extraction of protein from agri-food by-products such as: rapeseed (Wang et al., 2016), plum seeds (González-García et al., 2014), peach seeds (Vásquez-Villanueva et al., 2015), cauliflower leaves (Xu et al., 2017), olive stones (Vergara-Barberán et al., 2014), and pumpkin seeds (Liu et al., 2017). More recently, UAE has also been used to extract proteins with improved functionalities from *A. platensis* (Taragjini et al., 2022). Regarding coffee by-products, UAE has been studied in the obtention of phenols (Al-Dhabi et al., 2017; Andrade et al., 2012), biodiesel and ethanol (Rocha et al., 2014), proteins (Wen et al., 2021), from spent coffee grounds; fibers (Gallina Toschi et al., 2014) and caffeine (Guglielmetti et al., 2017) from coffee silver-skin. World coffee production reached 163.7 million 60 kg bags in 2019/2020, down from 172.5 million 60 kg bags in 2018/2019. Most of the world's coffee is produced by South America, especially Brazil (Statista, 2021). Roasters select coffee green beans to eliminate those that do not meet the required quality standards (non-compliant). Non-compliant coffee beans are beans that are discarded as black or brown, immature, damaged by insects or broken. They correspond to 15 to 20% of coffee production but are not used for beverages as they affect the quality of coffee preparation (Ramalakshmi et al., 2007). However, the protein content of the non-compliant CGB is considerable, around 15.3% on dry matter as previously reported (Prandi et al., 2021a). However, research in this regard is still quite limited, and non-compliant CGBs are certainly not fully exploited for the recovery of their protein fraction.

Another innovative extraction technique is microwave-assisted extraction (MAE), which induces cell wall destruction caused by forced overheating of trapped water molecules and continuous collisions within the matrix. This cellular breakdown causes sudden exudation of components within cells into the surrounding solvent (Varghese & Pare, 2019). MAE has been used to extract proteins from rice bran (Bedin et al., 2019), sesame bran (Gorguc et al., 2019), sunflower cake (Nathia-Neves & Alonso, 2021), peanut flour (Ochoa-Rivas et al., 2017) and others. UAE and MAE have been applied for the extraction of proteins from the silverskin of coffee (Wen et al., 2021) and could potentially also be exploited for the extraction of proteins from other coffee by-products. In fact, by-products and waste from coffee processing are a widely available, low-cost, and good quality resource for producing packaging and biofuels (Sisti et al., 2021), suitable for application in the production of biomaterials and encapsulation products for various industrial purposes (Ballesteros et al., 2014). However, they are still underused for protein recovery.

The extraction medium for protein recovery with UAE or MAE is usually aqueous or alkali (Vilkhu et al., 2008). Frequency, power, time interval, temperature, pH, ratio, and intensity of the ultrasound are essential factors for the efficiency of the UAE (Kumar et al., 2021). However, during ultrasonication proteins can undergo several types of modifications: physical, chemical, structural, functional, and nutritional (Almeida et al., 2022; Rahman & Lamsal, 2021). These changes can be attributed to a combination of several factors, such as acoustic energy, pH and temperature, among others. Analogously, the factors that may influence the performance of MAE are solvent nature, solvent-to-feed ratio, extraction time, microwave power, temperature, sample size and geometry, effect of stirring, among others (Chan et al., 2011). Therefore, it is important to optimize the process parameters to obtain the maximum extraction yield, while preserving the required protein properties (e.g., nutritional).

Exposure of food proteins to certain processing conditions induces racemization of L-amino acids into D-isomers, and this can compromise digestibility and nutritional quality (Friedmann, 1999). For example, green coffee has < 0.2% D-Glu, while 32–41% of D-Glu is found in roasted coffee (Palla et al., 1989). However, D-amino acid levels of 1–10% of the total amino acid content are not rarely found (Zagon et al., 1994). Peptide bonds with D-L, L-D or D-D configurations are resistant to proteolytic enzymes, thus inducing reduced digestibility and possibly the formation of oligopeptides with unknown biological activity (Marcone et al., 2020). In fact, it has been shown that the heat and alkaline treatment reduced prececal digestibility by up to 18% compared to native control proteins, changing in parallel with the D-amino acids content (de Vrese et al., 2000). A study conducted on various wheat products, oilseed cakes and lignin samples clearly showed that with increasing harshness of processing conditions, the amount of detectable amino acids decreases, while racemization rates increase (Horak et al., 2014). In particular, two main factors play a crucial role in determining the racemization of amino acids: the raising of the pH and the temperature of the process, as previously demonstrated for the alkaline deamidation of rice bran proteins (Guan et al., 2017). To our knowledge, a detailed study on the racemization of amino acids as a function of ultrasound and microwave protein extraction parameters has never been carried out. This study will provide important knowledge on the process parameters that most influence the racemization of amino acids and will provide useful information aimed at preserving the enantiomeric purity of amino

