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Valorization of soursop (*Annona muricata*) seeds as alternative oil and protein source using novel de-oiling and protein extraction techniques

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ABSTRACT

In this research, for the first time, the valorization of soursop seeds, a poorly studied biomass, was investigated to simultaneously produce oil and proteins within a biorefinery approach. A new mild protein extraction method (Pressurized Water Extraction, PWE) was developed and optimized *via* response surface methodology and compared with conventional alkaline extraction. Furthermore, the impact of the de-oiling technique, *i.e.*, *n*-hexane or cold pressing, was investigated. The extracted proteins were evaluated in terms of physicochemical composition, amino acid content, and techno-functional properties. The type of de-oiling technique was found to have a minor impact. Instead, considerable differences were observed between the two protein extraction methodologies. At optimized conditions (pH 8.1 and 40 °C), PWE resulted in a more protein-rich extract (48 *vs.* 30 wt% for alkaline water), better preservation of the amino acid content, and higher solubility and *in vitro* digestibility. A mixed behavior was found for the techno-functional properties. Emulsification and oil holding capacity were similar for both techniques, while improved foaming was observed after alkaline water extraction. This research showed that the non-exploited soursop seeds could be a valuable source of oil and protein. Moreover, the novel PWE enables protein extraction with reduced impact on the protein structure.

1. Introduction

Meat production is responsible for 54% of the greenhouse gas (GHG) emissions produced by agricultural activities, with an expected increase to 59% by 2030. The environmental impact of meat production is even more significant when water consumption is included. The water footprint for the production of fruits (322 L kg^{-1}) and vegetables (962 L kg^{-1}) is significantly lower than that of chicken, pork, sheep, and beef (4325, 5988, 8763, and 15415 L kg^{-1}, respectively) (van der Zee, 2018). Therefore, it is crucial to identify new protein sources that are less environmentally demanding but still have adequate characteristics to satisfy consumers' nutritional and sensory demands.

Hence, science and industry joined efforts to investigate plants as novel protein sources to reduce the reliance on animal proteins. Although these plant-based proteins could represent a viable alternative in terms of environmental impact, they often are deficient in some essential amino acids (EAA) and have reduced digestibility compared to animal proteins. Blending proteins from different plant sources to balance the amino acid composition could tackle these nutritional deficiencies (Berrazaga et al., 2019). To achieve this, detailed knowledge of the protein content and composition of different vegetal-origin biomasses is essential. Moreover, novel protein fractions might have interesting techno-functional properties making them potential technological adjuvants in food preparations.

Scientific literature has demonstrated that legumes, algae, and some oilseeds are rich sources of proteins. The nutritional value and techno-

Abbreviations: PWE, Pressurized water extraction; GHG, Greenhouse gases; EAA, Essential amino acids; SDS PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DM, Dry matter; FID, Flame ionization detector; TCD, Thermo conductivity detector; DH, Degree of hydrolysis; OPA, *o*-phtaldialdehyde; NAC, N-acetyl-cysteine; OHC, Oil holding capacity; PLE, Pressurized liquid extraction; RSM, Response surface methodology.

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functional properties (*e.g.*, foaming, emulsifying, solubility) of these plant proteins position them as valuable for nutrition and as texture-enhancer in food products (Avelar et al., 2021). Moreover, some by-products from food production, such as seeds, have been identified as a potential protein source for animal or human consumption (Kumar et al., 2022). Seeds can represent up to 55% of the discards generated during the food production chain, bringing new possibilities to use this biomass for developing novel food products depending on their composition (Villacís-Chiriboga et al., 2020).

Annona muricata (soursop) is a tropical fruit that has caught attention owed to its taste and nutritional features. However, around 30% of the whole fruit consists of peels and seeds, discarded as non-edibles. Whereas the peels might be a suitable alternative fiber source, the protein content of the seeds (2.4–27.3%) (Solís-Fuentes et al., 2011) makes them attractive as a potential novel protein source. Combined with a high oil content (18.3–37.7%), these seeds have thus potential for application in food, feed, and pharmaceutical industries after isolating these compounds through a biorefinery process. Despite these positive aspects, soursop seeds have been scarcely studied, especially their protein and oil content.

Different techniques are applied to extract oil from seeds in a biorefinery frame. Given its simplicity, cold or screw pressing has been used for a long time. No skilled operator is needed in this technique, and no solvent or other hazardous contaminant is present in the oil. Nevertheless, between 8 and 14% of the oil remains in the pressed meal, resulting in poorer de-oiling performance. In contrast, organic solvents (usually non-polar, such as *n*-hexane) can extract more oil from the seeds, as only around <0.1% persists in the meal. Despite this advantage, the volatility and flammability of *n*-hexane pose safety issues. Moreover, the extracted oil needs purification to avoid the presence of hazardous traces of solvent (Bhuiya et al., 2020). The protein quality in the remaining seed meal can also be affected by the type of de-oiling technique applied. Studies have demonstrated that *n*-hexane results in protein denaturation (Lee et al., 2021), exposing in this way hydrophobic groups, which results in better techno-functional behavior (Kim et al., 2021), such as an increase in protein foaming (Gravel et al., 2021). Nevertheless, the exposure of hydrophobic groups can also result in aggregate formation, reducing protein solubility and, consequently, also protein digestibility (Tibbetts et al., 2016).

As for protein extraction, alkali or acids are usually applied to improve protein solubility, degrade the cellular structure, and ease their release in solution. However, using these chemicals leads to partial hydrolysis, degradation, aggregation, and denaturation of proteins, resulting in deficient functional properties (Accardo et al., 2022). Also, several compounds naturally present in the matrix (polyphenols, organic acids, terpenes) could be co-extracted, decreasing the product quality (Wang et al., 2003). With all these disadvantages, searching for mild protein extraction technologies with sufficient overall quality is imperative to guarantee a safe biomass ready for consumption obtained by an environmentally benign procedure.

Pressurized water extraction (PWE) has become an efficient flow-based alternative to traditional methods. In this technique, only water is used as solvent. The high pressure facilitates cell wall disruption, enhancing solvent penetration and facilitating the solubilization of the target compounds. If moderately high temperatures are used during extraction, the time can be decreased due to an improved mass transfer (Poojary et al., 2022). Despite its high potential, few authors have studied PWE for protein extraction. Burdějová et al. (2021) investigated protein extraction from *Viscum album* leaves, and Šalplachta and Hohnová (2017) from *Sambucus nigra* L. branches. However, these studies primarily focused on protein characterization via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and proteomic tools. No information on the nutritional and techno-functional properties of the PWE proteins is given, and no comparison is made with more traditional approaches.

Considering that soursop seeds are currently discarded during the industrial processing of the fruit and are expected to be valuable sources of oil and proteins, it is not only key to have detailed knowledge of their composition but also to develop a biorefinery that allows their efficient recovery. In this context, the comparison of conventional technologies with new emergent technologies that are potentially less chemically aggressive and able to deliver more intact ingredients is essential. This does include knowledge of the impact of the processing on the chemical characteristics and also on their techno-functional properties. With these premises, this study aimed (1) to evaluate the suitability of soursop seeds for protein extraction with co-valorization of oils in a biorefinery approach. (2) to develop a milder protein extraction method via PWE and compare its performance against a more conventional water extraction at alkaline pH, and (3) to assess the impact of the oil extraction technique and the protein extraction method applied on the extracted oil quality and the molecular and techno-functional properties of the extracted proteins.

2. Materials and methods

2.1. Materials and storage

The soursop seeds were kindly donated by Austrofood (Quito – Ecuador). After manually removing the non-seeds elements (*e.g.*, branches, peels, pulp), the seeds were dried at 75 °C for 72 h or until final moisture of 10%. Then, the particle size was reduced with an industrial Pin Mill (Alpine, 400t, Hosokawa – Alpine, Offenbach an der Queich, Germany) and sieved to exclude particles bigger than 500 μm. The flour obtained was stored under vacuum in aluminum bags at −18 °C.

2.2. Step 1 biorefinery: oil extraction

2.2.1. Hexane extraction

Two g of powder sample was placed inside a thimble and loaded into the Soxhlet system. As solvent, *n*-hexane was used at reflux conditions during 3 h. The solvent was removed in a rotavapor at reduced pressure and 35 °C. The oil was stored in amber glass vials at −18 °C.

