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Synergy of cold-induced aggregation and centrifugation to enhance microfiltration performance and protein purity in water-based pea protein extraction

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ABSTRACT

Avoiding the use of chemicals during the extraction of commercial pea proteins is crucial to preserve protein nativity and ensure sustainability, which is best achieved through water-only extraction coupled with membrane filtration. To minimize membrane fouling during filtration, this study evaluated various storage and centrifugation pretreatments of the pea extract to remove suspended solids prior to pilot-scale microfiltration (MF). Inducing aggregation via storage followed by centrifugations improved the flux significantly, where the cold-induced aggregation and centrifugation of the extract achieved nearly four times higher steady flux than the control. This flux improvement was due to the selective removal of aggregated legumin proteins during cooling and centrifugation, resulting in coextraction of pellets with protein purity >90%. Ultrafiltration (UF) of the same treated sample showed slightly improved flux compared to the control, suggesting that fine-tuning the feed pretreatments with the membrane pore size is of importance. It was confirmed that the proteins had no major secondary structure changes across all pretreatments. Measured viscoelastic properties indicated that all the supernatants have formed stronger gel networks than pellets. Overall, coupling storage and centrifugation conditions as a pretreatment of pea extract before MF enhances filtration performance and enables the coextraction of highly pure native proteins using only water, laying groundwork for a scalable approach for sustainable pea protein extraction.

1. Introduction

Pea protein has recently started to replace soy and animal-based protein sources due to its high nutritional value, low allergenicity, sustainability, availability, and low cost of production (Barac et al., 2010; Boukid et al., 2021; Lam et al., 2018). However, commercial pea proteins are generally denatured (Bu et al., 2022; Hansen et al., 2022) due to harsh extraction conditions (Pam Ismail et al., 2020), which hinders their application to many types of foods. Differences in extraction conditions contribute to differences in the protein profile and structure, ultimately impacting functionality (Hansen et al., 2022). To broaden the application of pea proteins and minimize the adverse impact of harsh extraction conditions on protein quality, mild and efficient processing methods are needed.

Recently, membrane separation processes have emerged as a milder alternative to conventional pea protein extraction to preserve the protein nativity and enhance the functional properties of pea proteins via (mild) alkaline-acid or salt extraction methods (Boye et al., 2010; Fredrikson et al., 2001; Gao et al., 2001; C. Kornet et al., 2020; R. Kornet et al., 2022; Mondor et al., 2012; Taherian et al., 2011). Lately, a novel processing route to extract native pea proteins with superior functionality has been studied, utilizing only water throughout the extraction process, which ultimately relies on membrane separation (Geerts et al., 2017; Möller, Li, et al., 2022). However, membrane processing of the pea extract obtained from water-only extraction suffers from fouling (Alemu et al., 2025), which requires feed pretreatment to enhance the filtration performance.

The most commonly used method to increase the efficiency of membrane separation of protein solutions is to chemically pretreat the feed by adjusting the pH above/below the isoelectric point of the proteins to increase the solubility (Ding et al., 2019; Huisman et al., 2000). This necessitates the exploration of novel sample pretreatment routes that eliminate the use of chemicals and improve the membrane separation performance.

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Centrifugation as a sample pretreatment before membrane filtration is used in different applications, such as wastewater treatment (Turano et al., 2002) and as one of the unit operations during the extraction of pea proteins (Alemu et al., 2025; Gravel et al., 2023; Möller, Li, et al., 2022; Mondor et al., 2012), which makes it easily integrable for sample pretreatment of the extract. Despite this potential, a systematic study of different centrifugation conditions as a pretreatment for pea protein extract before membrane filtration remains unexplored. Particularly, the influence of coupling storage-induced aggregation of the pea extract with centrifugation is absent in the literature. Storage of pea extract at room temperature and neutral pH causes aggregation of pea proteins (globular) (Durand et al., 2002). This aggregation process may be intensified by cold temperature storage, as Helmick reported evidence of structural destabilization of pea globulins at sub-zero temperatures, contributing to aggregation and phase separation (Helmick et al., 2021. We hypothesized, therefore, that cooling will induce the formation of larger protein aggregates that can be more easily removed by centrifugation, which will in turn reduce the impact of these proteins on membrane fouling during filtration of the supernatant (Huisman et al., 2000). At the same time, the coextracted aggregate obtained may constitute a valuable pea protein fraction, which can have a novel functionality for food applications, as suggested by Helmick et al. (2021). Given that this fraction is extracted mildly, it will provide native versatility for non-thermal post-extraction modification of pea proteins to modulate their functionality for wider application (Rout & Srivastav, 2024).

Therefore, this study systematically investigated the effect of different storage-induced aggregation and centrifugations during the wet extraction of pea proteins on the performance of membrane filtration of pea extracts and the co-extraction of novel pea protein fractions. Compositional and rheological characteristics of the supernatants and pellets obtained during pea protein extraction were also compared.

2. Materials and methods

2.1. Materials

Dried whole yellow pea (*Pisum sativum L.*) was purchased from Alimex (Sint Kruis, Netherlands) and Milli-Q water (resistivity 18.2 MΩ•cm, Merck Millipore, France) was used for wet extraction.

2.2. Milling of the pea seed

An adapted milling protocol from *Pelgrom* et al. was used to ensure maximum separation of the protein bodies and starch granules during aqueous protein extraction (Pelgrom et al., 2013). Briefly explained, the peas were pre-milled to reduce the size of the peas into grits using a pin mill (LV 15 M, Condux-Werk, Wolfgang Bei Hanau, Germany) at room temperature. Subsequently, the grits were further milled into fine pea flour by a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany). The operating parameters were set as follows: the impact mill speed at 8000 rpm, the classifier wheel speed at 4000 rpm, the airflow at 52 m^3/h, and the feeder rotating at 2 rpm (approximately 0.75 kg/h). A thermometer inside the mill was mounted to monitor the operating temperature, which does not exceed 33 °C. The flour was then stored at −20 °C before use.

2.3. Wet extraction/ sample preparation before membrane experiments

The wet extraction process was adapted from (Möller, Li, et al., 2022), with a modified centrifugation step. Briefly, the flour and water are mixed with a 1:10 ratio, which is 300 g of flour with 3 L of Milli-Q water. The mixture is stirred using an overhead stirrer mixer at a speed of 1600 rpm for 1 h at room temperature. The suspension was then subjected to subsequent sample pretreatments to assess their effect on the membrane performance.

2.4. Experimental design

The experimental design used for exploring the effect of different storage and centrifugation conditions is depicted in Fig. 1. The first set of pretreatment conditions explores the effects of various centrifugation speeds on the resulting fractional composition and permeate flux during membrane filtration of the supernatants (Fig. 1, route 1, dotted red lines), which are denoted in the figure as A, B, and C, respectively. During this process, starch and insoluble proteins settled at the bottom and were collected using a spatula after the supernatant had been gently poured into a separate container.

For the second set of pretreatment conditions, the supernatant obtained at a centrifugation speed of 10,000 ×*g* (control sample that benchmarks the control of the first pretreatment condition and standard centrifugation speed used for pea protein extraction) was used to further explore the effect of storage conditions combined with centrifugation prior to MF (Fig. 1, route 2-blue solid lines). These pretreatments include: (1) overnight storage at 20 °C followed by centrifugation at 10,000 ×*g* at 20 °C (warm storage-warm centrifugation), (2) overnight storage at 20 °C followed by centrifugation at 10,000 ×*g* at 4 °C (warm storage-cold centrifugation), and (3) overnight storage of the supernatant at 4 °C followed by centrifugation at 10,000 ×*g* at 20 °C (cold storage-warm centrifugation), which are denoted as D, E, and F, respectively, in Fig. 1. The terminology of the conditions stated in the brackets/ Fig. 1 is used in the sections below. All membrane experiments in the experimental design are conducted at least in duplicate.

The fractions obtained from all experiments were freeze-dried using a pilot-scale freeze dryer (Chris Epsilon 2- 6D, Osterode am Harz, Germany) and used for analysis.

2.5. Membrane experiment

A pilot-scale membrane system was used for the filtration experiments of the pea extract. The layout of the pilot-scale membrane set-up is presented in (Alemu et al., 2025). Polyvinylidene fluoride (PVDF) MF membranes with an average pore size of 0.1 μm (Synder Filtration, Vacaville, CA, USA) and Polyethersulfone (PES) UF membranes with MWCO 50 kDa (Synder Filtration, Vacaville, CA, USA) were used. All membranes used are in a spiral wound configuration and have a 31 mil spacer. Filtration experiments were performed in recirculation mode at a transmembrane (TMP) of 1 bar and a crossflow velocity of 0.1 m/s at 20 °C. The complete characteristics of the membranes are shown in Table 1.