acids during protein extraction with UAE or MAE. The aim of this work was to assess the integrity of the protein extracts as a function of the extraction conditions, i.e., their degree of racemization and the presence of high molecular weight aggregates or low molecular weight degradation products.

Materials and Methods

Raw Material

Coffee green coffee beans (*Coffea arabica* L.) from different origins (Central and South America, Asia, and Eastern Africa) that did not meet the required quality standards and the selection criteria established by Illycaffè S.p.A. (Trieste, Italy) were obtained by subjecting coffee lots from Central and South America, Asia, and Eastern Africa to electronic sorting by means of color sorting as previously described (Full et al., 1999). Not-compliant coffee green beans (CGB) were sampled in September 2018 and stored frozen (−22 °C).

Sample Conditioning

To maximize the protein extraction yield, CGB were cleaned, milled, and defatted according to the protocol described by Prandi et al. (2021a).

Alkaline-acid Protein Extraction

Alkaline-acid extraction was carried out by mixing 25 g of non-compliant CGB with 300 mL of the selected solvent (either NaOH at different concentrations or phosphate buffered saline, PBS, as reported in Table 1). Then, microwave (2.4) or ultrasound assistance (2.5) was applied on the mixture. After application, mixture was centrifuged at 10,000 rpm for 10 min, and then filtered to separate the n-CGB slurry from the filtrate. Protein-rich filtrate was precipitated by adding 25% HCl adjusting the pH value to 4.0. Protein precipitate was obtained by centrifugation (10,000 rpm for 10 min) and filtration. The protein precipitate was dried in a convective oven at 40 °C overnight until reaching a moisture content below 5%. Dried protein extract was storage until its characterization (Fig. 1).

Microwave-Assisted Extraction (MAE)

In a laboratory beaker, non-compliant CGB and solvent were mixed, then it was placed inside a laboratory scale CMC 30D microwave (Candy Hoover Group, Italy). The nominal microwave output power was of 900 W; however, the IEC 60705 (2010) (A method for measuring the performance of household microwaves ovens) was used to quantify the energy (W) transmitted to the mixture.

$$P = \frac{4.187 \times m_w \times \left(T_2 - T_1\right) + m_c \times (T_2 - T_0)}{t}$$

where, P is the microwave power output (W), m_w is the mass of mixture (g), m_c is the mass of the beaker (g), T_0, T_1, T_2, are the ambient, initial, and final temperatures of the mixture (°C), respectively. The microwave power output was rounded to the nearest 50 W. After characterization of the microwave, three different levels of microwave capacity were selected: 100, 50, and 30%, corresponding to the output powers of 650, 300, and 100 W, respectively. To deliver a similar amount of energy to all samples, the extraction was extended for 120 s (100%), 240 s (50%),

Table 1 Experimental trials performed by UAE and MAE technologies on defatted n-CGB samples, starting from 25 g of raw material and 250 mL of solvent

Test	Technology	Extracting solution	pH	T_0 (°C)	T_f (°C)	Protein mass (g)	Protein yield (% w/w)	D-Asp (%)	D-Glu (%)
1	UAE*	NaOH 0.0001 M	10.07	16	31	1.20	4.8	4.3	4.1
2	UAE*	NaOH 0.001 M	11.12	20	31	1.25	5.0	5.0	3.3
3	UAE*	NaOH 0.1 M	12.85	19	33	1.82	7.3	5.7	6.3
4	UAE*	NaOH 0.2 M	12.93	17	34	1.85	7.4	7.5	3.8
5	UAE*	NaOH 0.3 M	13.01	21	33	1.90	7.6	7.7	5.1
6	MAE	NaOH 0.1 M	12.85	24	77	1.45	5.8	11.3	2.8
7	MAE	NaOH 0.2 M	12.93	24	77	1.68	6.7	17.3	2.3
8	MAE	NaOH 0.3 M	13.01	24	77	1.76	7.0	20.7	5.2
9	UAE	PBS	7.20	22	83	1.39	5.5	5.3	4.9
10	UAE*	PBS	7.20	17	32	0.68	2.7	9.4	5.2
11	MAE	PBS	7.20	21	70	1.00	4.0	5.1	4.8

