



Cite this: *Sustainable Food Technol.*, 2025, 3, 1529

## Sustainable recovery of residual proteins from tofu whey by coupling nanofiltration with electromembrane processes and functional properties of resulting protein fractions

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In this study, a process was evaluated for the recovery of residual proteins from tofu whey. This process involved the coupling of a pre-concentration of tofu whey by nanofiltration (NF) at a 9× volume concentration factor, with demineralization by electrodialysis (ED), followed by electro-acidification through electrodialysis with bipolar membranes (EDBM) and centrifugation. The process resulted in a protein recovery of 25.1% in the final precipitate fraction. Molecular weight analysis showed that most proteins in tofu whey, retentate, precipitate and supernatant were under 1000 Da, with similar profiles suggesting no specific protein isolation in the precipitate. In addition, the functional properties of the fractions at each step of the process (Tofu whey, NF retentate, precipitate and supernatant) were assessed and compared with three commercial soy protein isolates (SPIs). For the first time, the functional properties of tofu whey proteins recovered by the NF + ED + EDBM process were systematically studied. Surprisingly, despite the residual proteins in tofu whey fractions having smaller molecular weights than the 7S and 11S proteins in SPIs, their functionalities were comparable, and in some cases, superior. The final supernatant and NF retentate exhibited better solubility, foaming properties, and emulsifying capacity than the SPIs, while the tofu whey and final precipitate demonstrated similar functional properties. These results suggest that all fractions could serve as valuable functional ingredients in food formulations, contributing to the valorization of tofu whey within a circular economy framework. However, it appeared that the EDBM step would not be necessary to valorize tofu whey in a more economical way.

Received 9th May 2025  
Accepted 24th July 2025

DOI: 10.1039/d5fb00202h  
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### Sustainability spotlight

Tofu whey, a by-product of tofu production, is discarded as waste despite containing residual proteins with potential functional properties. By recovering and valorizing these proteins, the tofu industry can create valuable and sustainable ingredients while minimizing environmental and economic impact. The sustainable advancement of this work lies in demonstrating a viable method to valorize tofu whey, turning an overlooked by-product into functional protein ingredients. The study shows that protein recovery can be achieved with comparable or superior functionality to commercial alternatives. This contributes to waste reduction and supports the development of a circular economy and a sustainable food system. This aligns with the 12th UN sustainable Development Goal: Responsible consumption and production.

## 1. Introduction

Soy ingredients, such as soy protein isolates, soy protein concentrates and soy flours are widely used in the food industry due to their functional properties. These properties include

foaming and emulsifying properties, making them valuable ingredients in various food formulations.<sup>1–3</sup> In this context, previous studies highlighted the potential of electrodialytic processes as an innovative approach for the recovery of residual soy proteins from tofu whey through electrodialytic processes.<sup>4,5</sup> Indeed, tofu production results in substantial volumes of tofu whey, with approximately 9 kg generated for every 1 kg of tofu.<sup>6</sup> Recovery of proteins from tofu whey represents a crucial opportunity for the soy industry, addressing environmental concerns by creating added value protein ingredients.

Recently, researchers have investigated the efficacy of a pre-concentration step using nanofiltration to enhance protein recovery during a coupled electrodialysis (ED) and

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electrodialysis with bipolar membrane process (EDBM). This study revealed that increasing the volume concentration factor (VCF) of tofu whey by nanofiltration (NF) (from 1× to 3×) significantly improved the protein recovery yield, and higher VCF could further enhance protein recovery yield.<sup>5</sup> Indeed, NF allows the concentration of a solution through the selective removal of water and monovalent ions.<sup>7</sup> Hence, molecules with a molecular weight higher than the cut-off of the membrane, typically between 100 and 500 Da, are retained. Additionally, the charge on the membrane surface enables the retention of multivalent ions, as they interact with the membrane.<sup>8</sup> Consequently, the coupling of NF prior to ED + EDBM would have many advantages: (1) the concentration of divalent ions increases the conductivity of the solution, improving the efficiency of the further ED step,<sup>5,9,10</sup> (2) NF concentrates the proteins, increasing the hydrophobic protein–protein interactions during the EDBM step,<sup>5,11,12</sup> and then promoting the protein precipitation, and (3) the demineralization step by ED removes most of the divalent ions ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) preventing membrane scaling during the EDBM step.<sup>13,14</sup> However, to valorize these residual proteins recovered as valuable protein ingredients and highlight the coupled process interests, their functional properties need to be studied and compared with those of commercial ingredients. Indeed, very few studies reported the functional properties of minor soy proteins in tofu whey, as existing literature primarily focuses on the major fractions: 7S and 11S or on soy ingredients such as soy flours, concentrates or isolates. Furthermore, to the best of our knowledge the functional properties of residual proteins from tofu whey recovered by NF + ED + EDBM process have never been studied.

In this context, the aim of this study was to valorize tofu whey by recovering proteins and, the objectives were to (1) study the impact of the pre-concentration of tofu whey by nanofiltration at a 9× FCV on the recovery of the residual protein during an ED + EDBM process, (2) characterize the fractions composition at the different steps of the process, (3) evaluate the functional properties (solubility, foaming and emulsifying properties) of the protein recovered in the different fractions and (4) compare their functional properties with those of commercial soy isolates (SPIs).

## 2 Materials and methods

### 2.1 Materials

Tofu whey was provided by Unisoya (Saint-Isidore-de-Laprairie, Quebec, Canada). It was obtained after the coagulation of tofu by calcium chloride and magnesium chloride as coagulants. The commercial soy protein isolates (SPIs) used for comparison of functional properties were Supro 120, Supro 500E, and Supro XT-40, manufactured by Solae LLC (St. Louis, MO, USA). The composition of the three commercial isolates is detailed in Table 1.

### 2.2 Protocol

After the reception of tofu whey, it was filtered using a cheese-cloth to remove any solid tofu particles. During the process

Table 1 Composition of commercial soy protein isolates

g/100 g	Supro 120	Supro 500E	Supro XT-40
Protein	$82.5 \pm 0.3$	$83.0 \pm 0.9$	$73.9 \pm 0.8$
Fat	$2.5 \pm 0.2$	$1.3 \pm 0.1$	$0.8 \pm 0.1$
Ash	$4.0 \pm 0.6$	$3.7 \pm 0.4$	$12.5 \pm 0.7$

(Fig. 1), tofu whey was concentrated by nanofiltration (NF) at 9×. Briefly, tofu whey was pre-heated at 45 °C. NF was performed on a L-pilot plant GEA filtration system (Hudson, WI, USA), with a Synder NFX-3B-2540M spiral wound membrane made of Polyamide thin-film composite (PA TFC) (Synder Filtration, Vacaville, CA, USA), in order to concentrate divalent ions and proteins in the retentate. The molecular weight cut-off was 150–300 Da and the total membrane area was 2.15 m<sup>2</sup>. NF was performed at 200 psi. The VCF of 9X was calculated according to the permeate mass.

Then, successive ED and EDBM (Fig. 2A and B) were performed in triplicate on NF retentate (NFR), as carried out in a recent study.<sup>5</sup> First, 700 mL of tofu whey was treated by ED until 70% of demineralization was achieved.<sup>15</sup> This step removes most of the divalent ions, which helps minimize membrane fouling during the EDBM process.<sup>13,14</sup> Additionally, it reduces the solution's ionic strength, promoting protein precipitation during electro-acidification.<sup>5,11,12</sup> For both electrodialytic processes, tofu whey, recovery solution (700 mL, 2 g L<sup>-1</sup> KCl), and electrode rinsing solution (800 mL, 20 g L<sup>-1</sup> Na<sub>2</sub>SO<sub>4</sub>) circulated between membranes at flow rates of 700, 700 and 1000 mL min<sup>-1</sup>, respectively. Voltage was maintained constant at 9 V throughout both processes. This voltage was established in a previous study after determining the limiting current density of the retentate and corresponded to about 70% of the limiting current density.<sup>5</sup> Then, 500 mL of the demineralized NF retentate (NFRd), was collected for electro-acidification by EDBM, for protein precipitation after centrifugation. EDBM was performed until NFRd reached pH 4.6, which is close to the pI of the proteins. PH and conductivity of the concentrated and demineralized tofu whey and recovery solution were measured during EDBM. After EDBM, the acidified and demineralized tofu whey were recovered, centrifuged (Avanti-J-E high-speed centrifuge, Beckman Coulter, Indianapolis, USA) and washed two times at 1000 g for 15 minutes, as carried out in a recent study.<sup>5</sup> After centrifugation and washing, the final precipitate and supernatant were obtained. The tofu whey, NF retentate, NF permeate, final precipitate and supernatant were recovered, lyophilized and kept at -18 °C before analyses. The moisture, ash, minerals, sugars, fibers, fat and protein content were analyzed.