2.6. Protein content determination

The protein content was determined by the Dumas nitrogen combustion method using a nitrogen analyzer (rapid N exceed-analyzer, Elementar, Langenselbold, Germany). The nitrogen measured was converted into the protein content by using a conversion factor of 6.25 (Mondor et al., 2012). Aspartic acid with known nitrogen content was used as a calibration curve to convert the nitrogen content measured for the samples into the protein content. The protein content of the samples was measured in duplicate.

2.7. Dry matter (DM) and ash content

The DM content of wet samples was measured using an infrared moisture analyzer (MA35, Sartorius AG, Germany) at 105 °C.

2.8. HPSEC analysis

Molecular weight distributions of the retentates and permeates from the membrane experiment were characterized using HP-SEC with two consecutive columns (TSK gel G4000SWxl, and TSK gel G3000Wx, Tosoh Bioscience GmbH Germany). The sample preparation is partly

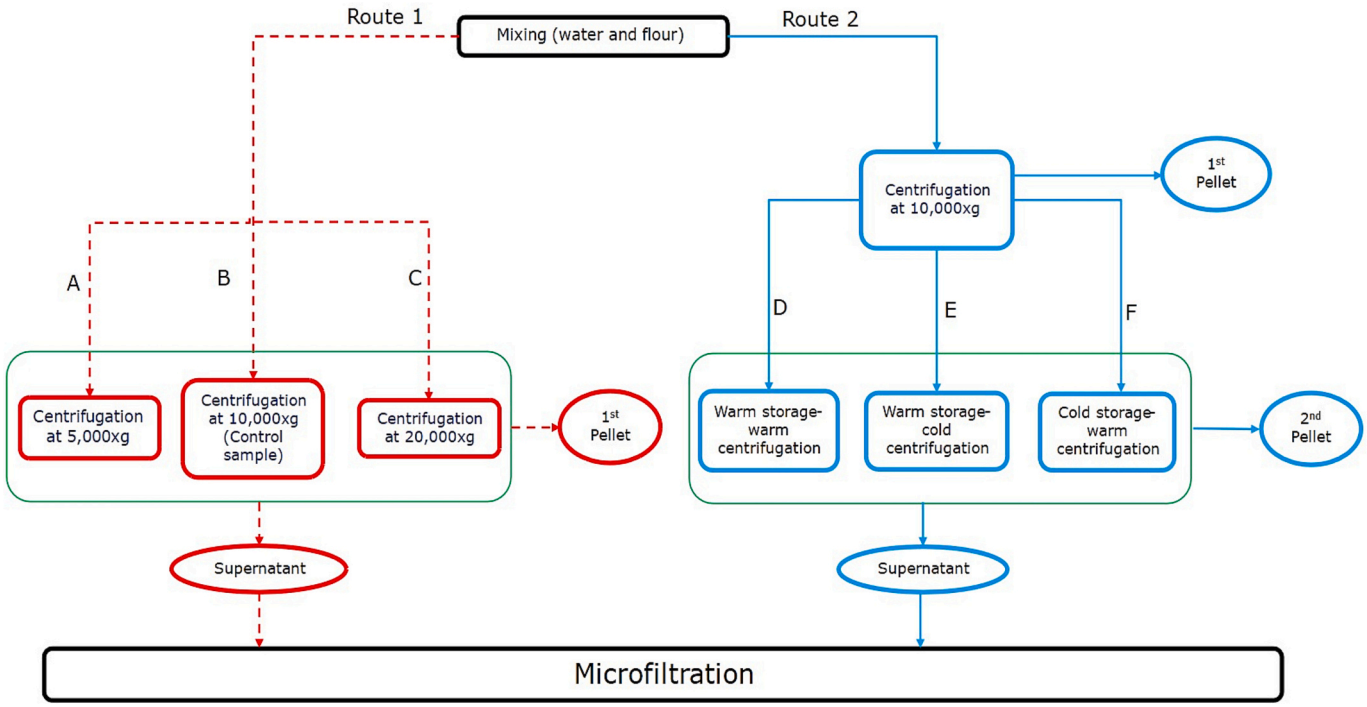

Fig. 1. Experimental design for exploring the effect of different pretreatments on the membrane performance and composition of the respective fractions. Route 1 (Red dotted lines) explores the effect of different centrifugation speeds, and route 2 (Blue solid lines) explores the effect of different storage followed by subsequent centrifugations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Characteristics of membranes used in this study.

Name	Supplier	Configuration	Membrane Material	pH resistance	Max. operating temperature (°C)	Max. inlet pressure (bar)	Membrane area (m^2)	Cut-off/ pore size
V0.1	Synder	Spiral wound	PVDF	2–10	55	8.3	0.3	0.1 μm
MQ-Max	Synder	Spiral wound	PES	2–10	55	8.3	0.33	50 kDa

adapted from (C. Kornet et al., 2020). The retentate and permeate samples were thawed from the freezer and vortexed for 2 min for better mixing. The vortexed samples were then centrifuged at 10,000 ×*g* for 10 min. The supernatant was then pipetted into the vials before HPSEC analysis.

The composition of the eluent for the HP-SEC was 30% (*v*/v) acetonitrile and 0.1% (v/v) tri-fluoroacetic acid. The columns were operated at 30 °C with a flow rate of 1.5 ml/min. The absorbance was detected at a wavelength of 214 nm. A calibration curve was made with known molecular weight compounds: Thyroglobulin (670 kDa), Bovine serum albumin (66.5 kDa), B-Lactoglobulin (36 kDa), A-Lactalbumin (14.5 kDa), Aprotinin (6.51 kDa), Bacitracin (1.42 kDa), and Phenylalanine (0.165).

2.9. Particle size distribution

Particle size analysis was conducted using a method adapted from (Tanger et al. (2022). The samples were analyzed by Malvern Mastersizer Hydro 3000E (Malvern Panalytical Ltd., Malvern, UK) by diluting them to 1% concentration. A refractive index of 1.45 for the particles and a refractive index of 1.33 for water (as a dispersant) were assumed. The absorbance was set at 0.001.

2.10. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The protein compositions of soluble and insoluble protein fractions were analyzed using SDS-PAGE (Bio-Rad Laboratories, Hercules, USA) under reducing and non-reducing conditions. Under reducing conditions, the sample buffer (2× Laemmli buffer (Bio-Rad Laboratories Inc., USA)) is mixed with Dithiothreitol (DTT) in a 1:19 ratio, while for the non-reducing condition, the sample buffer was used without mixing with DTT. The pea protein samples were prepared in a concentration of 2 mg/ml and were mixed with the respective sample buffer at a 1:1 ratio. The mixtures were heated to 95 °C for 10 min on a Thermomixer (Eppendorf AG, Germany) and cooled down for 30 min. Then, they were centrifuged at 10,000 ×*g* to remove the precipitates. Samples of 20 μl and standard marker of 10 μl (Precision Plus Protein standards 10–250 kDa, Bio-Rad, USA) were loaded onto the 12% precast gel (Mini-PROTEAN® TGXTM, Bio-Rad, USA). The electrophoresis was done at 200 V by inserting the loaded precast gel in a tank containing TGS running buffer. The gel is then removed and rinsed with Milli-Q water. Finally, Bio-SafeTM Coomassie G-250 brilliant blue (Bio-Rad, Germany) was used for staining the gel, and the stained gel was scanned using a gel scanner.

2.11. Fourier-transform infrared spectrometry analysis (FTIR)

An Invenio-S FTIR spectrometer (Bruker Optics GmbH & Co. KG.,

Ettlingen, Germany) was used for the FTIR analysis, which has a liquid nitrogen-cooled MCT detector. Ultrapure water was first measured, and the obtained spectra were used as a background (baseline) measurement. Then, 20 µl of a sample was loaded onto a BioATR II crystal (sample holder) controlled at 25 °C and was allowed to equilibrate for 2 min, followed by a subsequent measurement. The measurements were adjusted to take 128 scans at a resolution of 4 cm^{-1}. Structural changes of the proteins of each sample were compared by vector normalization and the second derivative of the *amide I* region of the spectra using OPUS software (version 8.5).

2.12. Rheological properties of the fraction

A protocol for measuring the rheological properties was adapted from (R. Kornet, Penris, et al., 2021). The linear viscoelastic response of the fractions was measured upon heating and cooling of the samples using an MCR301 rheometer (Anton Paar, Austria) fitted with concentric cylinder geometry. During this temperature sweep experiment, the samples were prepared at a normalized protein content of 10%, for which the dry matter of some low protein-containing samples was higher than those with high protein content to maintain a 10% protein content. After loading the samples into the rheometer, a highly liquid paraffin oil was applied to the surface of the sample to prevent evaporation during the actual measurement. The samples were then equilibrated for 5 min at 20 °C at zero oscillation, heated from 20 °C to 95 °C at a heating rate of 3 °C/min, held at 95 °C for 10 min, cooled to 20 °C at a cooling rate of 3 °C/min, and finally kept at 20 °C for 5 min. Storage (G') and loss (G") moduli were recorded as a function of temperature and time at a constant frequency of 1 Hz and strain amplitude of 1%.