*Temperature controlled by a chiller

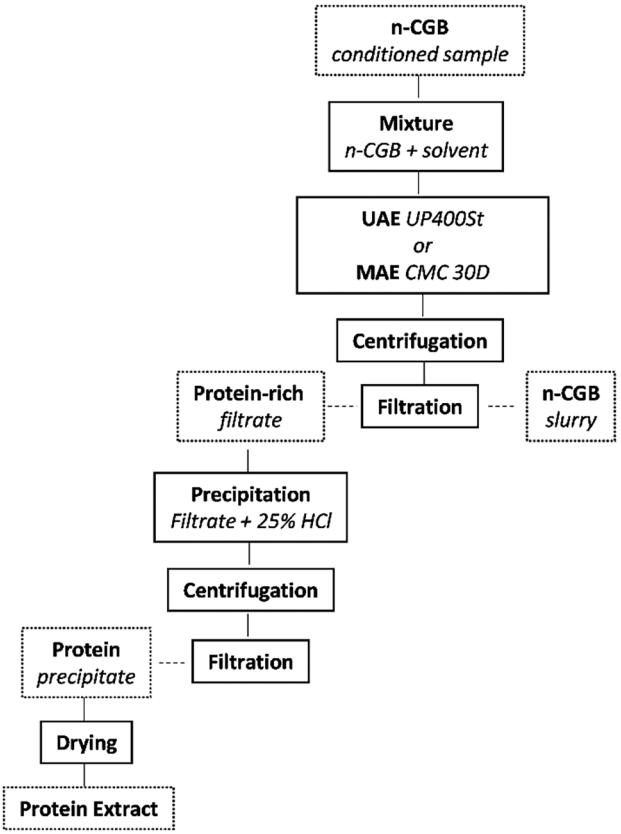

Fig. 1 Diagram of the alkaline-acid protein extraction assisted by ultrasounds (UAE) or microwave (MAE)

and 400 s (30%). For this study, the MAE was carried out at 50% capacity for 240 s. These conditions were selected after a preliminary study (results not shown).

Ultrasound-Assisted Extraction (UAE)

Ultrasound-assisted extraction was carried out at laboratory scale using an UP400St (400 W, 24 kHz) ultrasound device (Hielscher, Germany). A preliminary screening was carried out previously to evaluate the effect of the solid to extracting solution ratio (1:10–1:30) and UAE duration (5–15 min) on the extraction efficiency (results not shown). From this first screening, the solid to extracting solution ratio was set to 1:10 and the extraction length was extended for 12 min. The temperature of the mixture during the UAE was controlled using an external chiller at 6 °C. The parameters used can be seen in Table 1.

Protein Extracts Characterization

The obtained dried protein extracts were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and by the measurement of the degree of amino acid racemization (GC–MS). Both analyses were performed as previously described (Prandi et al., 2021b).

Protein extracts from non-compliant CGB (25 mg) were dissolved in 3 mL of 0.1 M HCl and 1 mL of acetonitrile. Protein content was determined with the Quant-iT Protein Assay Kit using the QBit Fluorometer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A volume equivalent to 30 µg of protein was dried under nitrogen flux. Polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed according to manufacturer's instruction (Bio-Rad, Hercules, CA, USA). The gels were stained by incubation with 0.1% w/v of Coomassie brilliant blue R-250 dissolved in 10% CH_3COOH, 40% CH_3OH, and 50% distilled water for at least 3 h. Any dye that was not bound to the proteins diffused by the gel during the de-staining steps (4 steps of 30 min each), when the gels were rinsed with a solution of 10% CH_3COOH, 40% CH_3OH, 50% distilled water to achieve the desired contrast. The images were acquired using the GS800 densitometer (Bio-Rad, Hercules, CA, USA).