### 2.3 Analyses

#### 2.3.1 Electrodialytic parameters

2.3.1.1 *Conductivity.* The conductivity of feed and recovery solutions was measured using a YSI conductivity meter (Model 3100, Yellow Springs Instrument, Yellow Springs, OH, USA) combined with a YSI-3252 electrode (cell constant of 1 cm<sup>-1</sup>).



## Process 1

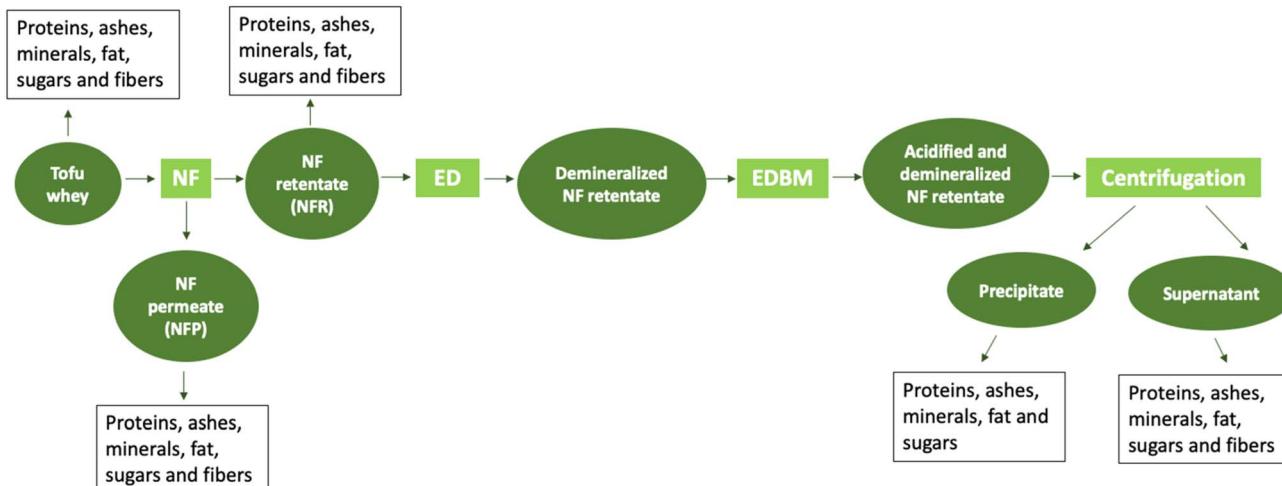


Fig. 1 Process diagram and analyses carried out on different fractions.

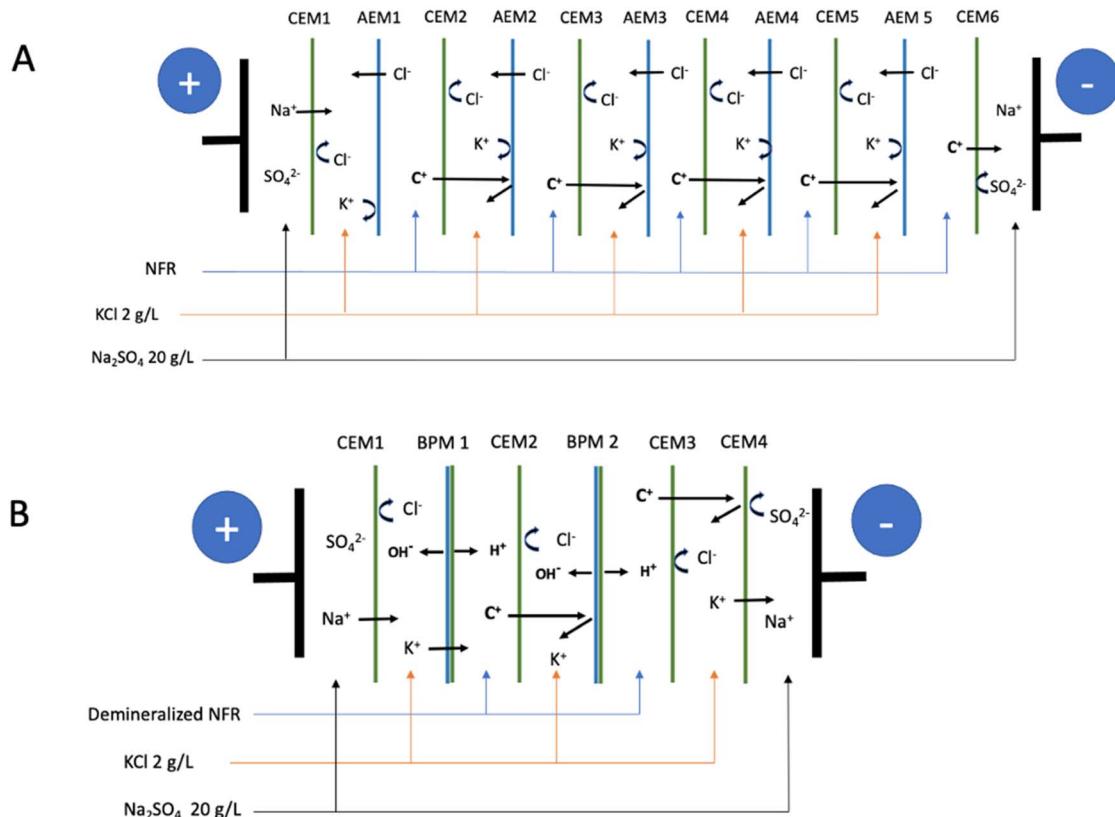


Fig. 2 Schematic representation of (A) ED cell configuration used for demineralization of nanofiltration retentate (NFR) and (B) EDBM cell configuration used for acidification of demineralized NF retentate.

**2.3.1.2 pH.** The pH of the feed and recovery solutions was measured using a VWR Symphony pH-meter model SP20 Thermo Orion (West Chester, PA, USA).

**2.3.1.3 Membrane thickness and electrical conductivity.** Membrane thickness and electrical conductivity were measured before and after each run, as described by Lemay *et al.* (2019).<sup>16</sup>

Prior to the analysis, membranes were soaked in 0.5 M NaCl solution for 30 min.

For thickness measurement, an electronic digital (Marathon Watch Company LTD, Richmond Hill, ON, Canada) was used. Six measurements were taken at different locations on the membrane, and the average thickness was calculated. The



membrane electrical conductivity was calculated using the membrane thickness measurements and the electrical resistance obtained from the membrane conductance ( $G$ ). Membrane conductance was measured using a YSI conductivity meter model 3100 Yellow Springs Instrument Co (Yellow Springs, OH, USA) equipped with a specially designed clip from the Laboratoire des Matériaux Échangeurs d'Ions (Université Paris XII, Créteil, Val de Marne, France). Six measurements were taken at different locations on the membranes, and the average conductance was used to calculate conductivity.<sup>16</sup>

The membrane electrical resistance was calculated according to Lteif *et al.* (1999) and Lebrun *et al.* (2003),<sup>17,18</sup> using eqn (1):

$$R_m = \frac{1}{G_m} = \frac{1}{G_{m+s}} - \frac{1}{G_s} = R_{m+s} - R_s \quad (1)$$

where  $R_m$  is the transverse electric resistance of the membrane (in  $\Omega$ ),  $R_{m+s}$  the resistance of the membrane and reference solution measured together (in  $\Omega$ ),  $R_s$  the resistance of the reference solution (in  $\Omega$ ).

The membrane electrical conductivity  $\kappa$  ( $\text{S cm}^{-1}$ ) was then calculated according to Lteif *et al.* (1999),<sup>17</sup> using eqn (2):

$$\kappa = \frac{L}{R_m A} \quad (2)$$

where  $L$  is the membrane thickness (in cm) and  $A$  the electrode area (1  $\text{cm}^2$ ).