2.2.2. Cold pressing

The powdered seeds (~25 g) were loaded into cotton fabric sacks and warmed for 2 h at 45 °C. Then, the samples were placed inside the stainless-steel cylinder of a hydraulic press Wickert & Söhne Maschinenbau (Landau in der Pfalz; Südliche Weinstraβe; Germany) and pressed at 980.66 [N]. The oil was stored in amber glass vials at −18 °C.

In both de-oiling processes, the oil recovery was calculated as follows [Eq. (1)].

$$\textbf{Recovered oil}\ [\%] = \frac{\text{Extracted oil [g]}}{\text{Dry matter weight of the initial biomass [g]}} * 100 \qquad [1]$$

Likewise, the oil yield was calculated as the extracted oil relative to the original oil content in the sample determined by the AOAC protocol (section 2.4.2.1 of this document).

2.3. Step 2 biorefinery: protein extraction

2.3.1. Pressurized water extraction

The extractions were performed in a Dionex Accelerated Solvent Extractor (Model 200). Four g of de-oiled dry seeds powder were loaded in a 33 mL capacity extractor vessel, and the empty space was filled with sea sand and celite. First, the cell with the sample was preheated to the desired temperature (40, 60, or 80 °C) for 5 min. Then, 60 mL of Milli Q water adjusted to the working pH (7, 9, or 11) were pumped at 15 MPa and heated for 5 min. Finally, a static period of 5 min was used before the collection of the extracts in 60 mL glass vials. Three cycles were performed in each cell. In view of the limited capacity of each cell in the lab scale equipment, several extractions were performed to produce

enough material for the subsequent experiments. The mixed supernatants were dialyzed against distilled water (cut-off = 12 KDa) for 48 h at 4 °C, freeze-dried, and stored at −18 °C in plastic vials. The channels were rinsed with water twice at the end of each extraction to avoid cross-contamination.

2.3.2. Conventional alkaline water extraction

Five g of sample were suspended in 100 mL of 0.15 M NaOH (1:20 *w*.v^{-1}) under stirring for 1 h (Sari et al., 2015). Then, the mixture was centrifuged (5000g for 20 min), and the pellet was resuspended in NaOH 0.15 M. Three extractions were performed. The mixed supernatants were dialyzed against distilled water (cut-off = 12 KDa) for 48 h at 4 °C and then freeze-dried.

In both extraction methods, the protein recovery was determined as the weight ratio between the extracted protein and the initial biomass (expressed in dry matter weight (DM)). The protein yield was determined by relating the weight of the extracted protein against the protein content of the initial biomass (AOAC method, see section 2.4.2.1). Finally, the mass yield represented the ratio between the extract and the initial biomass weight, expressed in DM content.

2.4. Chemical analysis

2.4.1. Oil-rich fraction

2.4.1.1. Fatty acid profile. The fatty acid profile was determined following the protocol of Satchithanandam et al. (2001). For the analysis, 0.05 g of oil were mixed with 1 mL of potassium hydroxide 0.5 M in MeOH and heated to 100 °C in a water bath for 10 min. After cooling down, 0.4 mL of 4:1 ($v.v^{-1}$) hydrochloric acid solution in methanol were added, and the sample was placed in a boiling water bath for 25 min. When the sample was at room temperature, 2 mL of double-distilled water and 3 mL of hexane were added, the mixture was stirred for 30 s in a vortex, allowed to stand, and the organic phase was recovered. This procedure was repeated three times in total, and the organic phases were combined. This final organic extract containing the fatty acid esters was dried with nitrogen at room temperature, re-dissolved with 2 mL of HPLC grade hexane, and placed in a 2 mL amber vial for analysis by gas chromatography (GC) coupled to a flame ionization detector (FID).

The separation was carried out on a SUPELCO SP TM 2560 capillary column of 100 mm × 0.25 mm × 0.2 μm, using a column oven temperature at 140 °C for 5 min, increasing the temperature at 4 °C/min up to 240 °C, using helium as carrier gas. The compounds were identified by comparing the retention times of the compounds against a SUPELCO FAME MIX (CRM 47885) standard from C_4 to C_{24}. Four replicates were measured in each sample. While for the quantification, the area of each sample and that of an external standard (C13:0-tritridecanoin) were compared. The results were expressed as g of each fatty acid per 100 g of oil.

2.4.2. Protein-rich fraction

2.4.2.1. Proximate analysis. The methodology described by AOAC (1995) was applied to determine moisture, ash, and oil. Moisture content was determined by drying two g of sample overnight at 105 °C or until constant weight. To quantify ash, two g of sample were burned at 550 °C for 24 h. One g of sample was de-oiled with *n*-hexane at boiling temperature for 3 h.

The protein content was determined based on the Dumas Combustion method. The sample was burned in an oxygen-rich environment at 950 °C, and the thermal conductivity by the presence of nitrogen was detected. All the burning gasses flowed through the reduction tube (helium as support gas) and were reduced to N_2, CO_2, H_2O, and SO_2. These different components were adsorbed at Selective Trap Columns and detected with a thermo conductivity detector (TCD) cell (Leni et al., 2020). A conversion factor of 5.5 was used to calculate the protein content based on the amino acid content as determined in this work and consistent with literature (Ezeagu et al., 2002). Total proximate carbohydrate content was calculated by difference. More detailed analyses of the proteins and residual saccharides are detailed below.

2.4.2.2. Protein purity. The purity of the extracted proteins was evaluated by the ratio between the amount of protein based on N content (see section 2.4.2.1) in the full extract and the dry matter (DM) content in the full extract [Eq. (2)]. For this, 2.5 g of liquid extract was dried overnight at 105 °C. Finally, the DM was related to the amount of protein.

$$\textbf{Purity}\ [\%] = \frac{\text{Total protein extracted [mg]}}{\text{Dry matter in the extract [mg]}} * 100 \quad [2]$$

2.4.2.3. Residual saccharides content and profile. To quantify the individual neutral saccharides, the samples were hydrolyzed following the Seaman hydrolysis procedure (Saeman, 1945). Briefly, 30 mg of sample in 1 mL of H_2SO_4 11M were shaken for 1 h at 37 °C. Then, the solution was diluted with Milli-Q water to H_2SO_4 1M and heated at 100 °C for 2 h under constant stirring. After cooling down, 1 mL of the hydrolyzed product was mixed with 0.5 mL of NaOH 400 mM and centrifuged. To determine acidic saccharides, one mL of a 5 mg mL^{-1} solution was incubated with 50 μL of Viscozyme®L (Sigma-Aldrich, Denmark) for 24 h at 45 °C, then the enzyme was inactivated by immersion in a water bath at 95 °C for 5 min (Babbar, Dejonghe, et al., 2016). The identification and quantification of individual sugars was performed following the protocol described by Babbar, Roy, et al. (2016). The final results were expressed as mg of sugar.g of sample^{-1} DM.

2.4.2.4. Degree of hydrolysis of the proteins. The degree of hydrolysis (DH) was calculated using *o*-phtaldialdehyde (OPA) (Butré et al., 2012; Spellman et al., 2003). To prepare the OPA/NAC (N-acetyl-cysteine) reagent, 10 mL of OPA diluted in methanol (50 mM), 5 mL of SDS (20%; $w.v^{-1}$), 10 mL of NAC (50 mM), and 75 mL of borate buffer (0.1 M, pH 9.5) were stirred together for 1 h protected from light with aluminum foil. Five μL of sample, or standard, were added to 215 μL of OPA/NAC reagent in a 96-well plate, shaken for 5 min, and the absorbance was recorded at 340 nm. Three control cells were used: 1) Standard: 5 μL H_2O + 215 μL of OPA. 2) Solvents: 5 μL of the main solvents (water at pH 7, 9, or 11) + 215 μL of OPA. 3) Samples: 5 μL of sample +215 μL of H_2O. L-isoleucine in concentrations ranging between 0 and 2 mg mL^{-1} constituted the standard curve. The DH was calculated as the ratio between the free nitrogen groups after hydrolysis and the total nitrogen groups [Eq. (3)]

$$\textbf{DH}\ [\%] = \frac{N_{free}}{N_{total}} * 100 \quad [3]$$

The first value was calculated by the OPA reactivity. The total moles of nitrogen atoms involved in peptide bonds before hydrolysis were calculated by the total g of proteins, obtained from total N content (paragraph 2.4.2.1), divided by the average of residual amino acids molecular mass (molecular weight 110).