Total amino acids were determined after acid hydrolysis as described in Prandi et al., 2021b. For the degree of racemization of the amino acids, 40 mL of solution coming from the standard acid hydrolysis of each protein extract is dried and the solid residue is dissolved in 2 mL of 2 M hydrochloric acid in 2-propanol. The resulting solutions are completely transferred into a 13 mm Pyrex glass tube fitted with Teflon-coated screw caps and heated at 90 °C for 1 h. The samples are then dried under nitrogen flow, dissolved in 1 mL of dichloromethane and 0.5 mL of trifluoroacetic anhydride and incubated for 30 min at 50 °C. The derivatized mixtures are dried under nitrogen flow and, only before the GC–MS analysis, dissolved in 1 mL of dichloromethane. GC–MS analyses are performed with an Agilent 7820A GC system coupled to an Agilent 5977B GC/MSD detector. The injector temperature is 250 °C, the transfer line temperature is 190 °C, and the quadrupole temperature is 150 °C. Samples (1.0 µL) are injected in split mode. The flow rate of helium, as a carrier gas, is set at 1.3 mL/min in constant flow mode. The separation of D/L amino acids is performed using a chiral capillary column (CHIR-L-VAL, 25 m × 0.25 mm ID, film thickness: 0.12 µm, Varian). The oven temperature program starts at 80 °C (held for 2 min), and increases at 2 °C/min up to 100 °C, then holds for 10 min; the temperature then increases by 5 °C/min up to 140 °C and is maintained for 1 min; the temperature increases by 10 °C/min up to 160 °C, and is maintained for 2 min; finally, the temperature increases by 10 °C/min or 180 °C, and is maintained for 3 min. MS conditions are as follows: ion source temperature: 230 °C; electronic impact: 70 eV; acquisition mode: full scan (m/z 40–400). The percentage of D-amino acids is calculated as follows: $A_D/(A_D + A_L) \times 100$, where A_D and A_L correspond, respectively, to the chromatographic area of the D or L enantiomer.

Results and Discussion

Microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) are becoming well-known techniques for recovering proteins and other valuable compounds from agri-food products and by-products. Their use, in combination with alkali extraction, has been shown to increase the protein extraction yield from partially defatted peanut flour by 77–100% (MAE-UAE), thanks to the increase in temperature (for MAE), or cavitation produced by ultrasonic waves (UAE) (Ochoa-Rivas et al., 2017). However, while data about techno functional properties are available, data regarding amino acid racemization are still missing. So, in this study, two different extraction methodologies were compared in terms of racemization: microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE). These techniques were applied to non-compliant CGB (a by-product of the coffee industry) to extract (and thus recover) their protein fraction. The composition of the initial feedstock has been previously analyzed (Prandi et al., 2021a) and it is reported in Table 2.

The aim of this work was to assess the integrity of the protein extracts as a function of the extraction conditions, i.e., their degree of racemization and the presence of high molecular weight aggregates or low molecular weight degradation products. These two parameters were chosen as indicators of the quality of the extracted proteins and the harshness of the extraction process. Indeed, it is well known that alkali treatment of proteins can cause extensive racemization of amino acids (Liardon & Hurrell, 1983). The protein profile was tested with gel electrophoresis under denaturing conditions as it is an immediate technique to observe the presence of high molecular weight aggregates or low molecular weight degradation products. For example, a smearing effect has already been observed in UAE-extracted proteins from edible bird nests, using water as an extraction solvent (Zukefli et al., 2017). The smearing effect can also be due to several reasons, such as the activity of proteases and oxidative enzymes, as well as other non-protein constituents. In particular, the main parameters influencing the degree of racemization, as already known from the literature (Friedman, 1999) are the temperature and the pH; therefore, these two parameters will be gradually modulated, and the integrity of the proteins will be accordingly evaluated.

Apart from the principle of the techniques, another important difference between UAE and MAE is the temperature reached during the protein extraction process. While in UAE there is the possibility to apply a refrigeration, fixing the upper temperature limit, in MAE the sample heating is unavoidable. As shown in Table 1, with UAE, the sample temperature at the end of the extraction process was in the range 31–34 °C; on the contrary, higher temperature is reached during MAE extraction, with 77 °C at the end of the process. One experiment was performed with UAE without thermosetting, with a final reached temperature of 83 °C.

The experiments shown in Table 1 could be summed up into five main segments: (i) effect of alkaline pH, with low extraction temperatures during UAE extraction (samples 1 to 5); (ii) effect of alkaline pH, with high extraction temperatures in MAE extraction (samples 6 to 8 and 11); (iii) effect of temperature at neutral pH during UAE extraction (samples 9 and 10); (iv) comparison of MAE and UAE at high temperatures and neutral pH (samples 9 and 11); (v) the effect of temperature at alkaline pH (samples 3–8).