**2.3.2 Proximal composition analyses.** The composition analyses were conducted at each step of the process on tofu whey, NF retentate, NF permeate as well as final precipitate and supernatant (Fig. 1). The fractions obtained immediately after ED and EDBM were not analyzed, as they correspond to intermediate steps in the overall ED + EDBM + centrifugation process, which is the focus of this study. Indeed, ED was carried out to enhance protein precipitation during EDBM, while centrifugation effectively separates the precipitated proteins after EDBM acidification. The analyses were performed in triplicate, and the results were expressed as g/100 g on a dry basis to facilitate the comparison of functional properties. Indeed, solutions for functional properties were prepared for each properties on the same protein concentration (1% m/v) as done in the literature<sup>19,20</sup>

**2.3.2.1 Moisture and ash contents.** The moisture and ash contents of each fraction were determined following the AOAC method 945.46.<sup>21</sup> Approximately 0.5 g of freeze-dried samples were weighed into a pre-weighted crucible and dried at 100 °C for 5 hours in a vacuum oven (Isotemp Vacuum Oven, Model 280 A, Thermo Fisher Scientific, Waltham, MA, USA). The moisture content was calculated using eqn (3):

$$\text{Moisture content}(\%) = \left( \frac{\text{sample mass before drying} - \text{sample mass after drying}}{\text{sample mass before drying}} \right) \times 100 \quad (3)$$

Then, to determine the ash content, the crucibles were placed in a furnace (Lindberg/Blue M Moldatherm Box Furnaces, Thermo Fisher Scientific, Waltham, MA, USA) at 550 °C for 18 h and weighed. The ash content was calculated using eqn (4):

$$\text{Ash content}(\%) = \left( \frac{\text{sample mass after incineration}}{\text{sample mass before incineration}} \right) \times 100 \quad (4)$$

**2.3.2.2 Minerals contents.** The mineral content of each fraction was determined using the method reported by Dufton *et al.* (2018).<sup>22</sup> Ash samples were solubilized in 2 mL of 25% nitric acid and 8 mL of miliQ water. Solutions were filtered with a 0.45  $\mu\text{m}$  PTFE filter (CHROMESPEC Syring Filter, Chromatographic Specialties, Brockville, ON, Canada). Calcium, magnesium, potassium, sodium, and phosphorus were determined using an Agilent 5110 SVDV ICP-OES (Agilent Technologies, Victoria, Australia). The analyses for all ions were carried out in radial and/or axial view, using the following wavelengths: 393.366; 396.847; 422.673 (Ca), 766.491 (K), 279.553; 280.270; 285.213 (Mg), 588.995; 589.592 (Na), 177.434; 178.222; 213.618; 214.914 (P).

**2.3.2.3 Sugar content.** Sugar content of fractions was analyzed by HPLC (Waters Corp., Milford, MA, USA), according to Ounis *et al.* (2008).<sup>23</sup> To determine raffinose and sucrose contents, about 0.02 g of samples were dispersed in 1000  $\mu\text{L}$  methanol and centrifuged at 5000  $\times g$  for 5 min. The supernatant was filtered with a 0.22  $\mu\text{m}$  nylon filter CHROMESPEC Syringe Filter, Chromatographic Specialties (Brockville, ON, Canada) and liquid samples were injected in a Sugar-Pak column Waters Corporation (Milford, Massachusetts, USA). To determine stachyose content, about 0.08 g of samples were solubilized in 2.5 mL HPLC-grade water and treated with Biggs-Szijarto solution to precipitate protein. Then, solutions were centrifuged at 5000  $\times g$  for 5 min. The supernatant was diluted in HPLC-grade water and filtered with a 0.22  $\mu\text{m}$  nylon filter CHROMESPEC Syringe Filter, Chromatographic Specialties (Brockville, ON, Canada). Then, liquid samples were injected in an ICsep-ION-300 column Trans-genomic, (Omaha, NE, USA) with a refractive index detector Hitachi (Foster City, CA, USA).<sup>23</sup>

**2.3.2.4 Fiber content.** Fiber content of fractions was analyzed according to the AOAC Method 991.43 using the kit and the procedure of Megazyme (Wicklow, Ireland). Due to insufficient quantities recovered, this analysis was not performed on the precipitate fraction after EDBM. Briefly, 1 g of dry samples was solubilized in 40 mL of MES-TRIS buffer.  $\alpha$ -amylase was added to samples and incubated in a water bath at 100 °C, then protease and amyloglucosidase were added successively followed by incubation at 60 °C. Samples were then precipitated with ethanol 95% and filtered washed with ethanol 78%, pre-dried with ethanol 95% and acetone and oven-dried at 105 °C. Residues were analyzed for protein according to the AOAC 991.20 Kjeldahl method,<sup>21</sup> and incinerated at 525 °C to determine ash content. The fiber content was calculated by eqn (5) and the results were expressed in g/100 g on a dry basis:

$$\text{Fiber content}(\%) = \frac{\frac{R_1 + R_2}{2} - p - A - B}{\frac{m_1 + m_2}{2}} \times 100 \quad (5)$$



where  $R_1$  is the residue weight 1 from  $m_1$ ,  $R_2$  the residue weight 2 from  $m_2$ ,  $m_1$  the sample weight 1,  $m_2$  the sample weight 2,  $A$  the ash weight from  $R_1$ ,  $p$  the protein weight from  $R_2$  and  $B$  the blank weight.

**2.3.2.5 Protein content and determination of molecular weight.** Protein content of each fraction was determined using the Dumas combustion method using a Rapid Micro N Cube (Elementar, Francfort-sur-le-Main, Germany). A nitrogen conversion factor of 5.71 was used (Krul, 2019).

To identify the molecular weight of proteins and peptides in the samples, RP-UPLC analyses were performed using a 1290 Infinity II UPLC (Agilent Technologies, Santa Clara, CA, USA), as described by Abou-Diab *et al.*<sup>24</sup> The equipment consisted of a binary pump (G7120A), a multisampler (G7167B), an in-line degasser and a variable wavelength detector (VWD G7114B) adjusted to 214 nm. Samples, at a concentration of 1% protein, were filtered with 0.45 µm PVDF filter into a 2 mL glass vial. They were loaded (1 µL) onto an InfinityLab Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 micron, Agilent, CA, USA). The column was operated at a flow rate of 0.4 mL min<sup>-1</sup> at 45 °C and maximum pressure was 600 bar. The gradient consisted of solvent A (LC-MS grade water with 0.1% formic acid) and solvent B (LC-MS grade ACN with 0.1% formic acid) where the column was equilibrated at 1% B. A ramp was applied until 45% B in 30 min, then 2 min at 95% B to end at 100% B for cleaning the column during 3 min. Finally, back to initial conditions for 5 min more for equilibration before the next injection.

A hybrid ion mobility quadrupole time-of-flight mass spectrometer (IM-Q-TOF, 6560 high definition mass spectrometry, Agilent, CA, USA) was used to identify the molecular weight of protein and peptides in the different samples.<sup>24</sup> All LC-MS/MS experiments were acquired using Q-TOF. Signals were recorded in positive mode at Extended Dynamic Range, 2 Ghz, 3200 *m/z* with a scan range between 100–3200 *m/z*. Nitrogen was used as the drying gas at 13.0 L min<sup>-1</sup> and 150 °C, and as nebulizer gas at 30 psi. The capillary voltage was set at 3500 V, the nozzle voltage at 300 V and the fragmentor at 400 V. The system was calibrated using an ESI-L low concentration tuning mix (Agilent Technologies, Santa Clara, CA, USA). Data acquisition and analysis was done using the Agilent Mass Hunter Software package (LC/MS Data Acquisition, Version B.09.00, Qualitative Analysis, Version B.07.00 Service Pack 2 with BioConfirm Software).

**2.3.2.6 Lipid content and characterization.** Lipid content of fractions was determined according to the Mojonnier method, AOAC 989.05,<sup>21</sup> in 10 ml of liquid samples for tofu whey, retentate and permeate, and 1.5 g of dry samples for precipitate and supernatant. Briefly, three extractions were carried out. The first extraction used 1.5 mL of ammonium hydroxide, 10 mL of ethyl alcohol, 25 mL of ethyl ether and 25 mL of petroleum ether, and the second and third extractions used 5 mL of ethyl alcohol, 15 mL of ethyl ether and 15 mL of petroleum ether respectively. The mass of extracted lipid was measured and compared to the mass of initial sample.

For lipid characterization, since lipids were all recovered in the NF retentate, the analysis was performed only on NF retentate due to insufficient quantities in the other fractions.

Lipids were extracted using the Folch method. Then, classes of lipids were separated using Thin-Layer Chromatography (TLC). In short, samples were dispersed in diethyl ether. Then, 3 drops were applied with a capillary to a TLC plate (Whatman, Maidstone, United-Kingdom). The plate was placed in a chamber with the elution solvent composed of hexane, diethyl ether and acetic acid (80 : 20 : 1.5). After elution, the plate was dried on a heating plate and placed in a chamber with iodine for staining. Photographs were taken immediately when the color appeared.<sup>25</sup> The standards used were canola oil (triglycerides) (ACH Food Companies, Inc., Oakbrook Terrace, IL, USA), β-sitosterol (Sigma Aldrich, Saint-Louis, MO, USA) and L-α-phosphatidylcholine (Sigma Aldrich, Saint-Louis, MO, USA). Then, the software ImageJ (Bio-Rad, Hercules, United-States) was used to determine the proportions of the separated lipids.<sup>26</sup> Briefly, for each sample, the integrated density of each dot was determined and compared to the integrated density of the total lipids.