2.13. Statistical analysis

Statistical analysis was performed using one-way ANOVA (SPSS, 30.0.0.0) to compare differences among samples obtained from different treatments, followed by Tukey's HSD post hoc test by setting the significance at $p < 0.05$. Results are reported as mean ± standard deviation (SD).

3. Results and discussion

Mixing of pea flour with water and subsequent centrifugation enabled the separation of pea extract from the rest. Storing and centrifuging of the pea extract resulted in pellets and supernatants. Before membrane separation of the resulting supernatants, the fractions from

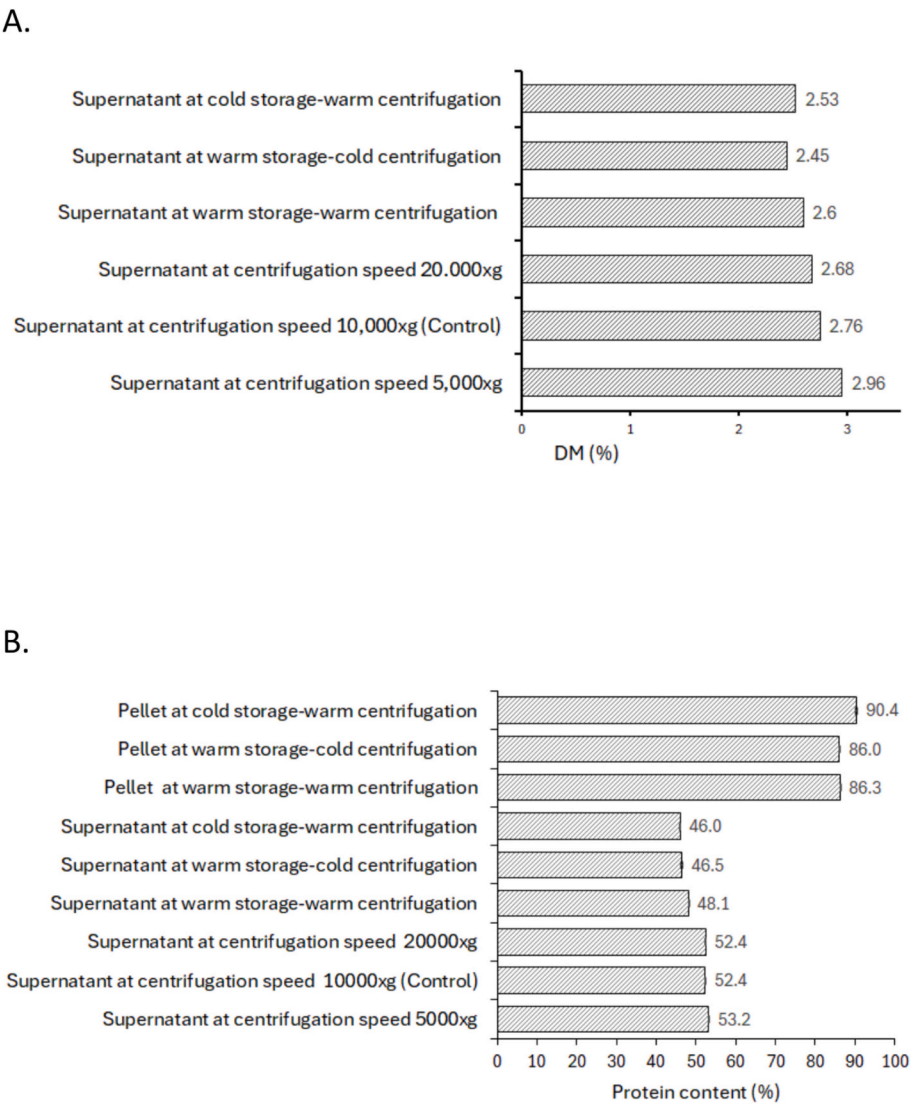

Fig. 2. Dry matter content (A) of all the supernatants and protein contents (B) of all pea extract fractions under different (storage and) centrifugation conditions. The dry matter content is based on the total volume, while the protein content is expressed based on total dry matter.

different pretreatment methods were characterized based on dry matter, protein content, SDS-PAGE, FTIR, Particle size, and viscoelasticity, as discussed below.

3.1. Dry matter and protein content of pea extract fractions

Dry matter content during exploration of the effect of centrifugation speed showed a logical order: the supernatant centrifuged at the highest speed exhibited the lowest dry matter content and vice versa (Fig. 2A). Supernatants obtained from combined storage and centrifugation

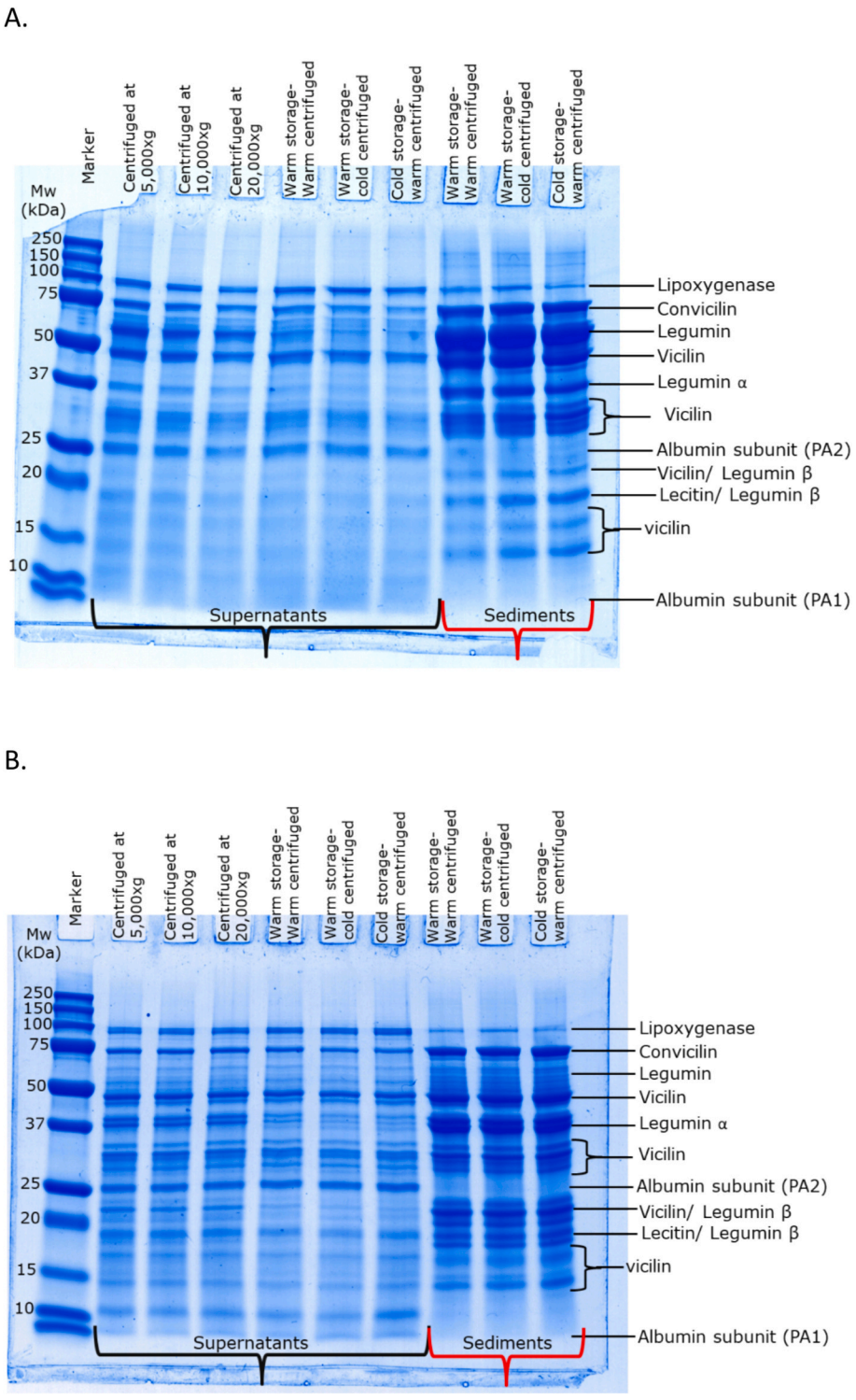

Fig. 3. SDS-PAGE analysis (A. Non-reducing and B. reducing conditions) of samples obtained from different centrifugation speeds and fractions obtained by different storage and centrifugation pretreatment conditions. The intensity of the bands for the polypeptides present in the pellets is more intense than in the supernatants, even when they are present in both fractions, because all the samples were prepared based on the same dry matter, which will cause higher protein concentration in the pellets, as they contain >80% protein.

treatments had, in general, lower dry matter than the control sample, with at least 6% lower than the control sample (2.76%). A consistent ≥6% difference in dry matter was obtained using a different pea batch between the control sample (2.50 ± 0.01%) and warm stored-warm centrifuged (2.35 ± 0.04%), warm stored-cold centrifuged (2.31 ± 0.01%), and cold stored-warm centrifuged (2.29 ± 0.06%) sample. Warm storage-cold centrifugation and cold storage-warm centrifugation samples have slightly lower dry matter content than the warm stored-warm centrifuged sample (2.6%). The cooling step involved in both treatments may have forced more suspended solids to settle in the pellet than without cooling.