Table 2 Composition of the non-compliant CGB used for the protein extractions, as reported in Prandi et al. (2021a)

Component analysis	n-CGB
Dry residue (%)	96.7 ± 0.0
Proteins (% g/100 g DW)	15.3 ± 0.1
Fibers (% g/100 g DW)	56.4 ± 0.1
Sugars (% g/100 g DW)	8.0 ± 0.8
Lipids (% g/100 g DW)	13.6 ± 0.2
D-Ala	2.0 ± 0.3
D-Asp	4.7 ± 0.4
D-Glu	1.9 ± 1.2
D-Phe	1.7 ± 0.3
D-Lys	2.3 ± 0.9
Total polyphenols (mg GA eq/g DW)	27.22 ± 1.03
Total flavonoids (mg CAT eq/g DW)	16.29 ± 0.65
Total flavanols (mg CAT eq/g DW)	5.26 ± 0.49
Tannins (mg/g DW)	2.46 ± 0.40
Caffeine (mg/g DW)	15.18 ± 0.49

i) *Effect of alkaline pH, with low extraction temperatures, in UAE extraction.* A significant positive correlation ($p < 0.05$) was found between the NaOH concentration (and, consequently, the pH) with the extraction yield (correlation coefficients 0.880 and 0.975, respectively). The high pH can, in fact, favor the extraction of proteins, thanks to an extensive degradation of the matrix. Similar results were also obtained by Roselló-Soto et al. (2015), who found a noticeable increase in protein recovery from olive kernels when the pH was raised. Hence, this observation made for the pretreatment with high voltage electrical discharges is also confirmed by our results using the ultrasound assisted extraction. However, there is also a significant positive correlation (Pearson's coefficient 0.961, $p < 0.01$) between the concentration of NaOH and the amount of D-Asp, confirming that an alkaline pH favors the racemization of the amino acid residues, such as Asp. Therefore, it is necessary to find a good compromise between the extraction yield (which is favored at high concentrations of NaOH), and the quality

of the extracted proteins, since high concentrations of NaOH lead to a high degree of racemization.

ii) *Effect of alkaline pH, with high extraction temperatures in MAE extraction*. Furthermore, in the case of the MAE extraction, as previously observed for the UAE extraction, there is a significant positive correlation between the NaOH concentration and the extraction yield for proteins (Spearman coefficient 1.000, $p < 0.01$). Therefore, as seen previously for UAE, also in the extraction with MAE the alkaline pH favors the extractability of the proteins, presumably favoring the disruption of the matrix and the release of the proteins in the extraction solution. At the same time, also in the MAE extraction, a significant positive correlation is observed between the concentration of NaOH, and the quantity of D-Asp detected in the protein samples (Spearman coefficient 1.000, $p < 0.01$). Therefore, regardless of the technique used (UAE or MAE), it is important to find an adequate concentration of NaOH that represents a compromise between a good extraction yield and acceptable racemization levels.

iii) *Effect of temperature at neutral pH during UAE extraction*. At neutral pH (kept constant using PBS at pH 7.2), the impact of the extraction temperature on the formation of D-amino acids during UAE extraction is negligible. During the UAE, the acoustic energy reaching the samples is transformed into heat and its effect could be mitigated by using a thermal chiller. However, the increment of the temperature during the extraction had benefits on the extraction yield. While test 9 reached a maximum temperature of 83 °C and the extraction yield was of 1.39 g, test 10, with thermal control, reached a lower temperature of 32 °C but exhibited a lower (50% less) extraction yield of only 0.69 g. Figure 2 shows the temperature profile observed in tests 9 (UAE with T control) and 10 (UAE without T control). The absence of temperature control causes an increase in temperature as a function of time; this increase is unavoidable, but it is much more contained in the presence of temperature control.

The temperature rise affected the viscosity of the solvent, helping the solvent to penetrate the plant matrix enhancing the extraction process. However, the drop of viscosity, turns into a dramatic loss of acoustic power, due to the sonotrode needing less power to oscillate at the desired amplitude. As shown in Fig. 3, the absence of temperature control leads to a decrease in the power delivered, as a function of time, while it remains constant with temperature control. In test 9, the loss of the acoustic power was 9%, while in test 10, the loss was 64%. To deliver the same amount of energy, this dramatic loss of acoustic power in test 10 meant an extension of the extraction time of 50% (Fig. 3).