### 2.3.3 Functional properties

**2.3.3.1 Preparation of soy protein dispersion.** All experiments were conducted on tofu whey, NF retentate, supernatant and precipitate. The NF permeate was not analyzed, since the protein content was insufficient (see Section 3.1) and this concentration does not make this fraction a good candidate for its valorization as a protein ingredient. These were compared to three commercial soy protein isolates (SPIs): Supro 500E, Supro 120 and Supro XT-40. Prior to functional properties analyses, these products were dispersed overnight in distilled water at a protein concentration of 1% w/v following the Dumas analysis results. The pH was then adjusted to 7 with 0.5 N NaOH.

**2.3.3.2 Solubility.** The solubility was determined according to Haque *et al.* with some modifications.<sup>27</sup> Briefly, 4 mL of the 1% solutions were transferred in 15 mL centrifugation tubes and pH was adjusted to values of 7.0, 6.0, 5.0, 4.0 and 3.0 respectively. These solutions were centrifuged at 1000×*g* and 20 °C for 10 min. Then, 2 g aliquots of the supernatant were transferred into pre-weighted microtubes and lyophilized.

Solubility (in percent) was calculated according to the following equation:

$$\text{Solubility}(\%) = \frac{\text{nitrogen in dry supernatant}}{\text{nitrogen in the initial solution}} \times 100 \quad (6)$$

Nitrogen concentration was determined using the Dumas method as described above after lyophilization of the samples.

**2.3.3.3 Foaming properties.** Foaming capacity and foam stability were measured according to the method described by Schwenke *et al.* with some modifications.<sup>28</sup> Briefly, 10 mL of the 1% w/v dispersions were transferred to a 50 mL tube and mixed for 2 min with an Ultra Turrax T25 basic, IKA-WERKE (Wilmington, NC, USA) at speed 2 (9500 rpm). The volume of the solution was measured immediately. The foam capacity (FC, in percent) was calculated according to the following eqn (7):

$$\text{FC}(\%) = \frac{V_B - V_A}{V_B} \times 100 \quad (7)$$



where  $V_A$  is the foam volume after whipping (in mL), and  $V_B$  is the solution volume before whipping (in mL).

To measure foam stability, the foam volume of the solution in the tube was recorded after 1, 3, 5, 10, 30, 60, and 120 min and was expressed as the percentage of the foam volume (mL) immediately after mixing compared to the foam volume (mL) at each time.

**2.3.3.4 Emulsifying activity.** Emulsifying activity was measured according to a slightly modified version of the method described by Neto *et al.*<sup>19</sup> The 1% w/v dispersions (10 mL) were added in 50 mL centrifuge tube with 10 mL of canola oil and homogenized with an Ultra Turrax T25 basic, IKA-WERKE (Wilmington, NC, USA) at speed 2 (9500 rpm) for 1 min. Then, the emulsions were centrifuged at 1100  $\times g$  for 5 min (IEC Centra CL2 Centrifuge, Thermo Scientific Inc., Waltham, MA, USA), and the volume of the total solution and emulsified layer were measured.<sup>19,29</sup> Emulsifying activity (EA, in percent) was calculated according to eqn (8):

$$EA(\%) = \frac{\text{height of emulsified layer (in mL)}}{\text{height of the total solution (in mL)}} \times 100 \quad (8)$$

**2.3.3.5 Emulsifying capacity.** Emulsifying capacity was measured by the method described by Mohanty *et al.* with slight modifications.<sup>29</sup> The 1% w/v dispersions (2 mL) were transferred in a 50 mL beaker. Canola oil was added gradually (about 5 mL min<sup>-1</sup>) while homogenizing with an Ultra Turrax T25 basic, IKA-WERKE; (Wilmington, NC, USA) at speed 4 (17 500 rpm). The quantity of oil needed for the inversion of emulsion, characterized by a sudden drop in viscosity, was recorded. The emulsifying capacity was expressed as the amount of oil (in mL) per 100 mg of protein.

**2.3.3.6 Emulsion stability.** Emulsion stability was measured with the method described by Stone and Nickerson with slight modifications.<sup>30</sup> The 1% w/v dispersions (5.5 mL) were added to a 50 mL centrifuge tube with 10 mL of canola oil and homogenized with an Ultra Turrax T25 basic, IKA-WERKE; (Wilmington, NC, USA) at speed 2 (9500 rpm) for 2 min. The volume of

the aqueous phase was reported after 24 h. The emulsion stability (ES, in percent) was calculated according to the following equation:

$$ES(\%) = \frac{V_B - V_A}{V_B} \times 100 \quad (9)$$

where  $V_B$  is the volume of the aqueous phase before emulsification (in mL) and  $V_A$  the volume of the aqueous phase after 24 h (in mL).

## 3. Results and discussion

### 3.1 Composition of tofu whey, retentate and permeate of NF

The composition of initial tofu whey, NF retentate (NFR) and NF permeate (NFP) is presented in Table 2. First and as expected, total nitrogen from tofu whey was concentrated in the retentate. Secondly, fat totally remained in the retentate, with no traces in the permeate. Regarding ash content, minerals were partially removed by NF, with a higher ash content in the permeate than retentate. Surprisingly, the majority of divalent ions were removed in the permeate. Indeed, NF is expected to remove only monovalent ions.<sup>7</sup> Sugars were removed in the permeate according to their size, with higher removal of sucrose (342 g mol<sup>-1</sup>) < raffinose (504 g mol<sup>-1</sup>) < stachyose (666 g mol<sup>-1</sup>). Conversely, fibers were almost totally concentrated in the retentate. Finally, the dry matter is also presented in Table 2, expressed in g/100 g of liquid. It represents the solid content of the fractions after all the water has been removed. The dry matter content allows for converting the composition values presented on a dry basis in Table 2 to a liquid basis, more representative of the final product composition in terms of concentration according to the volumes produced. As expected, the solids from tofu whey were concentrated in NF retentate.

Lipid characterization by Thin Layer Chromatography was performed only on the NF retentate as already mentioned. The lipids from NF retentate were separated according to their polarity (Fig. 3). The lipids were mainly composed of triglycerides (row 4) and phospholipids (row 1). They also contain phytosterols (row 3) and glycolipids (row 2).<sup>31</sup> According to the

Table 2 Composition of tofu whey, NF retentate, NF permeate, precipitate and supernatant

		Tofu whey	NF retentate	NF permeate	Precipitate	Supernatant
Protein (total nitrogen)	g/100 g (Dry basis)	15.1 ± 0.7 <sup>ba</sup>	21.0 ± 0.4 <sup>d</sup>	5.4 ± 0.4 <sup>a</sup>	43.6 ± 1.7 <sup>e</sup>	19.5 ± 0.4 <sup>c</sup>
Fat		2.0 ± 1.3 <sup>b</sup>	9.1 ± 0.2 <sup>c</sup>	0.0 ± 0.0 <sup>a</sup>	33.1 ± 3 <sup>d</sup>	2.3 ± 0.6 <sup>b</sup>
Ash		16.7 ± 0.3 <sup>d</sup>	6.9 ± 0.2 <sup>c</sup>	29.2 ± 3.3 <sup>e</sup>	1.0 ± 0.1 <sup>a</sup>	3.4 ± 0.2 <sup>b</sup>
Ca <sup>2+</sup>		0.75 ± 0.05 <sup>d</sup>	0.60 ± 0.01 <sup>c</sup>	1.06 ± 0.13 <sup>e</sup>	0.04 ± 0.01 <sup>a</sup>	0.16 ± 0.01 <sup>b</sup>
Mg <sup>2+</sup>		1.10 ± 0.03 <sup>d</sup>	0.78 ± 0.01 <sup>e</sup>	1.51 ± 0.15 <sup>d</sup>	0.04 ± 0.02 <sup>a</sup>	0.31 ± 0.02 <sup>b</sup>
K <sup>+</sup>		4.26 ± 0.1 <sup>d</sup>	1.52 ± 0.05 <sup>c</sup>	5.78 ± 1.38 <sup>d</sup>	0.04 ± 0.00 <sup>a</sup>	0.16 ± 0.05 <sup>b</sup>
Na <sup>+</sup>		0.65 ± 0.03 <sup>c</sup>	0.24 ± 0.01 <sup>b</sup>	0.99 ± 0.08 <sup>d</sup>	0.04 ± 0.02 <sup>a</sup>	0.25 ± 0.08 <sup>b</sup>
P		0.16 ± 0.01 <sup>b</sup>	0.15 ± 0.00 <sup>b</sup>	0.15 ± 0.03 <sup>b</sup>	0.12 ± 0.04 <sup>ab</sup>	0.08 ± 0.00 <sup>a</sup>
Stachyose		14.9 ± 0.1 <sup>c</sup>	17.4 ± 0.1 <sup>d</sup>	9.8 ± 0.3 <sup>b</sup>	3.8 ± 1.6 <sup>a</sup>	32.9 ± 3.2 <sup>e</sup>
Raffinose		2.5 ± 0.1 <sup>b</sup>	2.2 ± 0.2 <sup>b</sup>	3.3 ± 0.3 <sup>c</sup>	0.3 ± 0.0 <sup>a</sup>	9.6 ± 0.7 <sup>d</sup>
Sucrose		9.9 ± 0.5 <sup>c</sup>	5.1 ± 0.4 <sup>b</sup>	22.3 ± 1.3 <sup>e</sup>	0.8 ± 0.1 <sup>a</sup>	18.4 ± 1.0 <sup>d</sup>
Fibers		14.2 ± 2 <sup>b</sup>	26.1 ± 0.7 <sup>c</sup>	± 1.3 <sup>a</sup>	NA	12.2 ± 1.2 <sup>b</sup>
Moisture		3.6 ± 0.3 <sup>d</sup>	1.6 ± 0.1 <sup>b</sup>	12.3 ± 0.3 <sup>e</sup>	0.9 ± 0.2 <sup>a</sup>	2.8 ± 0.2 <sup>c</sup>
Dry matter	g/100 g (liquid basis)	3.2 ± 0.3 <sup>b</sup>	12.6 ± 0.4 <sup>c</sup>	1.39 ± 0.4 <sup>a</sup>	25.9 ± 0.8 <sup>e</sup>	8.3 ± 0.5 <sup>d</sup>