The protein content of the pea fractions at each condition is presented in Fig. 2B. The centrifugation speed has an insignificant impact on the protein content of the resulting supernatants, except for a slightly higher protein content at the lowest centrifugation speed. The supernatants found by combined storage and centrifugation of the pea extract have minor differences, with the warm storage-warm centrifuged sample having the highest protein content, while the other two supernatants have insignificant differences. The supernatants from combined storage and centrifugation have, in general, a little less protein content than those pretreated at different centrifugation speeds only. This is because the additional storage and centrifugation removed extra proteins (aggregates) from those supernatants.

It is interesting to observe that a very pure protein fraction can be extracted as pellets using only water and combined storage and centrifugation of the pea extract. This protein purity is much higher than that achieved by known mild processes such as dry fractionation, mild wet fractionation, and previous water-only extraction methods (Möller, Li, et al., 2022; Pelgrom et al., 2013). The proteins in the suspension formed aggregates during storage at room/cold temperatures, ultimately resulting in the removal of suspended solids upon centrifugation. The protein content of the pellet from the cold storage condition is even higher than that of the other pellets. The cooling step, therefore, caused more protein aggregation due to hydrophobic interaction (Dias et al., 2010; Helmick et al., 2021), and the aggregates were removed during centrifugation. Warm storage of the pea extract has also induced protein aggregation that was removed during the subsequent centrifugation step. The resulting pellet contained fairly comparable protein content to that from cold storage. This is due to the extraction steps at neutral pH already inducing aggregation of proteins, and longer storage time stabilizes the aggregates (Durand et al., 2002; Lu et al., 2020), which can be further intensified by additional weakening of the hydrophobic forces due to cold denaturation at a lower temperature (Dias et al., 2010; Helmick et al., 2021). The average amount of protein removed as pellets due to the pretreatments is approximated to be around 8% of the total dry matter in the supernatant.

3.2. SDS-PAGE results of the fractions

The polypeptide compositions of the different pea extract fractions, both the supernatants and pellets, obtained under different pretreatment conditions, were analyzed using SDS-PAGE under non-reducing and reducing conditions by preparing the samples at the same dry matter content (Fig. 3). To fairly compare the results, the same analysis was also done by normalizing the protein contents of all the samples (Appendix Fig. A1). Under non-reducing conditions, the compositions of the supernatants obtained at different centrifugation speeds are quite similar. This shows that polypeptides were not affected by the centrifugation, except that the intensity of the bands was slightly decreased with increasing centrifugation speed, which we attribute it to the removal of extra proteins. Supernatants from warm storage-warm centrifugation, warm storage-cold centrifugation, and cold storage-warm centrifugation exhibited similar compositions. However, legumin is depleted from them, whereas the legumin band is apparent in the SDS-PAGE of the pellets obtained from all these conditions. This indicates that the legumin proteins favored aggregate formation and were removed through subsequent centrifugation. Albumin subunits appeared more intensely in the supernatants than in the pellets, whereas high molecular weight but soluble proteins like lipoxygenase were also enriched in the supernatant and diminished in the sediment. This distribution reflects the higher solubility of these proteins at the extraction pH, which prevents their precipitation during storage. Similarly, convicilin and vicilin are present in both the supernatant and the pellet. This could be due to the slow precipitation of these proteins, for which a certain fraction will end up in the pellets while the remaining fraction stays in the supernatant.

Despite differences in pretreatment, all sediment fractions possessed a similar composition characterized by reduced levels of lipoxygenase and albumins, alongside intensified bands for convicilin, legumin, and vicilin. Under reducing conditions, the legumin fraction (with MW ~60 kDa) of all the samples is dissociated into acidic (~40 kDa) and basic (~21–23 kDa) subunits (Chen et al., 2019; Emkani et al., 2021). The legumin band is therefore missing in the SDS-PAGE under reducing conditions.

In summary, varying centrifugation speeds do not result in significant differences in polypeptide composition among the samples. At the same time, storage and subsequent centrifugations of the pea extract resulted in two distinct fractions (supernatant and pellet), which have the same polypeptide profile for each treatment method.

3.3. FTIR profiles of the pea extract from different treatments

FTIR analysis of the pea extract fractions from warm storage-warm centrifugation and cold storage-warm centrifugation was conducted to detect major changes in the secondary structure of pea proteins upon (cold) storage, as shown in Fig. 4. A fresh supernatant, just after mixing and centrifugation, was measured immediately. This same supernatant was then stored at room temperature and 4 °C overnight by stirring it until measurement, to prevent sedimentation of the aggregates during FTIR measurement. These two samples were centrifuged to remove the pellets, and their supernatants were measured for their FTIR spectra. The obtained characteristic peaks (Fig. 4 A and C) showed the presence of proteins, carbohydrates, and lipids in all supernatants. The absorbances of the supernatants are, however, a little lower than those of the supernatants before the second centrifugation, which corresponds with the amounts/concentrations in the sample (Bhuiyan et al., 2024). Detected components correspond to specific IR spectra wavelength ranges of 3000–2800 cm^{-1}, 1800–1500 $^{m-1}$, and 1200–900 cm^{-1}, which represent lipid, protein, and polysaccharides, respectively (Bhuiyan et al., 2024). The absorbances in the amide l and II (1700–1600 cm^{-1}) region showed insignificant differences between samples, suggesting comparable protein contents.

As shown in Fig. 4 B and D, the secondary structure of the warm and cold stored samples, together with their supernatants, was compared by determining the second derivative of the obtained FTIR spectra in the amide l region (1700–1600 cm^{-1}). The minimum obtained in the derived spectra at 1630 cm^{-1}, 1650 cm^{-1}, and 1615 cm^{-1} represents intramolecular beta sheet, alpha helices, and intermolecular beta sheets, respectively.

The results show that all samples retained their native conformation and were not denatured. Thus, the aggregation of proteins upon warm/cold storage is due to physical interaction among proteins without experiencing significant unfolding.

3.4. Particle size analysis

The particle size analysis of pea extracts treated at different centrifugation speeds showed a multimodal particle size distribution, and the largest particles, as large as 100 μm in diameter, had a higher volume density compared to the smaller ones. All samples exhibited the same peak profile within a reasonably comparable peak height. Therefore, different centrifugation speeds have an insignificant impact on the particle size distribution of the supernatants (Fig. 5 A). In all of the