Therefore, increasing the extraction yield by increasing the temperature would be a good alternative to using a strongly alkaline pH when extracting proteins with UAE.

iv) *Comparison between MAE and UAE at high temperatures and neutral pH*. At pH 7.2, there are no appreciable differences in the degree of racemization between UAE and MAE, with UAE giving slightly better extraction yields than MAE.

v) *Effect of temperature at alkaline pH*. From Fig. 4, it is possible to visualize the correlation between the concentration of NaOH and the amount of D-Asp detected in the proteins extracted using UAE or MAE technologies. As discussed above, the relation is significant ($p < 0.05$). Furthermore, the higher temperatures reached in MAE extraction cause more racemization of the Asp than with UAE. Therefore, while at neutral pH (point iii) the effect

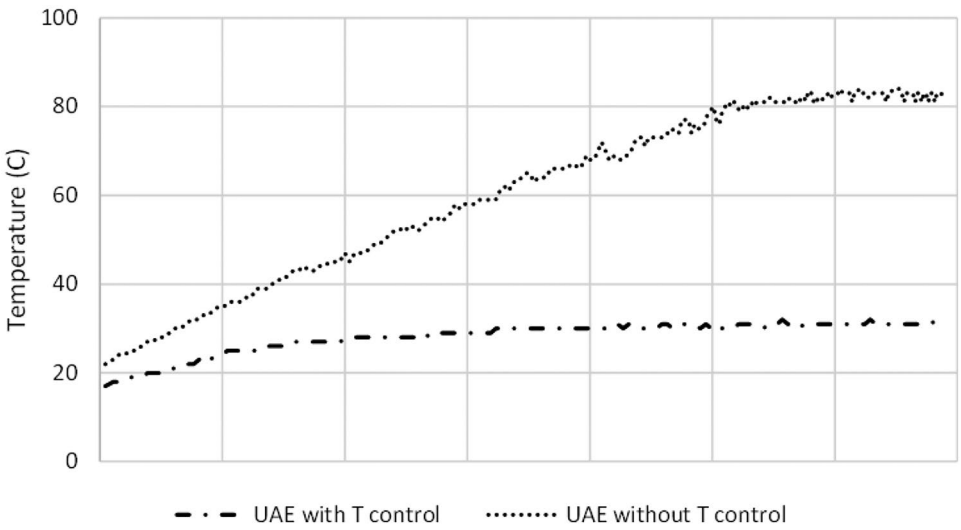

Fig. 2 Temperature profile during UAE

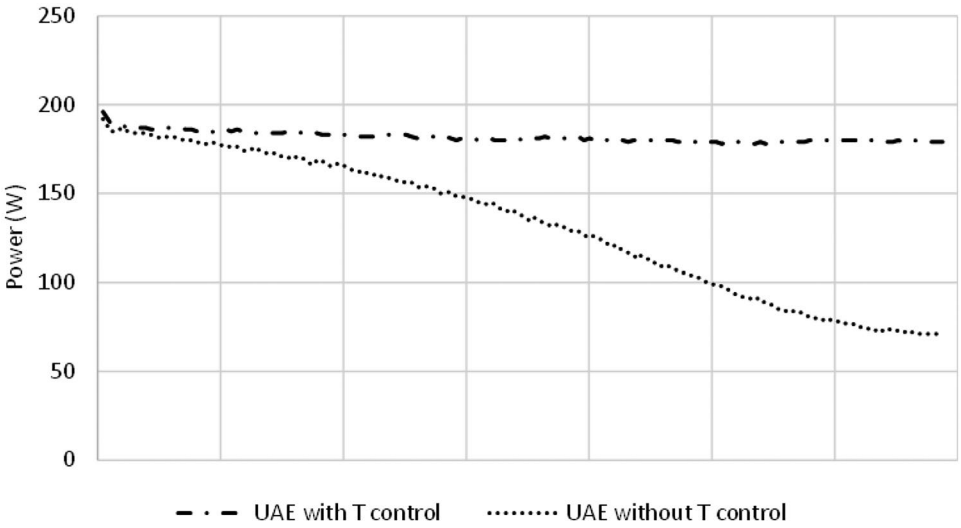

Fig. 3 Acoustic power profile during UAE

of temperature on racemization of amino acids is zero or limited, at alkaline pH an increase in temperature causes a visible increase in the degree of racemization.