<sup>a</sup> Letters that differ within the same row indicate statistically significant differences between the means, as determined by the Tukey test ( $P < 0.05$ ).



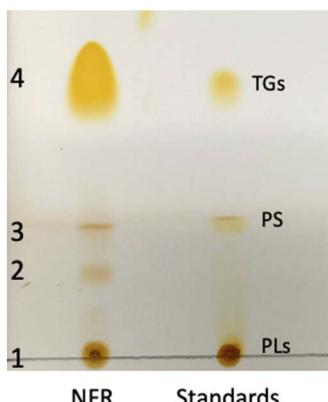


Fig. 3 Separation of lipids of NF retentate on Thin layer chromatography (TLC) plates.

integrated intensity of the dots, calculated with ImageJ software, triglycerides accounted for 59% of the total lipids, while phospholipids accounted for 20%.

### 3.2 Electrodialytic parameters

**3.2.1 Conductivity of NF retentate during ED and EDBM.** The initial conductivity of NF retentate was  $6.9 \pm 0.2 \text{ mS cm}^{-1}$  (Fig. 4). In the ED step, the conductivity decreased gradually until 70% of demineralization was achieved, corresponding to  $2.1 \pm 0.1 \text{ mS cm}^{-1}$ . The ED process took approximately 250 minutes. In the EDBM step, the conductivity of NF retentate remained stable, with final value of  $1.5 \pm 0.1 \text{ mS cm}^{-1}$ . This stability occurred due to the migration of cations through CEMs and the generation of highly conductive  $\text{H}^+$  ions from the bipolar membrane in the feed compartment.<sup>32</sup> This step took approximately 32 minutes.

**3.2.2 pH of NF retentate and recovery solutions during ED and EDBM.** During the ED step, the pH of the NF retentate slightly decreased, from  $6.0 \pm 0.2$  to  $4.9 \pm 0.1$ . As noted in

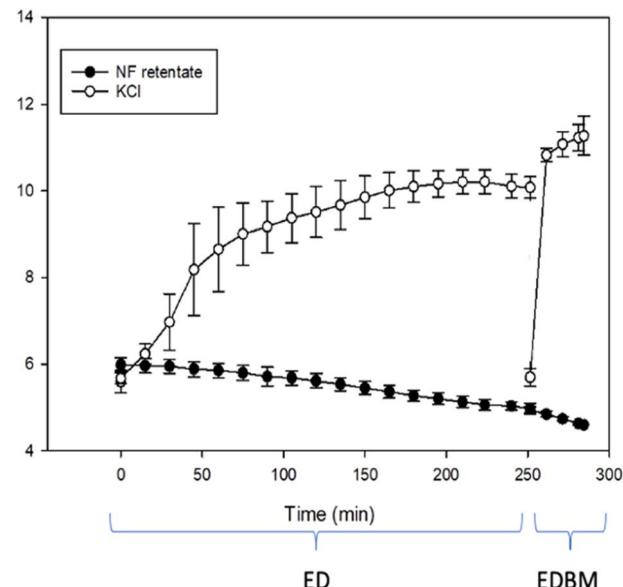


Fig. 5 pH evolution of NF retentate and recovery solution during ED and EDBM.

a previous study<sup>5</sup> (Fig. 5), this was limited by the buffering capacity of proteins in the solution. On the other hand, the pH of the recovery compartment gradually increased up to 4.5 pH units. The recovery solution used was KCl, which has no buffer capacity. As a result, the increase in pH was not proportional to that of the feed solution. Although the rise in pH may indicate water splitting, this is unlikely as the operating current density was much lower than the limiting current density. Furthermore, when water splitting occurs, there is typically a sharp rise in pH followed by a plateau, whereas, in this study, a gradual and consistent increase in pH was observed.<sup>5,33,34</sup> The increase in pH was consequently mainly due to the migration of conjugate bases of weak acids from the tofu whey solution. Tofu whey contains small amounts of organic acids, such as lactic, acetic, citric, formic, phytic, and oxalic acids.<sup>35</sup> At the pH of the process, some of these acids dissociate into their conjugate bases. Indeed, when the pH of the solution is higher than the  $pK_a$ , a larger proportion of the acid dissociates into its conjugate base and protons. Therefore, at the pH of the NF retentate (around 6.0), which is higher than the  $pK_a$  of most of these weak acids, they are predominantly present in their dissociated forms. In this form, the conjugate bases or their anionic forms, which are negatively charged, can migrate through the AEM into the recovery compartment, leading to an pH increase, as demonstrated in many studies.<sup>22,36-38</sup>

The EDBM step was performed until the pH decreased to 4.6 as intended for the precipitation of the proteins and due to the generation of  $\text{H}^+$  ions at the cationic interface of the BPMs. Conversely, the pH rise in the recovery compartment was caused by the generation of  $\text{OH}^-$  ions at the anionic interface of the BPMs. Furthermore, the KCl solution was changed for the EDBM step, which explains the drop of pH followed by the sharp increase, observed in Fig. 5.

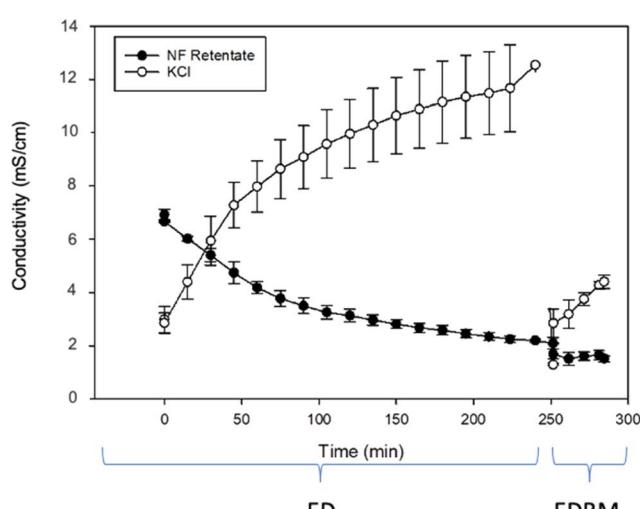


Fig. 4 Conductivity evolution of NF retentate and recovery solution during ED and EDBM.



**3.2.3 Membrane characterization.** To follow the IEM integrity during the different processes and electrodialytic steps, membrane's thickness and conductivity were taken before and after ED and EDBM treatment. No significant difference was observed for the thicknesses values, therefore the individual values were not presented. The average thicknesses were 0.148 mm for CEMs, 0.150 mm for AEMs and 0.243 mm for BPMs. However, some differences were observed for the conductivity.