2.4.2.5. Electrophoretic profile of the proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions as described by Gasparini et al. (2020). Briefly, ten mg of extracted proteins were solubilized in 1 mL of TrisHCl/SDS/DTT buffer and stirred for 10 min at room temperature. Then, thirty μg of protein were loaded into each well, and the reducing agent (20 ×) and diluted sample buffer (4 ×) were added to each sample, followed by an incubation period (5 min/95 °C). Each sample was loaded in 10% precast gel. A constant voltage of 150 V using a Criterion electrophoretic chamber was applied for the electrophoretic run. The protein bands were stained on the gel with Coomassie Blue. The approximate molecular weight of the bands in the samples was compared against a

molecular marker standard.

2.4.2.6. Identification of the proteins. The protein bands were excised from the gel and subjected to tryptic digestion in gel, according to Prandi et al. (2020). Briefly, the excised bands were cut into small pieces (about 1 mm) and washed several times to remove the Coomassie Blue. Then, protein reduction and alkylation were performed, and after several washes, the proteins were gel digested by incubation overnight at 37 °C with bovine trypsin (1749 BAEE units/mg solid).

The peptides were analyzed by micro-High Pressure Liquid Chromatography coupled to Linear Trap Quadrupole-OrbiTRAP detector (μHPLC-LTQ-OrbiTRAP) (Thermo Scientific, Waltham, MA, USA) according to Prandi et al. (2020). Identification was made using Peaks Studio software (Bioinformatics Solutions Inc., Waterloo, ON, Canada) and using *Annonaceae*, green plants, or the global database as a database.

2.4.2.7. Amino acid composition of the proteins. The total amino acids content, except for Trp, was determined, after acid hydrolysis and amino acid derivatization, by Reversed Phase Liquid Chromatography coupled to ElectroSpray Ionization Mass Spectrometry detection (RP-LC/ESI-MS), according to a previously published procedure by Prandi et al. (2019). For the determination of Trp, a different procedure making use of alkaline hydrolysis was performed, followed by RP-LC/ESI-MS analysis of free Trp, according to Prandi et al. (2019).

2.4.2.8. Amino acid racemization of the proteins. The enantiomeric purity of the amino acids was performed following a previously described procedure involving amino acid derivatization and analysis by chiral Gas Chromatography coupled to Mass Spectrometry detection (GC/MS), determining the percentage D/(D+L) % for Ala, Asp, Phe, Glu, and Lys, in view of their higher sensitivity to racemization (Accardo et al., 2022).

2.4.3. Nutritional assessment

2.4.3.1. In vitro digestibility. The *in vitro* digestibility was performed following the INFOGEST consensus protocol described by Brodkorb et al. (2019). The solutions to simulate the oral, gastric, and intestinal phases were prepared according to the protocol. The solutions were preheated at 37 °C, and the protein/digestive fluid reactions were performed in a water bath (Julabo SW22, Seelbach, Germany) at 37 °C with agitation of 40 rpm. The initial protein suspension (20 mg per mL of water) was incubated for 2 min with 0.5 mL of simulated saliva solution. Then, 1 mL of simulated gastric solution was added, and the pH was adjusted to 3 with HCl 6M. The suspension was incubated for 2 h. After this time, 2 mL of simulated intestinal solution was added. Subsequently, the pH was adjusted to 7 with NaOH 1M and incubated for 2 h. Finally, 40 μL of AEBSF (4-(2-Aminoethyl) benzene sulfonyl fluoride) hydrochloride (20 mg mL^{-1}) were added to stop the enzymatic activity.

The tubes were weighed and centrifuged at 5000 g for 5 min at room temperature. The N content and the DH were determined in the supernatant with the abovementioned methodologies.

2.4.3.2. Techno-functional properties of the protein fraction

2.4.3.2.1. Solubility. The solubilized protein (1% $w.w^{-1}$) was individually adjusted to pH 3, 5, or 7 with NaOH or HCl. The solution was shaken for 30 min. After this, the pH was re-adjusted if necessary and shaken for 30 min. Then, the solution was centrifuged at 5910g for 20 min at 4 °C, and the N content on the supernatant was determined as described above. The solubility was calculated as follows [Eq. (7)] (Leni et al., 2020):

$$\textbf{Solubility}\ [\%] = \frac{\text{N content in the supernatant}}{\text{N content in the pure protein extract}} * 100 \quad [7]$$

2.4.3.2.2. Emulsifying properties. A solution of 0.1% protein ($w.w^{-1}$) was mixed for 30 min. The supernatant was separated by centrifugation (3220 g/15 min/4 °C) and mixed with commercial corn oil in a ratio of 1:1 ($v.v^{-1}$). The mixture was emulsified with an ultra-turrax (11000 rpm/30 s). The emulsification was calculated as follows [Eq. (8)] (Leni et al., 2020):

$$\textbf{Emulsification}\ [\%] = \frac{\text{Height of the emulsified layer}}{\text{Total height of the solution}} * 100 \quad [8]$$

2.4.3.2.3. Foam capacity and foam stability. Protein was dissolved in demineralized water (1% $w.w^{-1}$), shaken for 30 min, and whipped with an ultra-turrax (11000 rpm/30s). The foaming capacity was calculated as expressed in [Eq. (9)], while foam stability was determined 30 min later and determined with [Eq. (10)] (Ding et al., 2020):

$$\textbf{Foaming capacity}\ [\%] = \frac{\text{Volume after whipping} - \text{Volume before whipping}}{\text{Volume after whipping}} * 100 \quad [9]$$

$$\textbf{Foaming stability}\ [\%] = \frac{\text{Volume after 30 min}}{\text{Volume after whipping} - \text{Volume before whipping}} * 100 \quad [10]$$

2.4.3.2.4. Oil holding capacity. One g of protein was mixed with 15 g of commercial corn oil, vortexed for 1 min, and allowed to stand at room temperature for 30 min. Then, the mixture was centrifuged for 30 min at 20 °C/3000 g. The supernatant oil was separated, and the remnant was weighed. The oil holding capacity (OHC) was calculated as follows [Eq. (11)] (Leni et al., 2020):

$$\textbf{OHC}\ \left[\frac{\textbf{g oil}}{\textbf{g protein}}\right] = \frac{\text{Sample after centrifugation [g]} - \text{Sample before centrifugation [g]}}{\text{Sample after centrifugation [g]}} \quad [11]$$

2.5. Statistical analysis

The optimization of the PLE extraction was performed by a design-of-experiments, using response surface methodology (RSM), whereby each extraction was performed in duplicate. The final model [Eq. (12)], was constructed with the outcomes of extraction yield, purity, ratio EAA/total protein, and degree of hydrolysis.

$$Y = \beta_o + \sum_{i=1}^{2} \beta_i A_i + \sum_{i=1}^{2} \beta_{ii} A_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{2} \beta_{ij} A_i A_j \quad [12]$$

Were Y represents the dependent variable to be modelled; A_i and A_j, the working parameters (temperature and pH), β_0 the intercept; β_i is the coefficient of linear effect; β_{ii} is the coefficient of quadratic effect, β_{ij} is the coefficients of interaction effect, and 2 is the number of variables.

All the outcomes obtained were included in a multiple-response optimization to determine the best extraction conditions. However, several boundary conditions were set. Thus, a minimal requirement of a protein recovery higher than 50 mg g^{-1} DM and a purity of at least 50% was specified. Then, the best conditions that gave an optimal overall result were chosen, including maximal protein content and purity (according to the described boundaries), maximal tryptophan content,

maximal EAA/total protein ratio, and minimal degree of hydrolysis. No discrimination of variables was set. Hence, the same weight (1.0) and impact (3.0) were assigned to all the responses.

The individual determinations (elemental characterization, DH, *in vitro* digestibility, nutritional and techno-functional assessment) on the nitrogen fraction extracted at the optimized conditions were performed in triplicate. Amino acid composition and racemization were determined in duplicate. STATGRAPHICS Centurion XVI was used for all statistical analysis, subjected to analysis of variance (ANOVA) with a 95% confidence level.