Another indication about the quality of the extracted proteins is given by their electrophoretic profile on SDS-PAGE (Fig. 5), compared with that of the initial feedstock. The proteins extracted with UAE at low temperatures (samples 1–5) showed a good protein profile, fully consistent with the original one. In the case of MAE (samples 6–8) instead, the reach of high temperatures during extraction, combined with the alkaline pH, leads to a deterioration of the protein fraction, with an absence of clearly defined bands and very bad resolution, probably due to the presence of aggregates or protein degradation products. Combined with the high percentage of D-Asp found with the GC–MS analysis, we can conclude that the conditions used for these MAE extractions (high pH, high temperatures) lead to the extraction of a low-quality protein fraction. Also keeping the pH constant and at a neutral value, UAE gave proteins with a well-defined profile on SDS-PAGE, that indicates a good preservation of the protein fraction during extraction.

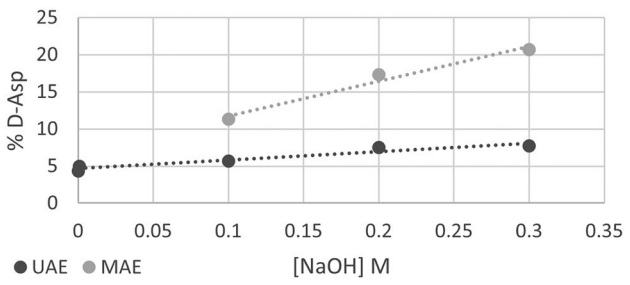

Fig. 4 Correlation between the concentration of NaOH and the amount of D-Asp detected in the extracted proteins

On the other hand, the profile of proteins extracted with MAE, even if at neutral pH, indicates a certain degradation during the extraction process, with low resolution of the protein bands.

The highest protein yields (1.85 and 1.90 g) were obtained using UAE with 0.2 M and 0.3 M NaOH as the extraction solvent (see Table 1). However, the degree of aspartic acid racemization was high (> 7%). Hence, the best compromise between the degree of racemization and the protein extraction yield is condition no 3, where 0.1 M NaOH was used as the extraction solvent for UAE and 1.82 g of protein was obtained. This condition was then chosen for the scale-up test.

Scale Up Trial

According to the information collected in the laboratory scale tests (Table 1), an additional experiment was

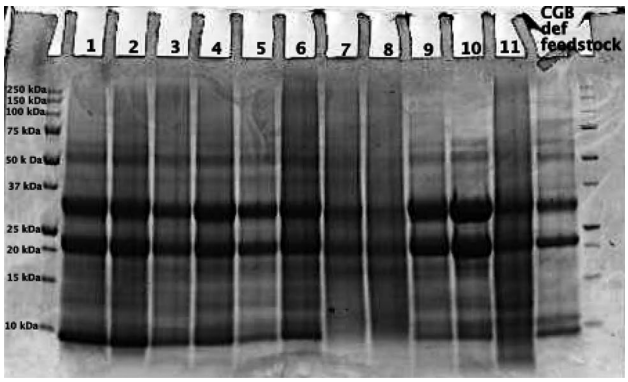

Fig. 5 SDS-PAGE of the proteins extracted from defatted coffee green beans, according to Table 1. "CGB def. feedstock" is the protein profile of the original starting material

Table 3 Results summary of UAE at higher scale

Test	Technology	Extraction time (min)	T_0 (°C)	T_f (°C)	Extract weight (g)	Protein content (%)
Scaled 1	UAE*	30	20	24	27.5	27.7
Scaled 2	UAE*	60	20	26	29.4	27.0

*Temperature controlled by a chiller

performed to validate not only the optimal extracting conditions but also to study the feasibility of a larger scale UAE. For each trial, 350 g of n-CGB was used, and the UAE extended for 30 and 60 min at the conditions chosen according to results shown in Table 1, entry 3. Table 3 summarized the main results of the extraction at higher scale.

Despite the duration of the extraction, the temperature was kept under 30 °C for both tests. The observed power loss was only 8.5 (30 min) and 10.1% (60 min). From the results in Table 3, it could be concluded that extending the UAE for 60 min promoted an increment of only 0.5% in the extraction yield compared to the 30 min UAE. These figures were 7.8 and 8.4% after 30 and 60 min of extraction, respectively. Both figures were in the range of the one observed at laboratory scale (7.3%). For both extracts, the protein content measured by using the Kjeldahl method (AOAC Method 976.05) was similar and rounded 27.3%.

These samples were also characterized for their amino acid composition, which is shown in Table 4. Comparing the results with the FAO/WHO amino acid score patterns, the amino acid score of the extracts is > 1 for all essential amino acids except sulfurates amino acids (cysteine and methionine), for which it is between 0.4 and 0.5, and which are therefore the limiting amino acids.