For the ED process, the average membrane electrical conductivity losses were 19% for CEMs and 43% for AEMs (Fig. 6A). First, when comparing AEMs and CEMs, the AEMs were more affected than CEMs, as previously reported by Deschênes-Gagnon *et al.* (2024).<sup>39</sup> This study on membrane fouling found that isoflavones, a type of polyphenol present in soybeans, are the primary molecules from tofu whey that interact with the membranes. Indeed, in this study, it was demonstrated that isoflavones can interact with AEMs through 3 types of interactions:  $\pi$ - $\pi$  stacking, hydrogen bond and electrostatic interactions, whereas only  $\pi$ - $\pi$  stacking, hydrogen bond are possible with the CEMs.<sup>39</sup> This explains the higher conductivity losses observed for the AEMs compared to the CEMs.

Regarding EDBM process, no change was observed for the conductivity of the BPMs before and after the treatments. The same behavior reported here was observed in a previous work<sup>39</sup> (Fig. 6B). In addition, CEM2 and CEM3 were particularly affected, with conductivity losses of 23 and 24% respectively. This is possibly due to their positioning in the electrodialysis cell, where they come into contact with divalent ions and  $\text{OH}^-$  ions generated by the BPMs. In an alkaline environment, divalent cations from the tofu whey can form hydroxides and precipitate on the membrane.<sup>13</sup> The conductivity losses can also be attributed to the presence of cations with lower conductivity, such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , in transit in the membrane during the demineralization, as previously reported.<sup>39,40</sup>

### 3.3 Protein recovery yield and composition of final precipitate and supernatant

The protein recovery yield was 25.1%. This yield increased compared to those obtained in a previous study using the same process (NF) but with lower VCF (1 $\times$ , 2 $\times$  and 3 $\times$ ). Indeed, the authors reported recovery yields of 16.2%, 16.9% and 19.4% for the 1 $\times$ , 2 $\times$  and 3 $\times$  VCF respectively, representing an increase of 20% from 1 $\times$  to 3 $\times$ .<sup>5</sup> In the present study, a recovery yield of 25.1% was obtained, representing an increase of 55% compared to the 1 $\times$  tofu whey, and 29% compared to the 3 $\times$ . The increase from 1 $\times$  to 3 $\times$  is similar to the increase from 3 $\times$  to 9 $\times$ , suggesting a proportional relationship between the concentration of tofu whey and protein recovery. This increase in protein concentration is explained by an increase in hydrophobic interactions between proteins at low ionic strength.<sup>12</sup> However, losses of fines were observed in the supernatant, possibly indicating that a conductivity of 2 mS cm<sup>-1</sup> was still too high for optimal protein precipitation. In the previous study of Deschênes Gagnon *et al.* (2023),<sup>5</sup> the final conductivities of tofu wheys after EDBM were 1.5, 1.6 and 1.8 mS cm<sup>-1</sup> for 1 $\times$ , 2 $\times$  and 3 $\times$  VCF, and no fines losses were observed in the supernatant.

The composition of the final lyophilized precipitate fraction and supernatant is also detailed in Table 2. The precipitate fraction was particularly rich in protein and fat, with concentrations of 43.6% and 33.1%, respectively. The protein purity in the final precipitate fraction is similar to the purity reported in a previous study by Deschênes-Gagnon *et al.* (2023).<sup>5</sup> In contrast, the supernatant contained approximately half the protein content of the precipitate, with a concentration of 19.5%. It also exhibited only trace amounts of fat (2.3%), highlighting a significant separation of lipids into the precipitate. The supernatant was notably rich in sugars, accounting for 60.9% of total sugars, whereas the precipitate retained only minimal sugar content (4.9% of total sugars). Furthermore, the ash content was higher in the supernatant (3.4%) compared to the precipitate (1%).

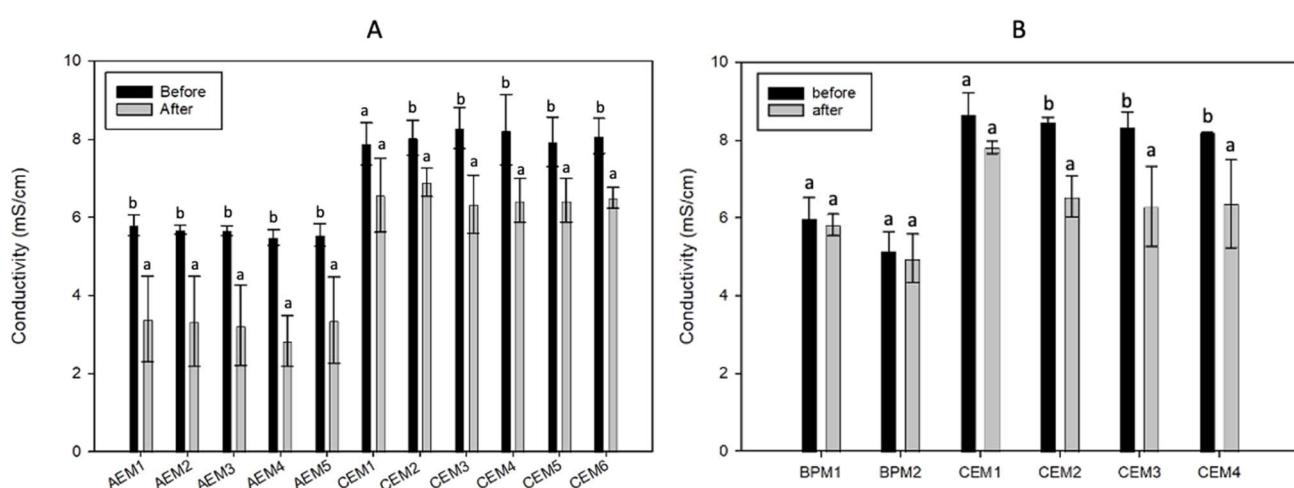


Fig. 6 Membrane conductivity before and after (A) ED and (B) EDBM. Different letters indicate significant differences between the means (Tukey,  $p < 0.05$ ).



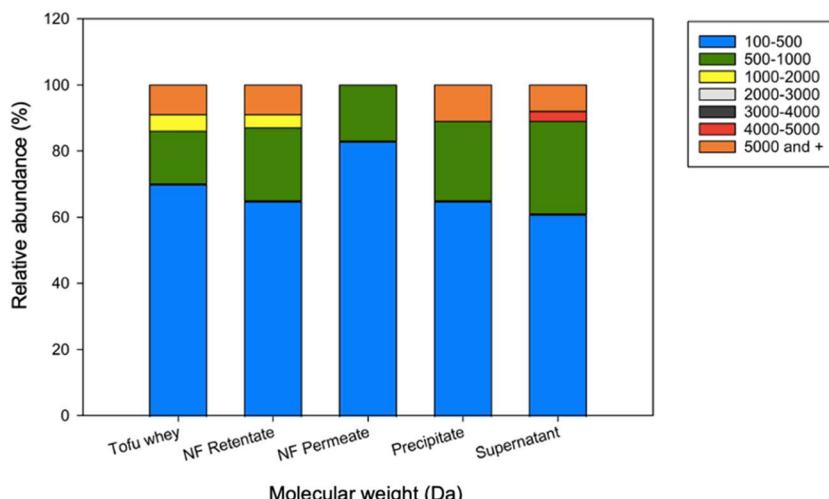


Fig. 7 Relative molecular mass distribution in tofu whey, NF retentate, NF permeate, precipitate and supernatant.

### 3.4 Molecular weight distribution

The molecular weight distribution of proteins and peptides in the initial tofu whey, NF retentate, NF permeate, final precipitate and supernatant is presented in Fig. 7. In the tofu whey, there was a higher relative abundance of peptides and small proteins with molecular masses ranging from 100–1000 Da. In addition, as molecular weight increased, the abundance decreased, since most of the large proteins coagulated to form tofu, leaving only smaller proteins/peptides in tofu whey. However, a slight increase in the abundance of proteins with masses higher than 5000 Da was observed. Overall, the NF retentate, final precipitate, and supernatant exhibited similar molecular weight distribution profiles to the initial tofu whey. In contrast, the NF permeate had a different profile, with a higher abundance of peptides or small proteins within the range of 100–500 Da, and no protein with masses higher than 1000 Da. This is consistent with the 150–300 Da molecular weight cut-off of the membrane. Despite some minor

differences, the protein profile was very similar for all the ingredients, indicating that precipitation by EDBM and the centrifugation did not selectively isolated specific proteins. Finally, this molecular weight profile highlights the interest for choosing an NF membrane over a UF membrane. These results are consistent with a previous study in which proteins from tofu whey were characterized using proteomic analysis. This analysis revealed that tofu whey proteins do not consist of the major soy globulins (7S and 11S), but rather comprise small, minor proteins poorly documented (Deschênes-Gagnon *et al.*, 2023).