3. Results and discussion

3.1. De-oiling procedures and composition of the extracted oils

Table 1 displays the results on yield and recovery and fatty acid characterization of the extracted oils. The use of *n*-hexane extracted a slightly higher ($p < 0.05$) amount of oil than cold pressing (19 wt% DM vs. 17 wt% DM, respectively). In both cases, the oil was constituted mainly by unsaturated fatty acids (between 69 and 76 wt% DM for cold pressing and *n*-hexane extraction, respectively), mostly oleic acid (47 wt % DM in *n*-hexane extraction, 43 wt% DM in cold pressing), followed by linoleic acid (28 and 25 wt% DM, respectively). The rest of the unsaturated fatty acids were quantified in concentrations of ~1 wt% DM. Saturated fatty acids constituted around 20 wt% DM of the oil fraction. Palmitic acid (14 wt% and 16 wt% DM in cold pressing and *n*-hexane extraction, respectively) and stearic acid (4.77 wt% and 5.22 wt% DM in cold pressing and *n*-hexane extraction, respectively) were the main compounds. Other saturated fatty acids were quantified in concentrations <1 wt% DM. The fatty acid composition of soursop is in good agreement with other species of the genus *Annona* (Mariod et al., 2017).

3.2. Protein extraction: optimization of the PWE parameters

As PWE is a flow-based extraction method in which the solvent flows continuously through the biomass, the optimal solvent/solid ratio was determined by evaluating the protein extraction in function of time, *i.e.,* cycles. It was noticed that the protein concentration in the extract decreased steadily with each cycle, going from 50 mg of protein per g of biomass DM in the first to only 2 mg of protein per g of biomass DM in the third cycle (Fig. 1). Therefore, three extraction cycles were set fixed for all subsequent experiments. The next step concerned the evaluation of pH (7, 9, and 11) and temperature (40, 60, and 80 °C) as the major working variables in the PWE extraction. The pressure (15 MPa), heating time (5 min), static period (5 min), and extraction time (5 min) were kept fixed. It was chosen to keep pressure high to have a maximal possible impact on cell disruption and temperatures below 100 °C to avoid hydrolysis. The extraction periods were also kept constant, as in the study of Šalplachta and Hohnová (2017), minimal effect was observed on the extraction of proteins from *Sambucus nigra* L. branches through PWE.

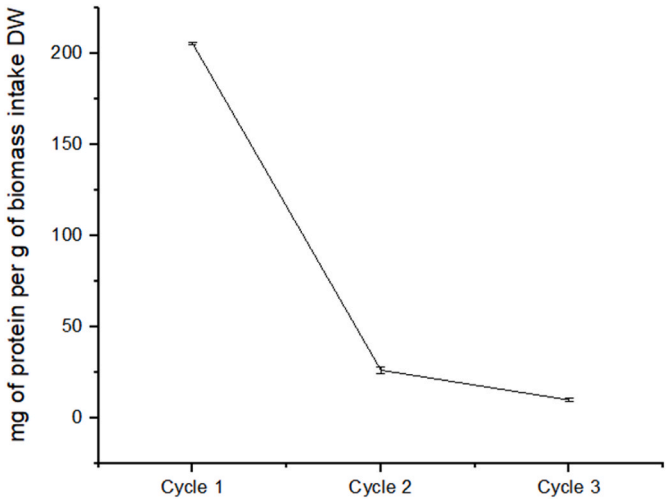

Fig. 1. Extraction kinetics of protein from soursop seeds via PWE.

The protein recovery decreased with an increase in the temperature (on average, 70 wt% less protein was extracted at 80 °C compared to 40 °C). Regarding pH, a slight increase (between 8 and 12 wt%) was evidenced when increasing the pH from 7 to 11 (see Supplementary Material SS.1).

The decrease in recovery at higher temperatures can be due to protein denaturation, aggregation, and precipitation. On the other side, the recovery increase with pH is likely due to the unfolding of the protein's tertiary structure, which enhances its flexibility due to the loss of side-chain interactions with proteins and non-protein components (Jiang et al., 2009; Kristinsson & Hultin, 2003).

The extracted protein's purity ranged between 38 and 63%, with decreasing purity at higher temperatures (see Supplementary Material SS.2). At 40 °C, the purity was between 10 and 30% higher than that obtained at 80 °C, while no significant differences were evidenced in purity as a function of pH variations. The decrease in protein purity with increasing temperature could be due to the co-extraction of other molecules from the biomass. Increasing temperatures are, for instance, known to facilitate the extraction of more saccharides from plant-materials in hot water (Yang et al., 2022). Hence, the lower purity could be due to the presence of saccharides in the extract.

As for DH, at pH 7, it ranged between 0.68 and 1.27% (indicating perfect protein integrity). At pH 11, a slightly higher DH was observed (between 4.61 and 6.62%), still a low degree of protein hydrolysis, mainly as a consequence of the pH and not of temperature (correlations of 0.93 and −0.06, respectively) (Supplementary Material SS.3).

The RSM generated for each response, and the Pareto charts are displayed in Supplementary Material SS.4. Temperature plays a significant, although negative, role in the extraction yield and purity

Table 1
Effect of Soxhlet and cold pressing extraction on the oil yield and fatty acid profile of soursop seed oil obtained in step 1.

Fatty acid composition of extract [g of FA per 100 g of oil]			
		Hex	CP
Saturated	**Palmitic acid C16:0**	14.08 ± 0.72^{b}	15.99 ± 0.76^{a}
	Heptadecanoic acid C17:0	0.07 ± 0.01^{a}	0.08 ± 0.02^{a}
	Stearic acid C18:0	5.22 ± 0.31^{a}	4.77 ± 0.32^{a}
	Arachidic acid C20:0	0.85 ± 0.02^{a}	0.88 ± 0.08^{a}
	Heneicosanoic acid C21:0	0.82 ± 0.08^{a}	0.81 ± 0.09^{a}
	Behenic acid C22:0	0.17 ± 0.01^{a}	0.17 ± 0.02^{a}
	Lignoceric acid C24:0	0.13 ± 0.02^{a}	0.14 ± 0.02^{a}
Unsaturated	**Palmitoleic acid C16:1**	1.00 ± 0.14^{a}	1.025 ± 0.09^{a}
	Oleic acid C18:1n9c	47.07 ± 1.37^{b}	42.68 ± 0.60^{a}
	Linoleic acid C18:2n6c	28.02 ± 1.14^{b}	24.67 ± 0.62^{a}
	Cis-11-eicosenoic acid C20:1	0.19 ± 0.02^{a}	0.21 ± 0.02^{a}
Extraction yield of step 1 [g extract per g initial, corrected for dry matter]			
		Hex	**CP**
	Mass yield	19.36 ± 0.37^{b}	17.32 ± 0.36^{a}
	Oil yield	62.96 ± 1.47^{b}	56.33 ± 1.42^{a}

The statistical analysis was carried out using one way ANOVA.
CP: Cold Pressing; Hex: *n*-hexane extraction.
All the values are expressed as mean ($n = 3$) ± SD.
Different letter in the same row indicates statistically significant differences in the amount of oil in the seeds or the concentration of the fatty acid in the oil as function of the extraction technique (95%).