The presence of free amino acids was also determined to evaluate a possible protein hydrolysis during extraction (results reported in Table 5). The amount of free amino acids is negligible (0.24% and 0.29% w/w), indicating that the extraction process did not affect the integrity of the proteins and did not cause any hydrolytic processes. Consistently, the degree of hydrolysis (measured by the *o*-phthalaldehyde method) is low: 6.8% and 5.4% for extractions of 30 min and 60 min, respectively. This means that of the total amino groups, only 6.8% and 5.4% are present in free form, while all the others are engaged in the peptide bond.

To compare the results of the scale up trial with those of the laboratory-scale experiments, the degree of racemization was measured (Table 6). In the scale up of the process, a slight increase in the degree of racemization was observed; in particular, the quantity of D-Asp slightly increased to 9.3% and 8.4% for the extractions of 30 min and 60 min, respectively. This could be ascribed to a slower temperature control action due to the greater quantity of processed material and extraction solvent.

A high temperature, strong alkalinity extraction causes unwanted changes including denaturation, racemization and lysinoalanine formation that reduce protein functionality, nutritional value and safety by creating non-metabolizable forms of amino acids (Ngamsuk et al., 2020). In fact, some amino acids can undergo chemical alterations during processing such as decomposition, racemization, dehydration and glycation (Álvarez-Viñas et al., 2021). The results presented here indicate that strict temperature control ($T_f < 35$ °C) is required during alkaline extraction to preserve the quality of the protein fraction. Increasing the pH of the extraction, by increasing the concentration of NaOH, the extraction yield of the proteins is improved, but the degree of racemization also tends to increase. Therefore, it is necessary to identify a trade-off between a high extraction yield and the quality of the extracted proteins. The UAE technique has generally proved to have less impact on the quality of the proteins, thanks to the possibility of refrigerating the system, while MAE shows a certain degree of degradation due to the high temperatures reached.

Table 4 Total amino acid composition of the protein extracts obtained with the optimized UAE conditions. Results are expressed as % w/w

Test	Gly	Ala	Ser	Pro	Val	Thr	Ile	Leu	Asp	Lys	Glu	His	Phe	Arg	Tyr	Cys	Met
Scaled 1	1.2	1.2	1.3	1.2	1.5	1.0	1.1	2.4	2.5	1.3	4.7	0.5	1.6	1.6	0.8	0.1	0.2
Scaled 2	1.2	1.3	1.3	1.2	1.5	1.0	1.1	2.4	2.4	1.3	4.7	0.5	1.6	0.4	0.8	0.1	0.3

Table 5 Free amino acid composition of the protein extracts obtained with the optimized UAE conditions. Results are expressed as ‰ w/w

	Gly	Ala	Ser	Pro	Val	Thr	Ile	Leu	Asp	Lys	Glu	His	Phe	Arg	Tyr	Asn	Gln	Met	Trp
Scaled 1	0.03	0.16	0.17	0.12	0.06	0.03	0.03	0.03	0.31	0.02	0.96	0.03	0.07	0.03	0.03	0.24	0.03	0.01	0.06
Scaled 2	0.05	0.14	0.25	0.21	0.16	0.09	0.12	0.25	0.25	0.09	0.78	0.02	0.17	0.06	0.06	0.15	0.05	0.01	0.03

Table 6 Amino acids racemization degree expressed as % D/(D + L)

	Scaled 1	Scaled 2
Asp	9.3	8.4
Phe	1.1	2.5
Glu	1.9	1.7
Lys	n.d	n.d

Conclusions

The application of an optimized UAE protocol allows to obtain extracts with good protein content and protein integrity (no secondary hydrolysis reaction). Therefore, an optimization step is necessary every time a good integrity of the protein fraction is desired, in terms of protein profile and degree of racemization. The results here highlight the importance of performing a detailed chemical and molecular characterization to select the best valorization strategy and extraction technique for every type of agri-food residue also in view of their possible final market exploitation.

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Author Contribution Barbara Prandi: writing original draft, writing—review, editing; Martina Di Massimo: formal analysis, methodology, data curation; Tullia Tedeschi: conceptualization, methodology, review and editing; Laura Rodríguez-Turienzo; conceptualization, methodology, review and editing, resources; Óscar Rodríguez: conceptualization, formal analysis, investigation and data curation, writing—review, editing.

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Data Availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of Interest We know of no conflicts of interest associated with this publication. The manuscript has been read and approved for submission by all the named authors.

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