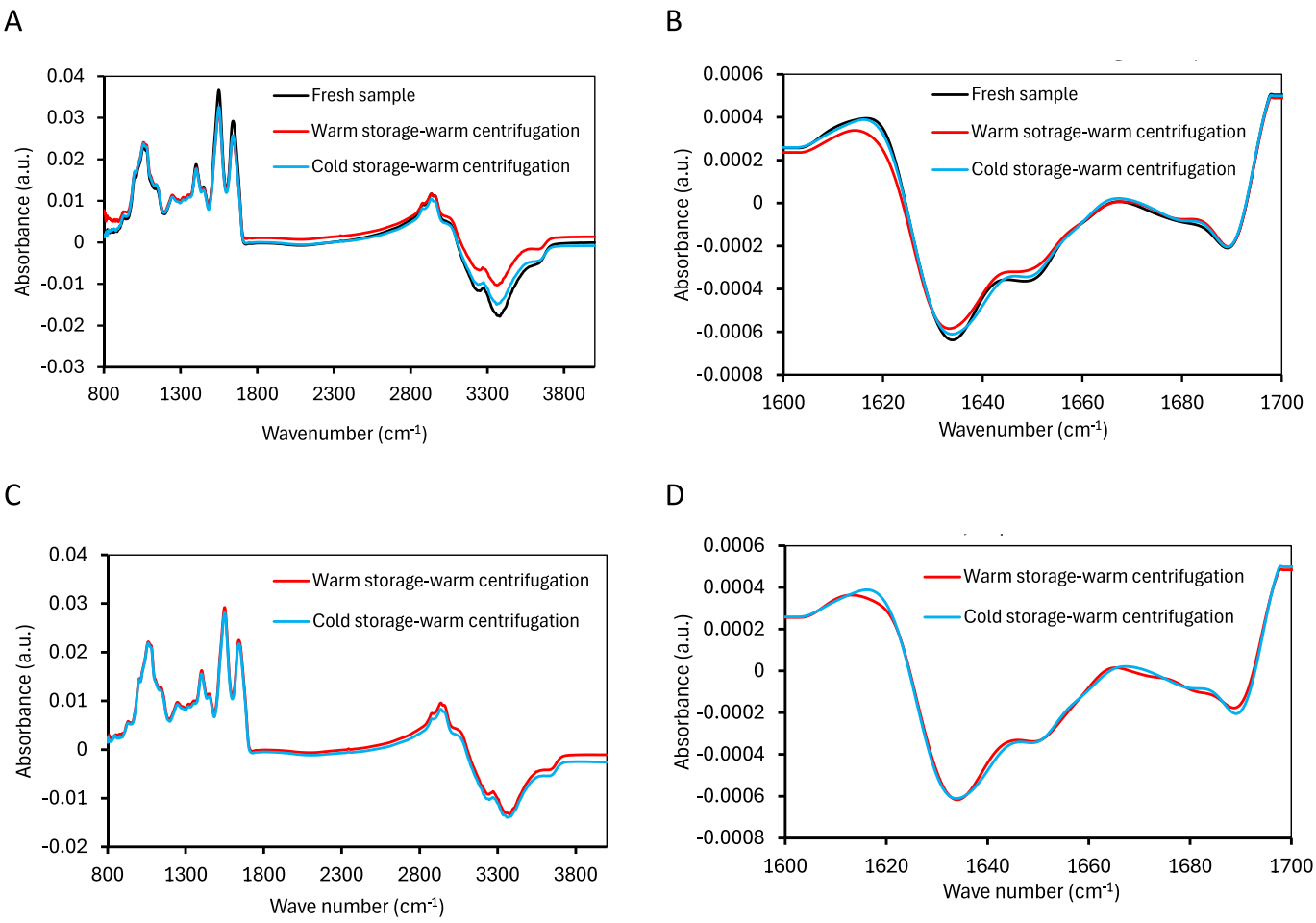

Fig. 4. FTIR spectra of the supernatants obtained after storing the pea extract at warm and cold temperatures (A), along with the corresponding second-derivative spectra in the amide I region. Panel (C) shows the FTIR spectra of those supernatants after a second centrifugation and their second derivative in the amide I region. These analyses were used to evaluate major changes in protein secondary structure under room-temperature and cold-storage conditions.

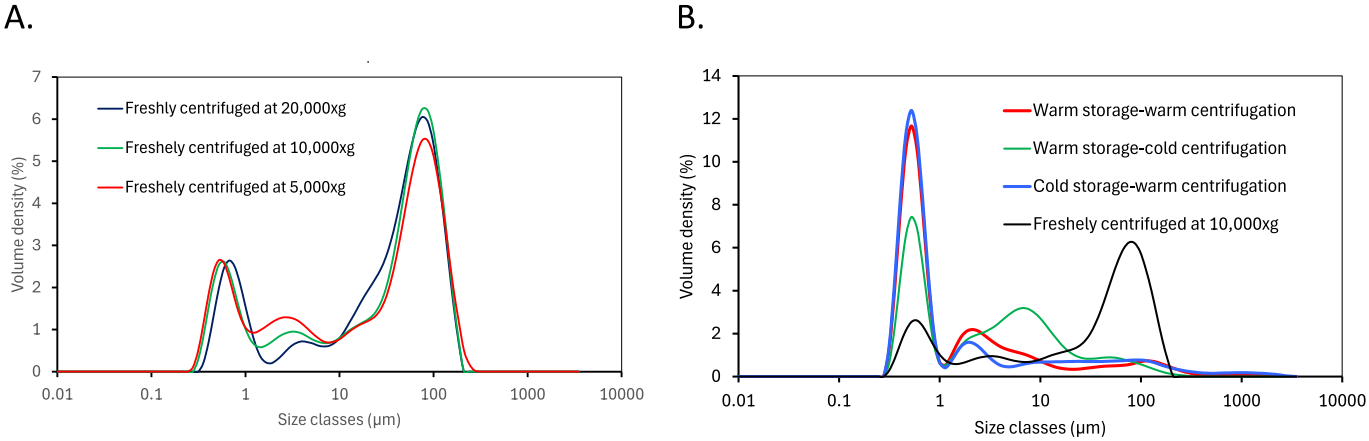

Fig. 5. Particle size distribution of the freshly prepared samples at different centrifugation speeds (A) and samples pretreated at different storage and centrifugation conditions (B).

extracts treated at combined storage and centrifugation conditions (Fig. 5 B), the peak of the biggest particles diminished strongly compared to the untreated sample centrifuged at 10,000 ×*g*. This confirms the removal of the largest particles/aggregates by storage and centrifugation, which were flocculated during room temperature/4 °C storage. The warm stored pea extracts showed more large particles (1–10 μm range) than cold stored one, which may have caused compositional variability.

3.5. *Viscoelastic properties of the extracted pea protein fractions*

Gelation is one of the properties of pea proteins that is used to create food texture (Lam et al., 2018). To characterize the viscoelastic properties of the fractions found by different treatments of pea extract, the

heat-induced gelation of each fraction was assessed by measuring G' and G" during ramping the temperature over time (Fig. 6). The protein content of each fraction was normalized to have the same protein content.

The G' and G" of supernatants obtained from different centrifugation speeds are the same, as shown in Fig. 6 A, B, and C. While at lower temperatures, G' was smaller than G", indicating viscous behavior. The final G' of these samples was larger than G" at around 1 kPa at higher temperatures, indicating gelation. Interestingly, the supernatants after storage and centrifugation also displayed similar viscoelastic properties as the supernatants created using different centrifugation speeds. These possessed similar gelling points (at which G' and G" increase sharply), which signifies the presence and importance of albumins in these fractions, as it is known to contribute to gelation (R. Kornet, Veenemans, et al., 2021; Möller, van der Padt, & van der Goot, 2022). Upon cooling, G′ increased further while G″ remained lower for all supernatant samples, indicating reinforcement of the gel structure upon cooling.

All the pellets obtained from different storage and centrifugation conditions demonstrated the formation of weaker gels. This is consistent with the albumins contributing significantly to the gelation of the supernatants. This will not be present in the pellets as they are well soluble, and the globulin-rich fraction, which is the main constituent of the pellets, is characterized by weak gelation properties and water-holding capacity (Chihi et al., 2018; Klost et al., 2020). The final G' of the pellets ranges between 7 and 105 Pa, with the pellet from cooling and centrifugation conditions showing the highest final G', which is still much lower than all supernatants separated from these pellets, and the supernatants from different centrifugation speeds. Additionally, the