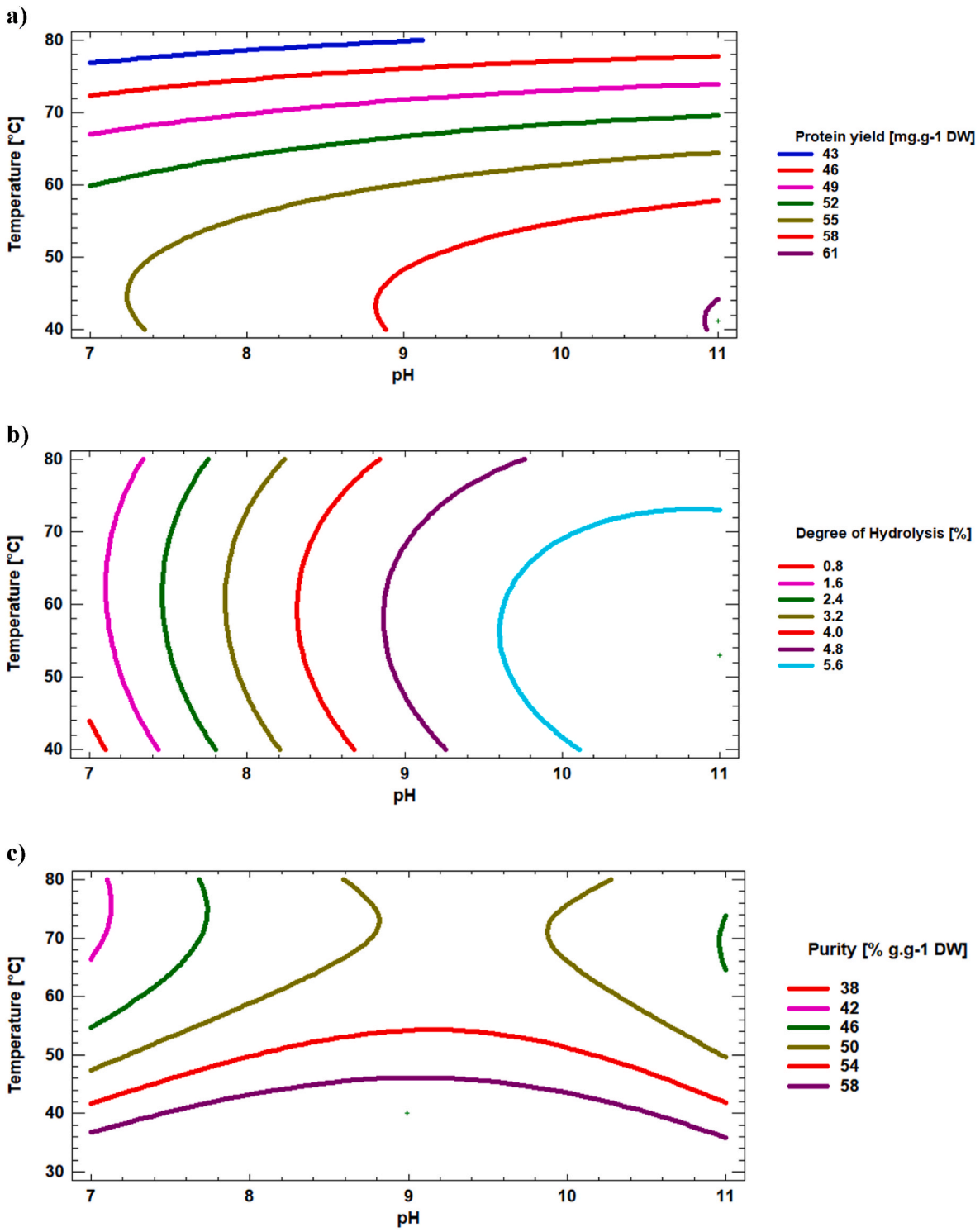

Fig. 2. Contours of Estimated Response Surface for protein yield [mg per g DM] (2a); degree of hydrolysis (2b) and protein purity (2c).

(−10.193 and −3.581, respectively). For the same responses, pH exerted a positive effect (3.443 and 0.937, respectively), although the impact was not significant in the last case. Finally, the degree of hydrolysis was severely influenced by the linear and quadratic effects of the pH (12.912 and −3.74, respectively).

The best overall conditions were determined through multi-response optimization (Fig. 2). As mentioned above, all the responses for protein recovery, purity, DH, tryptophan, and EAA/protein ratio were included. As boundary conditions, a minimal value for protein recovery of 50 mg per g DM and at least 50% purity were taken. With this condition, most of the responses (protein content in the extract, purity, tryptophan, and EAA/protein ratio) were maximized, except for DH, which was minimized. At 40 °C and pH of 8.1, the best compromise between the described variables was obtained. Similar conditions were determined by Burdějová et al. (2021) for the extraction of bioactive proteins from *Viscum album* leaves.

To validate the model, the extraction was performed at the optimal conditions. A good agreement was found between the predicted protein

recovery (56.80 mg g^{-1} DM) and the real protein recovery (between 56.63 and 58.03 mg g^{-1} DM). The optimal conditions to extract soursop seeds protein via PWE were applied in both types of de-oiled seeds.

The molecular, nutritional, and techno-functional properties of these proteins were thoroughly analyzed and compared with the reference alkaline protein extraction method.

The results are described in the coming sections.

3.3. Chemical characterization

3.3.1. Proximate concentration

The overall extraction mass yield was higher in alkaline water extracts than in PWE (~10.5 wt% and ~4 wt%, respectively) (Table 2). The protein content on the extracts ranged between 20 and 49 wt% DM, with significantly higher content in the extracts obtained via PWE, indicating that conventional alkaline solution extracted more proteins and more non-proteic compounds. Carbohydrates were the second most concentrated element, ranging from 40 to 69 wt% DM. The lipid content varied between 2 and 3 wt% DM, with similar content in all the cases. Ash content ranged between 1.19 and 1.75 wt%, and the moisture represented around 5.71 wt% DM of the freeze-dried protein extracts.

Higher protein purity was obtained with PWE (61–65% DM), while NaOH extracts presented a lower purity (52% DM). Basic environment increases the extraction yield at the expense of protein purity and nutritional characteristics. As mentioned, carbohydrates were among the major constituents of the extracts, mainly in base-extracted proteins, as they are easily extracted under basic conditions from oilseeds (Gerliani et al., 2019).

The carbohydrate characterization (data not shown) showed that glucose was the main saccharide. In line with previous results, the protein-rich fraction obtained via alkaline water from the cold-pressed defatted meal had an overall higher glucose content (~52 wt% DM), followed by the *n*-hexane defatted meal under the same protein extraction procedure (37 wt% DM). As for the extracts obtained after PWE, the glucose content was lower (~20 wt% DM) and independent of the de-oiling technique applied. Galactose was the second most recovered saccharide with a similar concentration in all the extracts (~10 wt % DM). PWE co-extracted xylose to a high extent independently of the de-oiling technique (~6 wt% DM). In comparison, for the alkaline water extraction, the xylose content was much lower (1.8 for hexane and 0.8 wt% DM for cold-pressing).

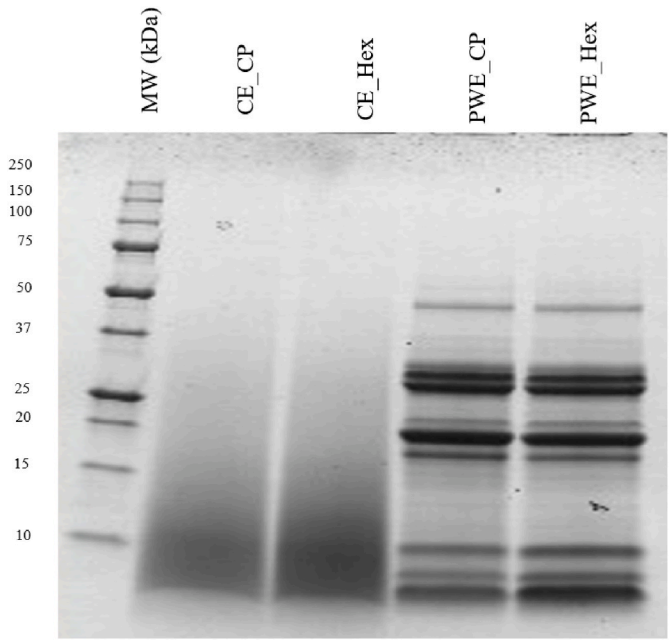

Fig. 3. SDS PAGE of the protein rich fraction. MW: molecular weight; CE_CP: alkaline water extraction + cold pressing; CE_Hex: alkaline water extraction + hexane; PWE_CP: pressurized water extraction + cold pressing; PWE_Hex: pressurized water extraction + hexane.

3.3.2. Degree of hydrolysis

A lower DH was evidenced in the extracts obtained via PWE than in the alkaline water extraction (2.6% vs. 11%, respectively) (data not shown). This indicates that an alkaline environment induces some peptide bond hydrolysis. Harsh conditions, *e.g.*, long extraction period or base concentration, could decompose the protein structure, degrade labile amino acids, and form complexes after alkaline extraction (*i.e.*, lysinoalanine), which explains the higher DH observed for alkaline water extraction (Hou et al., 2017), even if the effect is not too extensive. No effect of the de-oiling step was observed on the DH.