### 3.5 Functional properties

**3.5.1 Solubility.** Regarding the solubility of the products (Fig. 8), tofu whey, retentate and supernatant were the most soluble products, and that, whatever the pH values. As indicated before, NF permeate was not considered for functional properties analyses since its protein content was too low (Section 3.1). These three fractions had similar solubility values varying from

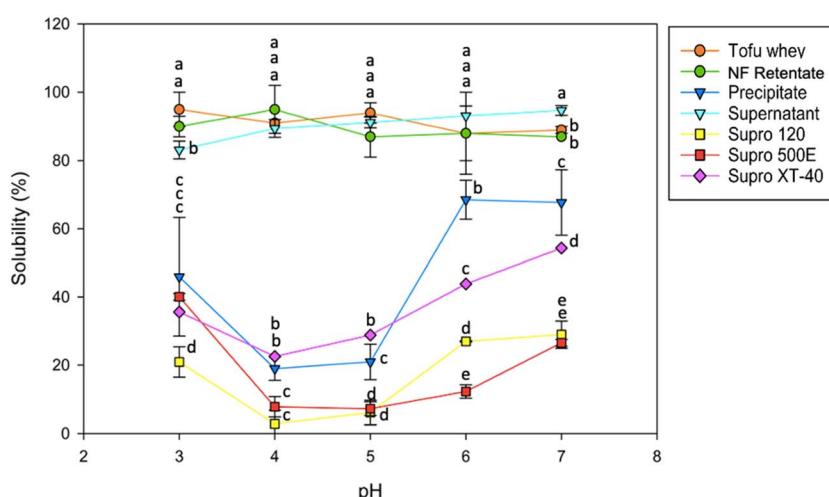


Fig. 8 Solubility of tofu whey, NF retentate, precipitate, supernatant and commercial SPIs. Different letters indicate significant differences between the means of each fraction at each pH (Tukey,  $p < 0.05$ ).



95% to 83%. This high solubility was expected since the protein in tofu whey and retentates are soluble protein after coagulation during tofu production and the proteins composing the supernatant remained soluble after the electroacidification step. In addition, even at a pH value corresponding to the isoelectric point of soy proteins (pH of 4.5), the proteins in tofu whey and retentate remained highly soluble compared to the precipitate fraction. This difference is likely due to the higher salt content in tofu whey and the NF retentate, which is 16.7% and 6.9%, respectively. In contrast, the salt content in the precipitate fraction is much lower, ranging from 1.0%, as most of the salts were removed during ED, EDBM and centrifugation steps of the process. As a result, the solubility of the protein in tofu whey and retentate remains high due to the salting-in effect. At low to moderate salt concentrations, the solubility of proteins is enhanced because the salt molecules interact with the proteins, reducing protein–protein attraction and preventing aggregation.<sup>41</sup>

The precipitate fraction and commercial isolates had solubility values that form a typical U-shaped curve according to the pH, with a minimum between pH 5 and 4, corresponding to the isoelectric point of the soy proteins.<sup>42,43</sup> Precipitate had a higher solubility than Supro 500E and Supro 120 but similar to Supro XT-40. In SPIs, the major proteins are 7S and 11S, having molecular weight of about 180–210 kDa and 320 kDa respectively.<sup>44–46</sup> However, the proteins found in tofu whey, and consequently in the precipitate, primarily consist of smaller proteins and peptides, most of which are under 1000 Da in size, that remained soluble during the tofu production.<sup>47</sup> These smaller proteins and peptides are expected to have better solubility than the larger, more complex globulins due to their higher surface-to-volume ratio and increased exposure of hydrophilic groups.<sup>48,49</sup> Finally, the drying method used to produce our ingredients was freeze drying, in contrast to the spray-drying process employed for commercial isolate.<sup>50</sup> Indeed, spray-drying tends to cause more protein denaturation due to thermal and air interface-related stresses, compared to freeze-drying, which can negatively impact protein solubility.<sup>51</sup> This could also explain the lower solubility of the SPIs. Solubility values of the commercial isolates were similar to those reported in the literature.<sup>52,53</sup> In comparison to the other two commercial SPIs, Supro XT-40 demonstrated greater solubility within the pH range of 4 to 7. According to the manufacturer, the XT serie, in contrast to the Supro serie, was designed specifically to improve dispersibility and solubility for beverage formulation. This explains the superior solubility of Supro XT-40 compared to Supro 120 and Supro 500E.

**3.5.2 Foaming properties.** The supernatant presented the higher foaming capacity and foam stability until 60 min, followed by the retentate (Fig. 9 and 10). First, those are the products with higher solubility with the tofu whey, which allows for better functionality. Furthermore, they contain higher amounts of salt and sugars compared to precipitate and SPIs, which are molecules that enhance foaming capacity. Indeed, it has been reported that higher sugar content can increase the solution viscosity and the foam stability of egg white proteins and whey protein isolate.<sup>54–56</sup> Furthermore, up to a maximum

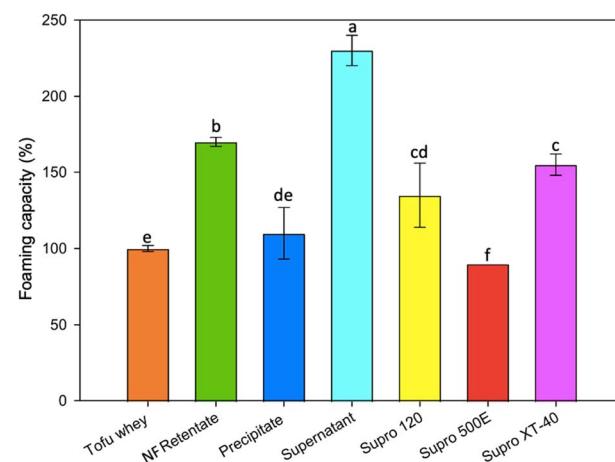


Fig. 9 Foaming capacity of tofu whey, NF retentate, precipitate, supernatant and commercial SPIs. Different letters indicate significant difference between the means of each fraction (Tukey,  $p < 0.05$ ).

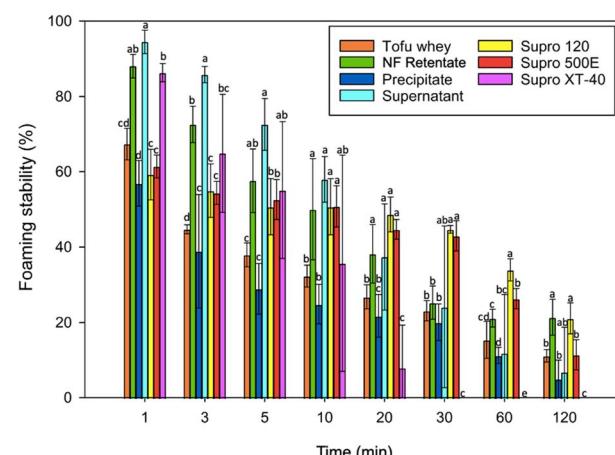


Fig. 10 Foaming stability of tofu whey, NF retentate, precipitate, supernatant and commercial SPIs. Different letters indicate significant difference between the means of each fraction at each time (Tukey,  $p < 0.05$ ).

concentration, salts act on the foamability of proteins by decreasing intermolecular electrostatic repulsion and enhancing surface activity, resulting in a thicker layer of protein adsorbed at the interface and higher foaming capacity.<sup>57,58</sup> Therefore, these differences in composition could explain the higher foaming properties of the supernatant and retentate. However, although tofu whey contains a high sugar concentration, the salt concentration ( $16.7 \pm 0.3\%$ ) is likely too high compared to the protein concentration ( $15.1 \pm 0.7$ ), which affects the foaming properties of tofu whey.<sup>49,57,59,60</sup>

For precipitate, despite its higher solubility compared to commercial isolates, it had similar foaming capacity to Supro 120 and 500E, and lower than Supro XT-40. It also had a lower foam stability. This might be caused by its higher fat content. Indeed, precipitate contain 34% of fat, which is known to reduce foaming properties. This has been reported for many protein sources such as milk proteins, chickpea, pea and