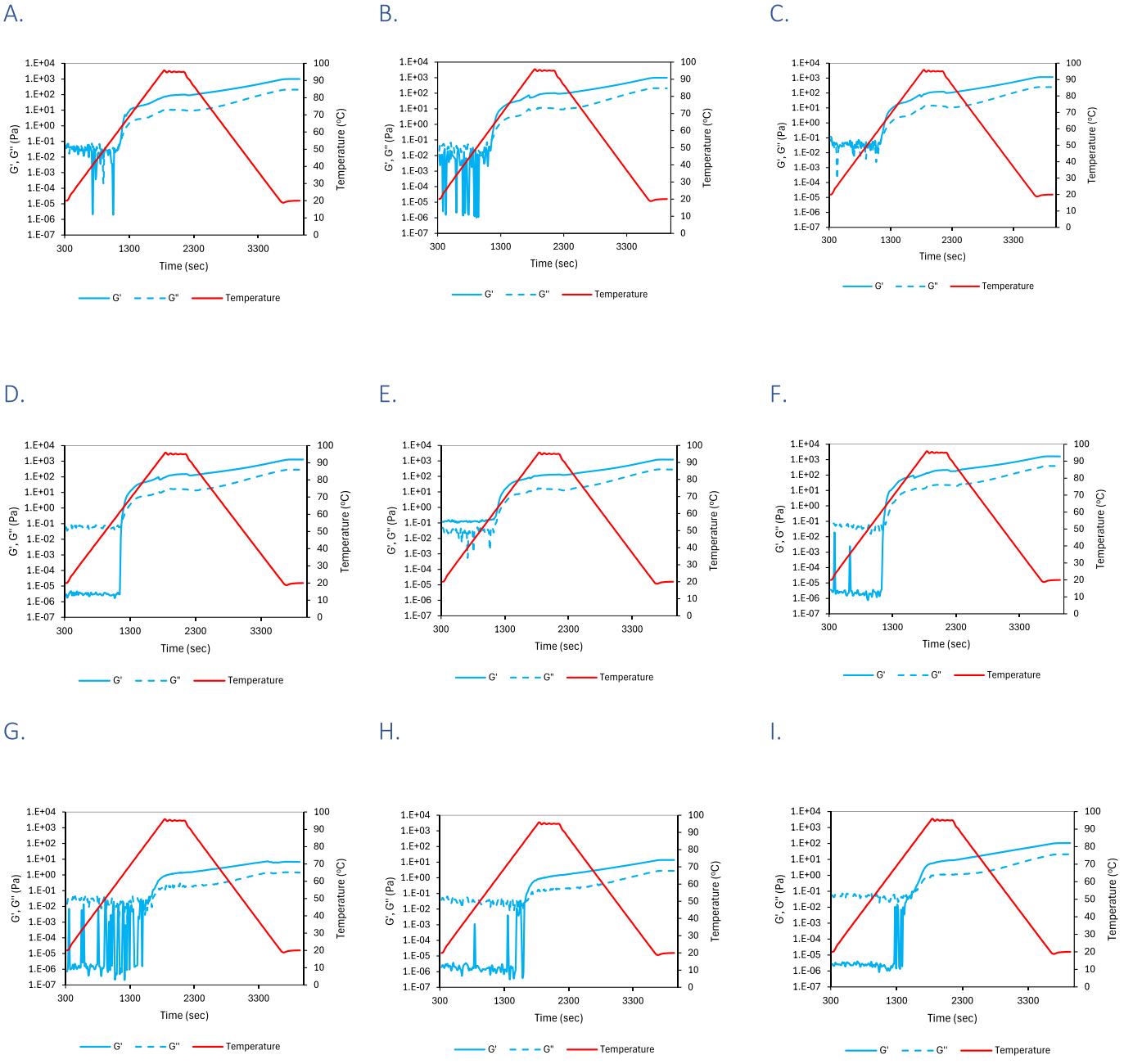

Fig. 6. Rheological response of different pea extract fractions found under different pretreatment conditions, determined by measuring Storage modulus (G') and loss modulus (G") through ramping the temperature over time. (A), (B), and (C) are pea extracts obtained by centrifuging the flour and water mixture at 5000 ×g, 10,000 ×g, and 20,000 ×g, while (D), (E), and (F) are the supernatants obtained by warm storage-warm centrifugation, warm storage-cold centrifugation, and cold storage-warm centrifugation, respectively. And (G),(H), and (I) are their respective pellets obtained after the treatments.

pellets required a longer time and a higher temperature before gelation started.

It can be concluded that the differences in rheological properties between the pellets and supernatant are principally due to the differences in protein composition (R. Kornet, Penris, et al., 2021), as all samples were normalized to have the same protein content during preparation. The differences in their viscoelastic properties are highlighted by requiring a higher temperature and a longer induction time to form, and a lower final G' of the pellets compared to the supernatants, which is consistent with the poor gelation properties of globulin-rich fractions (Möller, van der Padt, & van der Goot, 2022).

3.6. MF flux behavior

We discuss now the effect of the pretreatments on membrane separation. We first look at the effect of centrifuging the pea extract at different centrifugation speeds. Although there is a slight difference in dry matter in the samples, their membrane separation performance was basically the same, as shown in Fig. 7A. Higher centrifugation speeds did not remove particles responsible for membrane fouling.

Fig. 7B shows the effect of combining centrifugation with storage. The cold-stored + warm-centrifuged pea extract gave a significant flux improvement compared to other conditions. This is due to the removal of protein aggregates that block the MF membrane (see also Fig. 5). Removal of protein aggregates is known to improve flux during membrane filtration (Huisman et al., 2000).

Samples stored at room temperature (warm storage) have lower fluxes than that stored at cold condition, but still higher fluxes than the control. Application of centrifugation after storage of the extract, even at room temperature, results in the removal of a certain amount of sedimented pellets, although not as much as after cold storage. This could be due to the extraction step at neutral pH and longer storage time, which prompted hydrophobic interactions between proteins (Durand et al., 2002; Lu et al., 2020). Both warm storage condition samples show comparable flux profiles, despite being centrifuged at cold and warm temperatures. It is therefore the storage that induces most of the aggregation, allowing their removal with centrifugation at any temperature.

To verify whether the MF flux improvement of the cold-stored + warm-centrifuged supernatant was due to reduced dry matter, an MF experiment was carried out by diluting the control sample to the same dry matter content as the cold-stored and warm-centrifuged sample. As shown in Fig. 8, the flux of the diluted sample (2.35% DM) has almost the same flux behavior as the control sample (2.7% DM), except a slight increase initially. This confirms that the higher flux during MF of cold-stored and warm-centrifuged samples is due to the selective removal of specific membrane fouling agents, and not due to the dry matter reduction. The fouling agents are therefore identified as the legumin fraction of the pea extract, as confirmed by the protein content analysis of the pellets.

The effect of cold-storage + warm-centrifugation has also been measured using a UF membrane (Fig. 9). Although the fluxes of MF and UF of the control sample were quite similar (from Fig. 7A and Fig. 9), the UF flux of the cold-stored + warm-centrifuged sample did not show a

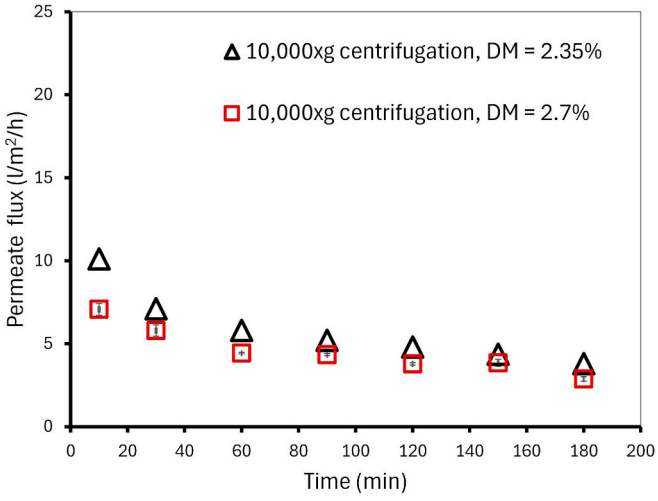

Fig. 8. MF flux of the sample prepared at the control condition (at a centrifugation speed of 10,000 ×*g*) and a diluted sample with a dry matter value equal to the cold storage-warm centrifuged sample. The MF of the diluted sample is a single run.

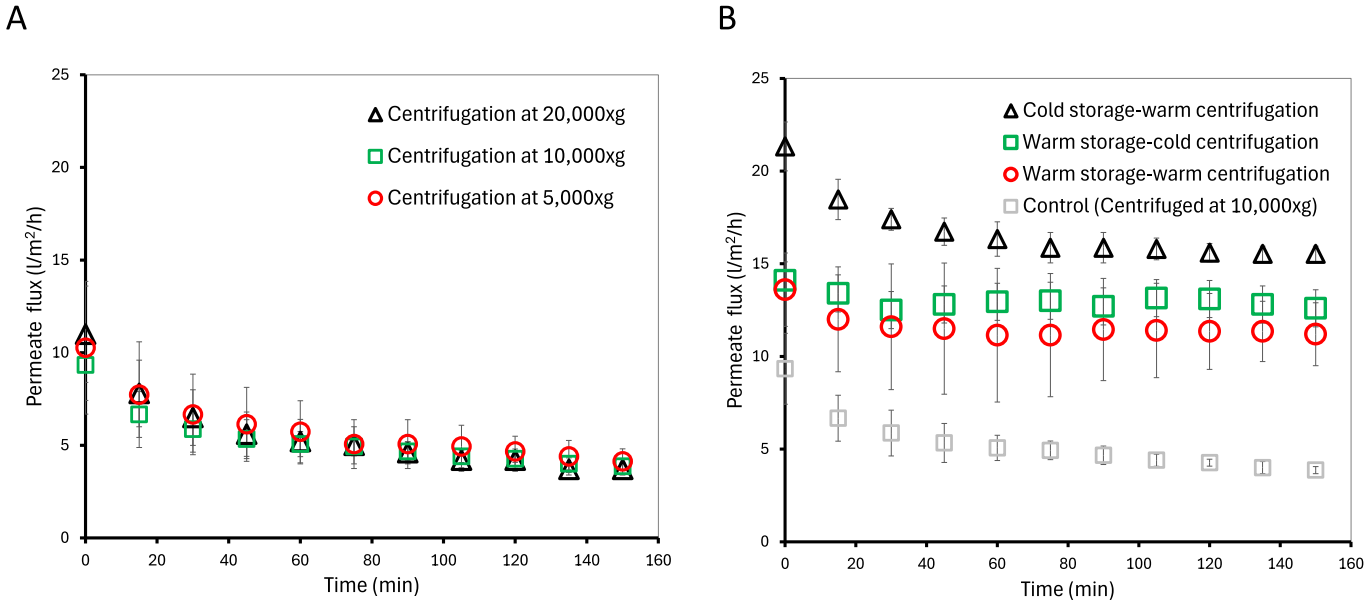

Fig. 7. Effect of pretreatment conditions on MF flux. Figure (A) shows the effect of different centrifugation speeds, and Figure (B) shows the effect of storage conditions coupled with centrifugation on the flux of MF of the pea extracts. The control sample shown in Figure (B) (light gray squares) is plotted using the same experimental data as in Figure (A) for plotting the flux at 10,000 ×*g* centrifugation.