3.3.3. SDS-PAGE analysis

Significant differences in the distribution of protein bands were seen as a function of the extraction (Fig. 3). The alkaline water-extracted

Table 2
Effect of the process on the yield and proximate composition of the extracts obtained in step 2.

	Proximate analysis of the extract [g per 100 g of extract mass]			
	CE		PWE	
	Hex	CP	Hex	CP
Moisture	6.25 ± 1.15^{a}	5.64 ± 0.38^{a}	6.11 ± 0.34^{a}	4.83 ± 0.26^{a}
Ash	1.75 ± 0.29^{b}	1.62 ± 0.16^{ab}	1.50 ± 0.23^{ab}	1.19 ± 0.03^{a}
Oil	2.83 ± 0.25^{ab}	3.33 ± 0.38^{b}	2.32 ± 0.28^{a}	2.77 ± 0.25^{ab}
Protein	37.7 ± 0.32^{b}	20.42 ± 1.21^{a}	49.18 ± 0.32^{c}	47.13 ± 0.57^{c}
Carbohydrates	50.96 ± 0.62^{c}	68.70 ± 0.60^{d}	40.21 ± 0.49^{a}	43.43 ± 0.87^{b}
	Extraction yield of step 2 [g extract per g initial, corrected for dry matter]			
	CE		PWE	
	Hex	CP	Hex	CP
Mass yield	9.75 ± 0.55^{b}	11.19 ± 0.79^{c}	3.85 ± 0.08^{a}	4.35 ± 0.11^{a}
Protein yield	67.47 ± 1.76^{b}	67.02 ± 0.75^{b}	45.13 ± 2.19^{a}	45.11 ± 2.10^{a}

Statistical analysis was performed in a one-way ANOVA.
CP: Cold Pressing; Hex: *n*-hexane extraction.
CE: alkaline extraction (NaOH 0.15 M); PWE: Pressurized Water Extraction.
Values are expresses as mean ± SD ($n = 3$).
Different letters in the same column represent statistically significant differences in the components of the proteins based on the extraction method.
All analysis were performed following the protocols described by AOAC

proteins were characterized by the lack of defined bands and a large smear below 20 kDa, indicating protein degradation and aggregation. In contrast, clear, well-defined bands were obtained with the PWE-extracted proteins, mainly at MW between 20 and 35 kDa. These bands likely belong to globulins and albumins, which are better extracted with water and saline solutions. In addition, globulins are the main protein in vegetal storage tissues (Baptista et al., 2017). No effect of the de-oiling step was observed on the gel profile. In line with our results, proteins extracted from *Sambucus nigra* L. branches via pressurized hot water extraction showed a high concentration in regions close to 30 KDa (Šalplachta & Hohnová, 2017).

Proteomic analysis was attempted to identify the main bands starting from the gels obtained by the PWE extraction (in the other case, no bands could be identified). This was performed in gel tryptic digestion of the main single bands followed by high resolution MS peptide identification and protein database matching of the identified sequences. Unfortunately, reliable identifications were rarely achieved using the *Annonaceae* proteins or the green plants' proteins database. The only consistent high-quality identification with both databases concerned the intense band at the lowest molecular mass, lesser than 10 kDa, which could be reliably identified as actin, a protein present in many plants that is fundamental for ensuring vegetal cells' structural integrity. Quite interestingly, actin isoforms in all plants usually have much higher mass (in the range of 40–50 kDa). This means that actin proteins in the analyzed samples were present as fragments, which could be a consequence of the disruptive process used for protein extraction by PWE (even if, as already observed, the degree of hydrolysis was pretty low in those conditions). Indeed, many of the identified peptides were also present with oxidative modifications, another sign of possible cell stress. The lack of reliable identification for all the other bands might then be due to extensive protein modifications induced by the process or by the fact that being soursop an unknown biomass, its seed proteins are not present in the databases used, nor do they resemble other seed proteins of genetically related plants.

3.3.4. Amino acid analysis

In general, Glu, Arg, Asp, and Leu excelled among the 18 amino acids detected (Table 3). The amino acid content was between 1.6 and 1.8 times higher in protein extracted with PWE. As the SDS-PAGE analysis suggested, this is likely due to the protein degradation induced by the basic environment in alkaline water extraction. The amino acids most affected were Ser, Thr, Cys, and Lys, all amino acids involved as nucleophiles in reactions in basic environment (Accardo et al., 2022). In PWE, the amino acids were better preserved, but the least preserved ones were Phe, Tyr, His, Met, and Trp. The common characteristic of all those amino acids is that they are easily oxidized, thus suggesting that some oxidative stress was induced to the protein fraction during the PWE procedure, even if this extraction method better preserves proteins than alkaline water extraction.

Table 3
Amino acid profile of the extracted proteins expressed as mg of AA per g of extracted protein DM.

	CE		PWE	
	Hex	CP	Hex	CP
Gly	20.8 ± 1.7^{a}	25.8 ± 13.1^{ab}	39.7 ± 1.3^{b}	36.8 ± 1.0^{b}
Ala	23.8 ± 1.0^{b}	18.1 ± 1.0^{a}	36.7 ± 3.3^{c}	38.4 ± 0.5^{c}
Ser	17.0 ± 1.2^{b}	13.4 ± 0.1^{a}	30.4 ± 0.9^{d}	27.8 ± 0.4^{c}
Pro	19.2 ± 0.8^{b}	15.5 ± 0.3^{a}	36.0 ± 2.0^{c}	36.9 ± 0.2^{c}
Val	19.2 ± 0.3^{b}	15.3 ± 0.0^{a}	28.0 ± 1.0^{c}	27.9 ± 0.8^{c}
Thr	9.7 ± 0.5^{b}	7.5 ± 0.1^{a}	19.6 ± 0.7^{d}	18.1 ± 0.4^{c}
Ile	17.5 ± 0.6^{b}	13.1 ± 0.2^{a}	23.4 ± 0.8^{c}	22.2 ± 0.7^{c}
Leu	38.8 ± 1.6^{b}	29.3 ± 0.8^{a}	50.8 ± 1.5^{c}	51.5 ± 1.5^{c}
Asp	43.1 ± 0.5^{b}	31.2 ± 4.1^{a}	62.7 ± 6.2^{c}	68.1 ± 2.6^{c}
Lys	14.4 ± 0.0^{a}	11.4 ± 0.9^{a}	23.6 ± 1.8^{b}	23.5 ± 2.1^{b}
Glu	77.7 ± 0.2^{b}	60.4 ± 6.2^{a}	134.5 ± 8.1^{c}	127.7 ± 2.3^{c}
His	12.2 ± 0.7^{a}	12.3 ± 0.2^{a}	15.0 ± 009^{b}	13.5 ± 1.2^{ab}
Phe	19.9 ± 1.8^{ab}	16.8 ± 0.0^{a}	22.4 ± 1.6^{b}	23.2 ± 1.8^{b}
Arg	43.0 ± 2.3^{b}	31.4 ± 1.3^{a}	81.6 ± 2.1^{d}	72.8 ± 0.8^{c}
Tyr	19.2 ± 2.2^{a}	16.6 ± 0.5^{a}	24.5 ± 1.7^{b}	23.6 ± 1.5^{b}
Met	8.9 ± 0.3^{b}	7.4 ± 0.3^{a}	12.6 ± 0.0^{c}	9.7 ± 0.6^{b}
Cys	8.4 ± 0.2^{a}	7.7 ± 0.7^{a}	16.9 ± 0.3^{c}	13.9 ± 0.7^{b}
Trp	2.4 ± 0.1^{b}	1.9 ± 0.2^{a}	3.4 ± 0.1^{c}	3.6 ± 0.2^{c}

Results are expressed as average ± standard deviation (SD).
CP: Cold Pressing; Hex: *n*-hexane extraction.
CE: alkaline extraction (NaOH 0.15 M); PWE: Pressurized Water Extraction.
$n = 2$.
Different letters in the same row represent statistically significant differences in the amino acid content of soursop proteins as function of the extraction conditions ($p < 0.05$).

Also as per the de-oiling method, the higher amino acid content in proteins extracted from seeds previously de-oiled with *n*-hexane could be likely due to the bond disruption between phospholipids and structural proteins (Shen et al., 2020).