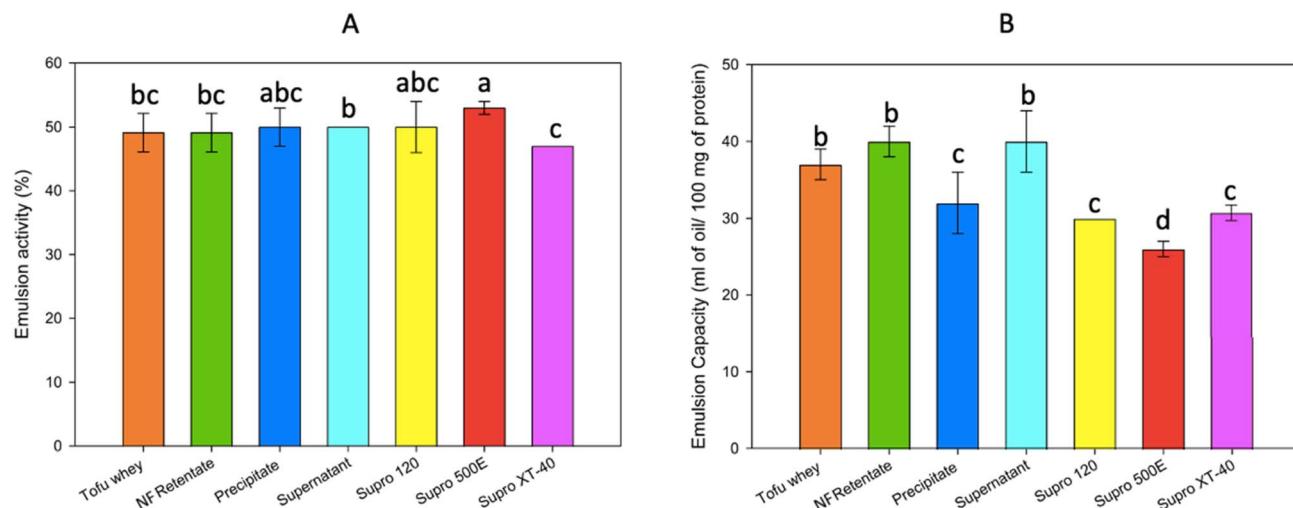


Fig. 11 (A) Emulsion activity and (B) emulsion capacity of tofu whey, NF retentate, precipitate, supernatant and commercial SPIs. Different letters indicate significant differences between the means of each fraction (Tukey,  $p < 0.05$ ).

mealworm proteins.<sup>61–64</sup> In another study comparing the foaming properties of SPI and soy whey proteins (from SPI manufacture), similar results were observed. Soy whey proteins allowed the formation of foams more quickly and with greater density and showed better stability at pH 7 than SPI, because of their better solubility.<sup>49</sup>

**3.5.3 Emulsifying properties.** In general, the emulsifying properties are quite similar among the different products (Fig. 11A and B and 12). However, there were some differences observed regarding EC and ES. Firstly, the tofu whey, retentate and supernatant had a higher EC than the precipitate and the three commercial isolates, which is likely linked to their better solubility (Fig. 11B). Indeed, higher solubility leads to a better adsorption of the proteins at the water–oil interface to decrease the interfacial tension.<sup>65,66</sup> In addition, smaller proteins and peptides diffuse quickly at the interface compared to larger proteins.<sup>67,68</sup> As demonstrated earlier, the ingredients from

whey are composed of a majority of proteins under 1000 Da. Conversely, commercial SPIs contain a majority of 7S and 11S proteins which are larger proteins. In addition, these proteins have a complex tertiary and quaternary structure, limiting their emulsion properties as they cannot unfold in their native state.<sup>67,68</sup> Regarding the ES, values were similar for each ingredient until 1 h (Fig. 12). Then, the emulsion stability was lower for Supro XT-40 but very similar for other ingredients. Indeed, as mentioned earlier, the Supro XT serie is formulated for better dispersibility and solubility, while Supro 120 and 500E are both known to provide good emulsion stability.

## 4. Conclusion

In this study, we explored a process for recovering residual proteins from tofu whey, in order to valorize this by-product in a circular economy framework. This new process involved the pre-concentration of the tofu whey by NF at a 9× VCF, a demineralization by ED followed by an electro-acidification through EDBM and a final centrifugation to separate the insoluble protein from the rest of the soluble ones. This approach allowed for a 25.1% recovery of tofu whey proteins, which is about 29% higher than a previous study using the same process but with a 3× VCF. This improvement highlights the significant impact of the volume concentration factor on protein precipitation, emphasizing the importance of concentrating tofu whey and reducing its salt content (ionic strength) to enhance protein–protein interactions. Additionally, our study identified that the majority of proteins in tofu whey are smaller than 1000 Da, making NF membranes ideal for retaining and concentrating proteins in the retentate.

Furthermore, the functional properties of all fractions generated at every step of this new process were analyzed for the first time and compared to three commercial isolates. Surprisingly, these fractions, composed of small minor soy proteins, exhibit functional properties that are comparable to, and in some cases even better than, those of commercial soy isolates made up of 7S and 11S globulins, well-known for their functionalities. The

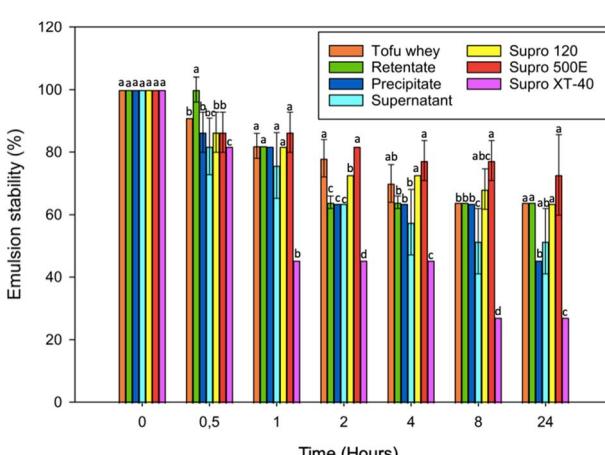


Fig. 12 Emulsion stability of tofu whey, NF retentate, precipitate, supernatant and commercial SPIs. Different letters indicate significant differences between the means of each fraction at each time (Tukey,  $p < 0.05$ ).



supernatant and retentate exhibited better solubility, foaming properties, and emulsifying capacity than the SPIs, while the tofu whey and precipitate demonstrated similar functional properties as the commercial isolates. The higher fat content of the precipitate and the higher salt/protein ratio of the tofu whey may explain their lower foaming properties. According to these results, all of these fractions generated by this coupled process could be used as functional ingredients in food formulation, such as frozen desserts and meat substitutes, providing added value to tofu whey. However, the protein content of supernatants is only about 20% with a majority of sugars, which could present some limits in food formulations. The other products generated in the process, with no protein or low protein content, could also be valorized: The recovery solution of ED enriched in cations ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) from tofu whey could be reused as coagulants in further tofu productions. Additionally, in order to valorize the NF permeate, a demineralization step by ED would allow the separation of salts and sugars. The demineralized NF permeate, could therefore be used as a culture media for the growth of microalgae for example.<sup>69,70</sup> Thus, this enters the model of a circular economy, and it would be possible to generate added-value ingredients or products from this waste.

Since the NF retentate was one of the products with the highest functionality, a simplified process without the EDBM step could be more economic and functionally interesting. Indeed, a significantly high added-value is required to justify the implementation of the complete NF and electromembrane process. A simpler approach, such as optimizing the concentration with diafiltration, may be sufficient to produce a protein fraction with good purity and desirable functional properties, especially since it has been observed that the protein profile remains consistent between the initial tofu whey and both the precipitated and supernatant fractions. Such an optimization of the proposed process is currently underway in our laboratory.

## Ethical statement

This work did not involve the use of human and animal subjects.

## Author contributions

Conceptualization, R. D. G., M.-È. L., F. L. and L. B.; methodology, R. D. G. and L. B.; software, R. D. G.; validation, R. D. G. and L. B.; formal analysis, R. D. G.; investigation, R. D. G.; resources, R. D. G., M.-È. L., F. L. and L. B.; data curation, R. D. G. and L. B.; writing—original draft preparation, R. D. G.; writing—review and editing, R. D. G., M.-È. L., F. L. and L. B.; visualization, R. D. G. and L. B.; supervision, L. B.; project administration, L. B.; funding acquisition, L. B. All authors have read and agreed to the published version of the manuscript.

## Conflicts of interest

The authors 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

Data is contained within the article. Please contact the corresponding author for further information.

## Acknowledgements

This research was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC), Alliance Grant: «Integrated valorization of coproducts by ecoefficient food technologies in the context of a circular economy (Consortium VITALE) » (Grant ALLRP561008-20 to Laurent Bazinet), the NSERC Discovery Grant Program (Grant SD RGPIN-2018-04128 to Laurent Bazinet) and the Consortium de Recherche et Innovations en Bioprocédés Industriels au Québec (CRIBIQ; Québec, QC, Canada) [2020-050-C67 to L. Bazinet]. The technical assistance of Jacinthe Thibodeau and Véronique Perreault research professionals at Université Laval is gratefully acknowledged. The authors also thank Véronique Richard, Patrick Leclerc, Françoise Nadeau and Diane Gagnon for their assistance in HPLC, ICP-OES, TLC and Micro-Leco analyses and Pascal Lavoie for his technical support during the NF process.

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