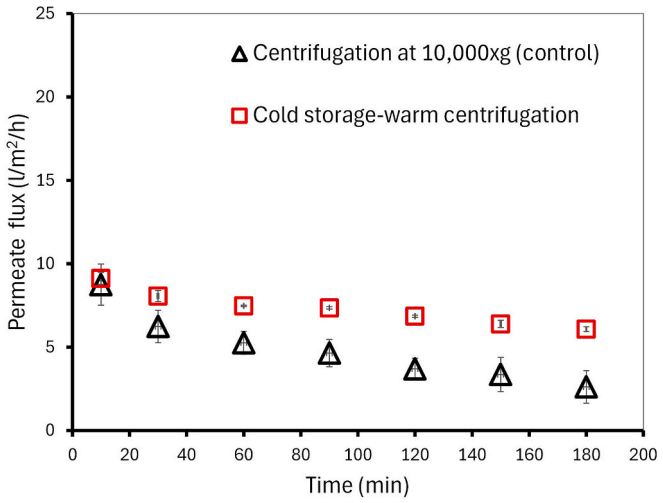

Fig. 9. UF of supernatant prepared at 10,000 ×g (black triangles) and the sample obtained with the same condition, which underwent cold storage and subsequent centrifugation at 10,000 ×*g* (Red squares). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant flux rise compared to MF, but it did show a slower rate of flux decline over time. This indicates that the removal of the aggregates has reduced the cake layer deposition on the surface of the UF membrane, which is the main cause of UF fouling (Kim et al., 1992; Marshall et al., 1993). In contrast, removal of the aggregates resulted in reduced pore blocking in MF, which is manifested with much higher flux increase from the start, as MF is more prone to pore blocking (Marshall et al., 1993).

3.7. HPSEC results of the retentates and permeates

HPSEC revealed that the MF membrane basically acts as a UF membrane: it retained larger proteins while smaller ones permeated, as shown in Fig. 10, for which the permeate of all conditions contained >75% of proteins less than 9 kDa, and > 54% of proteins in the retentate are larger than 9 kDa (Appendix Fig. A2). The retentate and permeate from MF at different centrifugation speeds (Fig. 10 A and B) showed the same profiles, except that at 20,000 ×g, the absorbance is lower compared to the other treatments, indicating lower concentrations. The higher centrifugation speed may have reduced the dry matter content. This is also observed in the permeate streams. All retentates have higher absorbances for the larger molecular weights, which are globulins/aggregates (Möller, van der Padt, & van der Goot, 2022). The permeates of all MF runs show higher absorbance for lower molecular weight proteins, which may correspond to vicilin subunits and albumins (C. Kornet et al., 2020). The HPSEC chromatograms of the retentates and permeates from MF of stored plus centrifuged conditions showed insignificant differences. This shows that the composition of the proteins is not affected by the pretreatments, indicating that the underlying filtration mechanism is probably the same. This is also supported by the SDS-PAGE analysis of each fraction, as the feed streams possess quite the same protein composition (Fig. 3).

The retentates from different centrifugation speeds and stored + centrifuged samples exhibit small variations for high molecular weights, while the permeate streams are almost the same at all conditions. This shows that the removal of the aggregates after storage affected only the larger proteins to a small degree, and the smaller proteins remained in the solution during the pretreatment and filtration experiments. These small peptides, of course, can be further recovered by membranes with lower molecular weight cut-offs, and could have different functional properties in food applications (Möller, Li, et al., 2022).

3.8. Protein rejection during MF

To evaluate the rejection profile of the MF of different supernatants, the protein content of the retentate and permeate was determined on a dry basis. Protein rejection was calculated as $R = 1 - (concentration\ in\ the\ permeate/conentration\ in\ the\ retentate)$. The samples from different centrifugation speeds showed the same protein rejection, with an average value of around 0.7 (Shown in the appendix Fig. A3). The stored plus centrifuged samples exhibited a somewhat lower (comparable) rejection (0.6) than the samples that had not been stored and centrifuged. The HPSEC results have already confirmed that the retentate and permeates of all the conditions have similar molecular weight distributions. This implies that the remaining proteins in the supernatant after storage and centrifugation treatments still contribute to aggregate-induced blocking and cake formation during MF. Therefore, the removal of the protein aggregates has probably reduced the blocking/cake layer formation, but did not completely avoid it, leading to similar HPSEC profiles of retentate and permeate and protein rejection. One could therefore speculate that a more thorough removal of the aggregates, by stronger centrifugation or another method of removal, should lead to lower MF rejections/fouling.

4. Conclusions

In this study, the pea extract obtained from water-only extraction was pretreated by centrifugation at various centrifugation speeds and by combinations of storage-induced aggregation and centrifugation. Storage-induced aggregation plus subsequent centrifugation reduced membrane fouling during filtration and led to an additional fraction that is quite pure in protein. Storage leaves time for the formation of larger protein aggregates after the extraction, which can be removed more easily with subsequent centrifugation, reducing pore plugging of the MF membrane. Unlike conventional methods, this approach preserves protein nativity and improves microfiltration (MF) performance. The MF flux improvement was due to the selective removal of the colloidally dispersed legumins during this pretreatment, as confirmed by the SDS-PAGE analysis. This legumin-rich fraction has a purity of around 90%, which otherwise can only be achieved by chemically intensive conventional extraction. Cold storage of the pea extract followed by centrifugation was the most effective pretreatment method, achieving both the highest MF flux and the highest-purity sediment. The flux improvement achieved by using the sample from this pretreatment is an economic benefit that can offset the cost of the pretreatment before filtration. Compared to the MF flux of this sample, the flux improvement of UF is limited, suggesting the need to tailor the sample pretreatment with an appropriate membrane pore size. Storing the pea extract at room temperature also promoted protein aggregation, which could be removed during centrifugation. The protein content of these aggregates (>85%) is slightly lower than with cold storage. The retentates can be further concentrated/diafiltrated, while the permeate streams can be further fractionated using ultra- and nanofiltration. The resulting fractions can be used for applications in food or pharmaceuticals. The similar protein profiles of permeates and protein rejection across all samples suggest a consistent filtration mechanism, further providing insights for the scaling of this method.

CRediT authorship contribution statement

Tadele Maru Alemu: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Anja E.M. Janssen:** Writing – review & editing, Supervision, Conceptualization. **Remko M. Boom:** Writing – review & editing, Supervision, Conceptualization.