3.3.5. Amino acid racemization

The conversion of L-amino acids into the D-form results from harsh protein extraction conditions. The results displayed in Fig. 4 indicate that racemization of proteins extracted with PWE is lower than alkaline water extracted proteins, where it reached almost 40% in the case of Asp, which is known to be one the most affected amino acids in harsh environments and can be considered as a marker to evaluate the extraction process (Prandi et al., 2019). In alkaline extraction, it reached up to 40% in cold pressing de-oiled seeds and 25% in *n*-hexane de-oiled seeds, as a direct consequence of the strong basic environment used in alkaline extraction. Among alkaline water extracted samples, cold pressed de-oiled meal presented a higher racemization degree, likely due to thermal stress during the pressing phase.

3.4. Nutritional characterization

3.4.1. In vitro digestion

The assessment of *in vitro* digestibility provides an approach to the behavior of foods after ingestion. Although not representing the exact human conditions, most critical factors (*e.g.*, transit time, temperature, pH, enzymes) are well reproduced. The outcome of this digestibility study provides information on the bioaccessibility of soursop seed proteins.

Digestibility, expressed as a percentage of solubilized nitrogen against total nitrogen, followed the order casein>soursop proteins extracted by PWE> soursop proteins extracted by alkaline solution (Fig. 5a). The digestibility evaluated for casein was between 3 and 13 times higher (90% N soluble after digestion), which indicates that the soursop proteins might be difficult to digest completely. Additionally, the high concentration of carbohydrates (33–45% DM) could also hamper the nutritional performance of proteins (Tibbetts et al., 2016).

Pressurized water allowed the extraction of more digestible proteins (~27% N soluble after digestion) compared to the base extracted proteins (~7.5% N soluble after digestion), which agrees with the data found by Accardo et al. (2022) and by the previously reported data on the amino acid degradation induced by the basic environment which leads to poor digestibility. Besides amino acid degradation, studies have stated that proteins form insoluble complexes with protein during extraction, significantly reducing protein digestibility (Bals et al., 2009). *In vitro* digestibility of protein extracted with pressurized water was considerably higher. The mild extraction conditions avoided the degradation and aggregation of the proteins. Hence, besides the higher amount of preserved amino acids, a better solubilization was achieved, which is also fundamental for the digestibility of proteins (Nissen et al., 2021; Rieder et al., 2021).

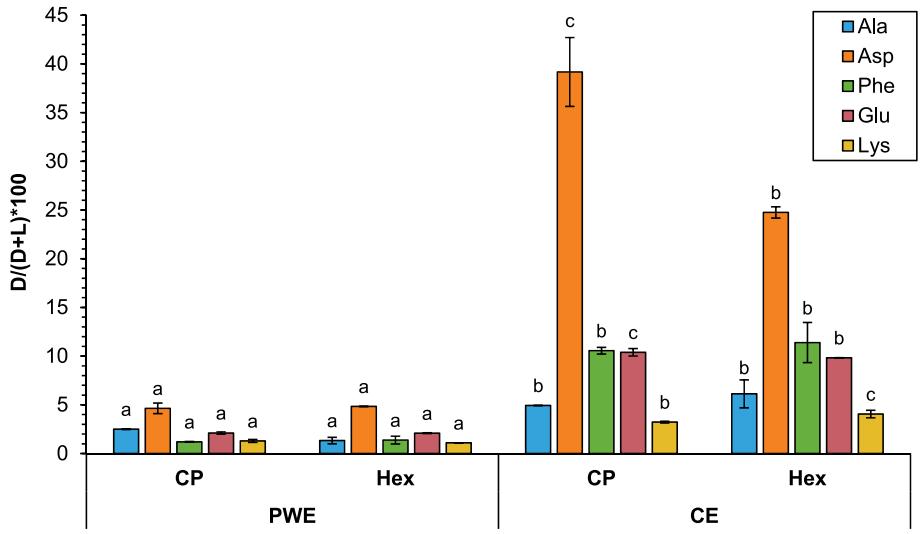

Fig. 4. Amino acid racemization. PWE: pressurized water extraction; CE: alkaline water extraction; CP: cold pressing; Hex: *n*-hexane extraction. Different letters mean statistically different racemization values (Fisher's LSD test, $p < 0.05$).

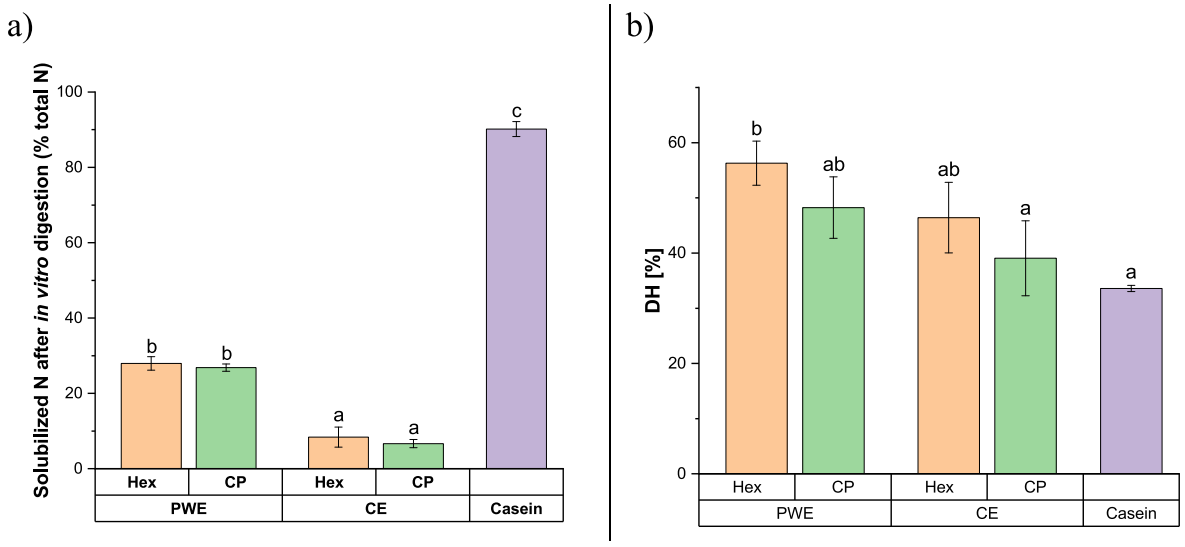

Fig. 5. (a) Solubilized nitrogen after *in vitro* digestion of soursop seed proteins, expressed as percentage of solubilized nitrogen against total nitrogen, and (b) Degree of hydrolysis of the solubilized proteins after *in vitro* digestion. PWE: pressurized water extraction; CE: alkaline water extraction; CP: cold pressing; Hex: *n*-hexane extraction. Different letters mean statistically different *in vitro* digestibility and DH values (Fisher's LSD test, $p < 0.05$).

The de-oiling technique did not significantly impact the digestibility ($p > 0.05$).

The digestion was also evaluated in terms of the DH of the solubilized proteins (Fig. 5b). Values between 33 and 56% were found (on the soluble fraction of the proteins), with lower DH for *in vitro* digested casein and higher for PWE-extracted protein de-oiled with *n*-hexane. In general, protein extracted from seeds de-oiled via the alkaline solution was evaluated with a lower DH (15% average). Additionally, the higher DH evaluated in the protein extracted from seeds de-oiled with *n*-hexane could be due to a previous denaturation during oil extraction. Studies have shown that post-*in vitro* digested plant proteins are characterized by a DH close to or higher than 60% (L. Jiang et al., 2019; Tang et al., 2022), while the digestion of casein after 3 h has shown a DH of around 40 and 50%, close to the result obtained in this study (Petrat-Melin et al., 2016).

3.5. Techno-functional assessment

Most of the techno-functional properties of proteins depend on solubility; therefore, good solubility indicates the potential applications of the protein in food formulations (Nissen et al., 2021). Fig. 6 displays the solubility of the soursop seeds protein at pH 3, 5, and 7. A better solubility was found at pH 7, while pH 3 and 5 did not drastically influence the solubility. Overall, lower solubilities were found in the proteins extracted from cold press de-oiled seeds. However, differences were found in the solubility of the proteins obtained via PWE and alkaline water. In the first case, up to 90% solubility was obtained. In contrast, the protein extracted with alkaline water exhibited a maximum solubility of 40% for the cold-pressing de-oiled meal and 19% for the meal de-oiled with *n*-hexane, again indicating a severe loss of integrity in the proteins extracted with basic solutions.

Proteins contain both lipophilic and hydrophilic sections. These groups can be exposed or hidden within the molecular structure depending on the conditions to which the protein is subjected. In this

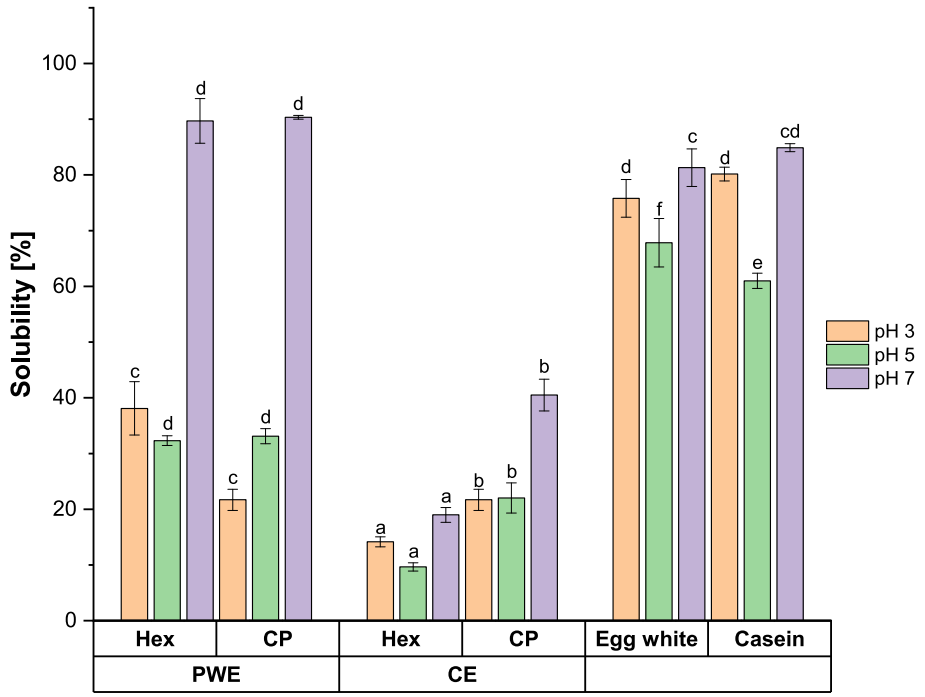

Fig. 6. Protein solubility. PWE: pressurized water extraction; CE: alkaline water extraction; CP: cold pressing; Hex: *n*-hexane extraction. Different letters mean statistically different solubility values (Fisher's LSD test, $p < 0.05$).

Table 4
Techno-functional properties of soursop seed protein.

Protein extraction	Defatting	Emulsifying activity [%]	Foaming capacity [%]	Foam stability [%]	Oil holding capacity [%]
PWE	Hex	17.17 ± 1.03^{a}	22.89 ± 2.69^{a}	NR	8.12 ± 0.31^{c}
	CP	16.33 ± 0.85^{a}	19.09 ± 2.80^{a}	NR	8.28 ± 0.19^{c}
CE	Hex	21.17 ± 1.31^{b}	59.10 ± 1.01^{cd}	86.34 ± 5.01^{a}	7.57 ± 0.14^{b}
	CP	18.83 ± 1.31^{ab}	57.01 ± 1.57^{c}	80.94 ± 4.84^{a}	8.38 ± 0.21^{c}
Egg white		48.89 ± 2.72^{c}	53.14 ± 3.18^{c}	121.30 ± 9.44^{c}	1.75 ± 0.04^{a}
Casein		39.26 ± 1.83^{c}	64.11 ± 5.82^{d}	86.74 ± 16.50^{b}	2.17 ± 0.17^{a}

Results are expressed as average ± standard deviation (SD).
CP: Cold Pressing; Hex: *n*-hexane extraction.
CE: alkaline extraction (NaOH 0.15 M); PWE: Pressurized Water Extraction.
$n = 3$.
Different letters represent statistically significant differences in the techno-functionality of soursop proteins as function of the extraction conditions ($p < 0.05$).

sense, no strong effect was attributed to de-oiling, but the protein extraction technique was deemed the most impacting factor. The improved interaction between water and protein possibly resulted from a limited unfolding of the protein due to the mild working conditions. However, when the proteins were extracted with NaOH, hydrophobic groups were likely exposed, while hydrophilic groups were hidden in the inner part of the molecular structure, resulting in reduced solubility at neutral and acidic conditions, as was also observed for rice protein extracted with alkaline solutions (Braspaiboon et al., 2020). In addition to the protein structure, the presence of oil through the formation of lipid-protein complexes influences the techno-functional properties of the proteins (Alzagtat & Alli, 2002).

As seen in Table 4, emulsification did not significantly vary as a function of the de-oiling. However, it was slightly higher in the proteins extracted from *n*-hexane de-oiled seeds. In contrast, proteins extracted with alkaline water developed a higher emulsifying activity (19–21%) than proteins extracted with pressurized water (16–17%). These values are significantly lower than those obtained for egg white and casein (49 and 39%, respectively). The modestly higher emulsifying activity of alkaline water extracted protein might be due to the complexation of proteins, forming insoluble aggregates, as already explained (Bals et al., 2009). Besides the protein structure, oil influences the emulsification of proteins. Lipoproteins surround the oil droplets in the form of solid films, which is further evidenced in a better emulsion compared to pure proteins in the absence of oil (Alzagtat & Alli, 2002).

The foam capacity and stability of the extracted proteins are displayed in Table 4. Protein extracted with an alkaline solution developed a significantly higher foaming capacity (57–59%) than the protein extracted via PWE (19–23%). The foams formed by alkaline water extracted proteins were similar to those obtained after whipping egg white and casein (53.14 and 64.11%, respectively). In both cases, no significant differences in the foaming capacity were found as a function of the de-oiling process. Similar behavior was seen after 30 min when the foam stability was assessed. The foam obtained with protein extracted via PWE had low stability. In contrast, the foams obtained with protein extracted with the alkaline solution, egg white, and casein showed similar stability with an average value of 94%, indicating that protein aggregation might increase protein techno functionality. As for the presence of oil, it negatively impacts the foam formation. Alzagtat and Alli (2002) explained that it might interfere between surface-active

polar lipids and the protein films by situating themselves at the air-water interface.

Table 4 shows that the proteins developed a similar OHC, with values ranging between 7.57 and 8.38 g of oil per g of protein DM. The comparison against egg white and casein showed that, on average, soursop seed proteins could retain 4.18-fold times more oil.

De-oiling did not drastically change the OHC of soursop seeds proteins. This could indicate that, in the case of soursop, OHC depends on the physical structure of the protein. Hence, it is possible that oil was trapped by the physical structure of the extracted protein. Moreover, the particle size and the presence of carbohydrates (specifically cellulose) could directly influence the amount of retained oil (Segura-Campos et al., 2014).

4. Conclusion

The results obtained in this study show that proteins extracted with PWE have a significantly superior quality and nutritional performance compared to proteins extracted via alkaline water, although the extraction yield was higher. The nutritional quality of the extracted protein displayed all the limitations of vegetal storage proteins, but this quality was further unpaired, in terms of amino acid composition, solubility, digestibility, and protein integrity, by the basic extraction environment. The techno-functionality of the proteins followed an opposite trend since foaming and emulsifying were better performed in proteins extracted via alkali solution. The impact of de-oiling was minor, and there were no significant differences in the fatty acid profile and protein quality of the extracts. For the first time, a study provides an in-deep assessment of the protein fraction of de-oiled soursop seeds. Additionally, pressurized water was demonstrated to extract better preserved proteins from a nutritional point of view compared to conventional alkali extraction.

CRediT authorship contribution statement

José Villacís-Chiriboga: Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Methodology. **Barbara Prandi:** Investigation, Formal analysis, Methodology, Writing – review & editing. **Jenny Ruales:** Writing – review & editing, Project administration. **John Van Camp:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition. **Stefano Sforza:** Conceptualization, Investigation, Supervision, Data curation, Writing – review & editing. **Kathy Elst:** Conceptualization, Methodology, Supervision, Data curation, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest. They declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114777.

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