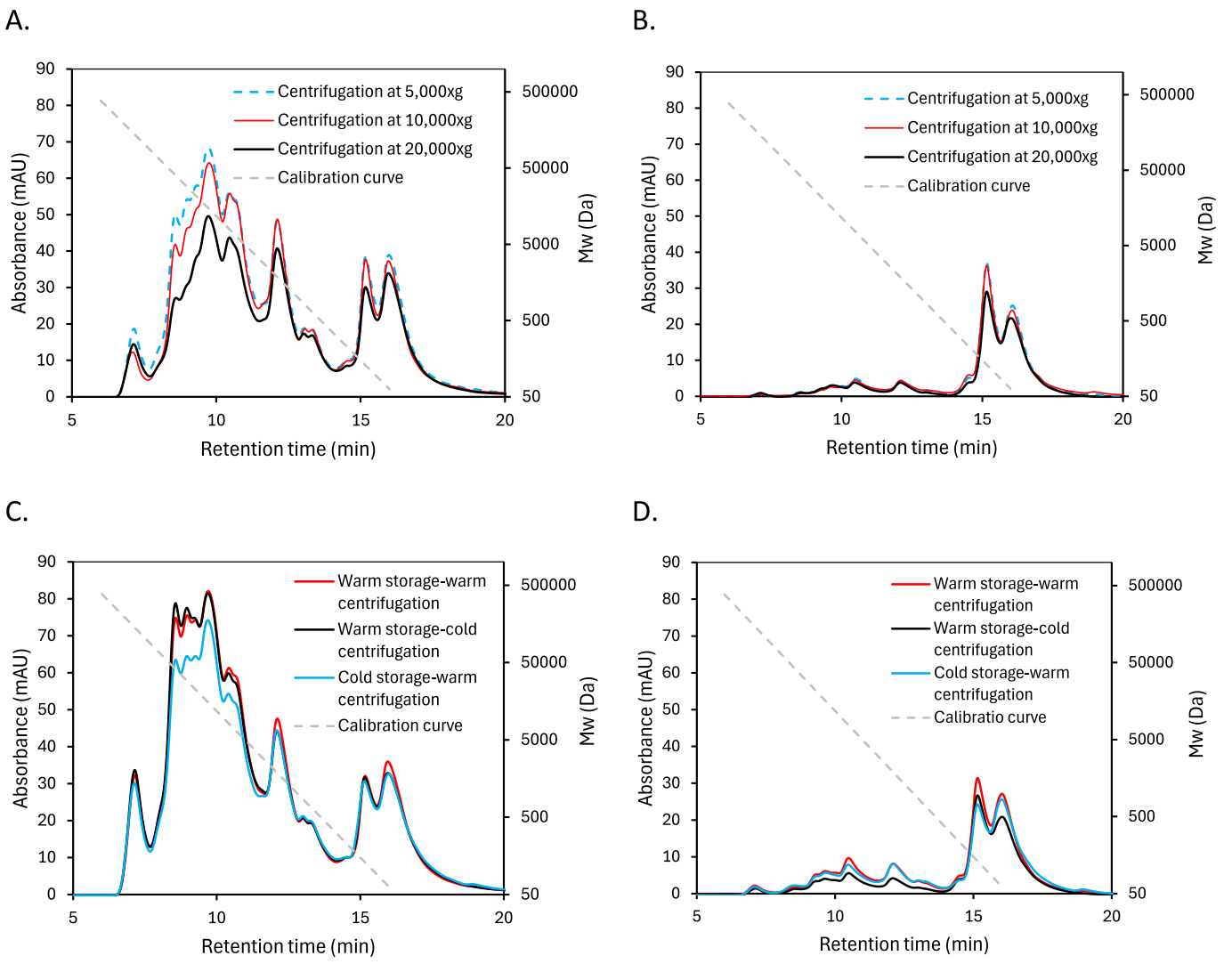

Fig. 10. Protein profile of retentate and permeate from MF of pea extract prepared using different pretreatments. Figures A and B represent the retentate and permeate profiles of MF of pea extract prepared at different centrifugation speeds, while C and D are the retentate and permeate of samples treated at various storage and centrifugation conditions, respectively. The dotted light gray lines are the calibration curve used to indicate the molecular weight at specific retention time and absorbance.

Declaration of competing 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.

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Appendix

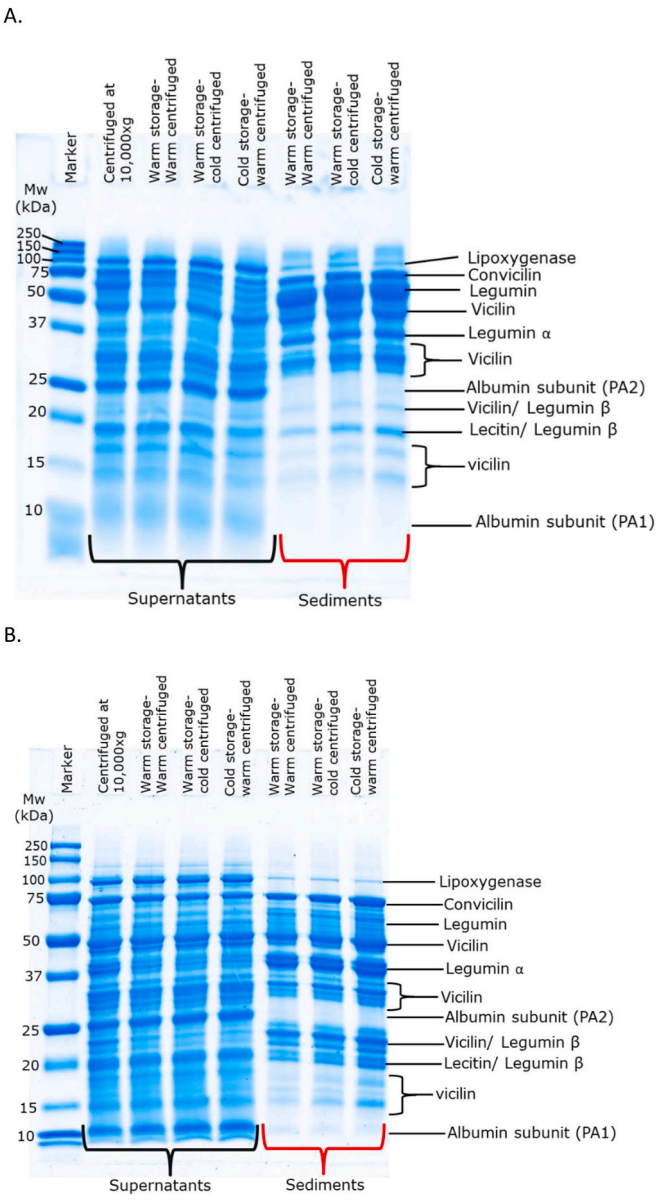

Fig. A1. SDS-PAGE results of the control sample(supernatant at 10,000 ×g), supernatants after pretreatments, and their sediments at non-reducing (A) and reducing (B) conditions. The samples are prepared by normalizing their protein content (all the protein contents are equal).

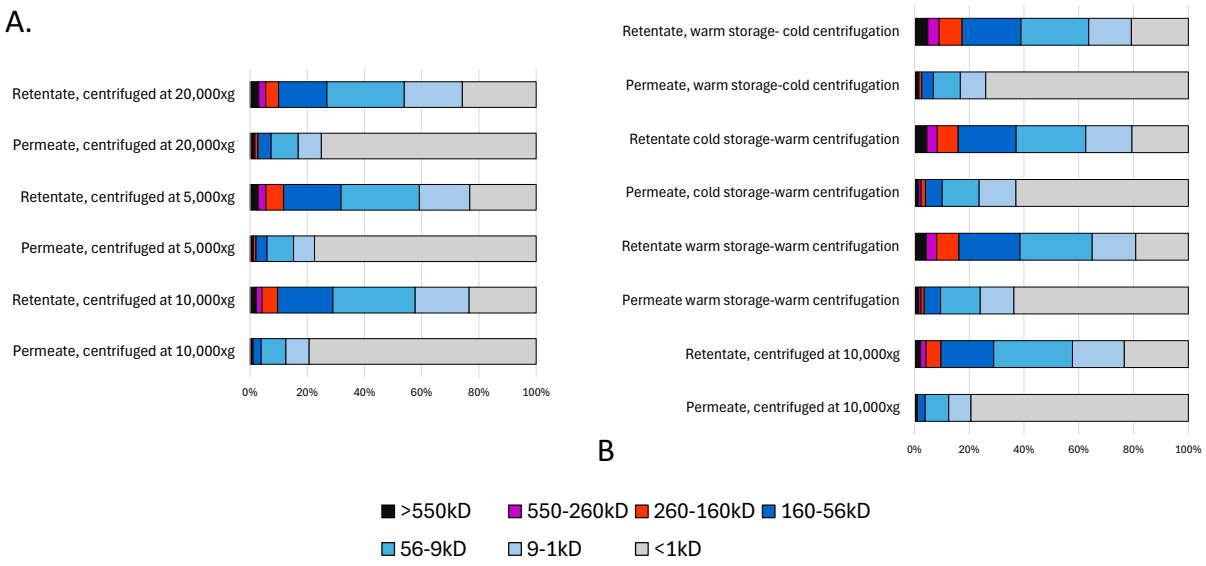

Fig. A2. HPSEC relative area of the retentate and permeate obtained from MF of samples from different centrifugation speeds (A) and storage and centrifugation conditions (B). The permeate and retentate for the samples centrifuged at 10,000 $\times g$ (control sample) in Figure B are the same measurements as in Figure A.

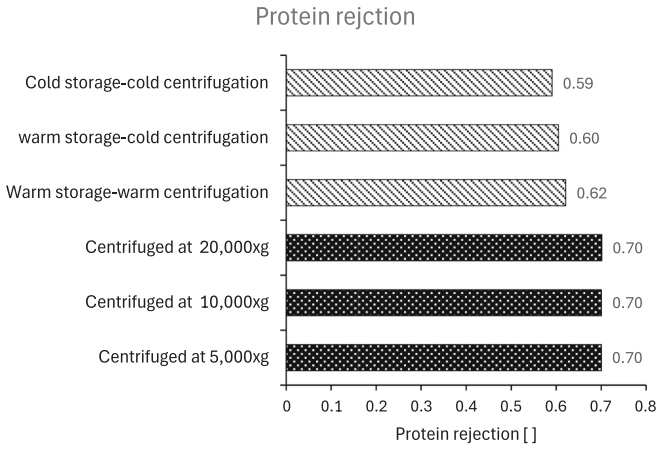

Fig. A3. Rejection of MF of pea extract treated at different centrifugation speeds (dark colored bars) and various storage conditions coupled with centrifugation (light colored bars).

Data availability

Data will be made available on request.

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